



September 7/9, 2003

2nd PROBIOTICS & PREBIOTICS
NEW FOODS



ATTI
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ANTIGENOTOXICITY: A NEW TOOL FOR PROBIOTICS

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Antigenotoxicity is now included among the numerous functional properties characterising probiotic bacteria. Numerous data have been accumulated, from epidemiological and experimental studies, supporting the implications that probiotics decrease colon cancer risk.^{5, 6, 17}

This evidence, which emerged from both human and animal studies, shows that the qualitative and quantitative alteration of gut microflora originated by probiotics may be associated with a diminished risk of intestinal carcinogenesis from environmental factors, such as dietary and endogenous xenobiotics.^{4, 8, 11, 16, 19}

In humans probiotics reduce the expression of numerous faecal bacterial enzymes (β -glucuronidase, nitroreductase, azoreductase, 7- α -dehydroxylase, etc) implicated in the production of genotoxic compounds which act as tumor initiators, or which are involved in mucolytic effects (bacterial glycosidases and proteases). Probiotic administration has been also associated with reduced colonic crypt proliferative activity in patients with high basal proliferation rates.¹

Animal studies showed that oral administration of probiotic bacteria is effective in reducing DNA damage by chemical genotoxins, in producing an inhibitory effect on the development of colon preneoplastic lesions and protecting against experimental tumors induced by AOM, AAC, IQ, and DMH treatment.^{11, 12, 22}

The inherent scientific importance from the above different evaluations underline direct and indirect implications of probiotic bacteria on antigenotoxicity. However - to date - the role of probiotics in reducing human colon cancer risk as regards the precise mechanisms by which they exert protective effects has not been completely established.^{5, 9}

A fundamental contribution to molecular mechanisms of probiotic-mediated antigenotoxicity come out from *in vitro* evaluations by short-term methods used in genetical toxicology. In particular, important suggestions have been obtained using different short-term assays with different targets, such as procaryotic cells (e.g. SOS-Chromotest for genotoxicity in *E. coli* and Ames test for mutagenicity in *S. typhimurium*) and eucaryotic cells (e.g. Single cell gel electrophoresis (SCGE) assay for genotoxicity in Caco-2 enterocytes).

The ability of bacteria to interact with xenobiotic compounds is well known. This prerogative, frequently found in environmental bacteria, also characterises certain probiotics which could be directly involved in deactivation of harmful compounds in the gastro-intestinal tract. Examples of bacterial species comprising strains able to inhibit DNA-reacting genotoxins may be found in different probiotics, such as *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus* and *Propionibacterium*.^{2, 6, 7, 10, 14, 18, 21}

Literature underlines that genotoxin deactivation is generally strain dependent and clearly related to microorganism-mutagen dualism. Consequently different mechanisms have been described. The more important theories for genotoxin deactivation are: (i) binding on bacterial cell components (peptidoglycan complex, polysaccharides), (ii) reaction with bacterial metabolites (peptides, polyamines), and (iii) bioconversion to unreactive moieties by different bacterial enzymes or genotoxin conjugation with bacterial metabolites. Live bacteria may be very effective in binding or permanently inhibiting genotoxins, whereas the effect of killed cells is lower, or absent, when compared with live cells. In the case of killed cells only a temporary inhibition has been observed. Therefore probiotic viability during and after genotoxin exposure is an important requisite for antigenotoxicity.^{6, 7, 14}

There is increasing evidence about the *in vitro* probiotic interactions with food related mutagens (heterocyclic aromatic amines, protein pyrolysates, nitrosamines, polycyclic aromatic hydrocarbons, aflatoxins) and other DNA-reacting compounds (nitroarenes, alkylating agents, etc).^{2, 10, 13, 15, 20, 23}

We showed high inhibitory activity against two reference direct acting genotoxins (4-nitroquinoline-1-oxide, 4-NQO and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, MNNG) in bacterial isolates belonging to different genera (*Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*) coming from dairy products (yoghurt, fermented milk), probiotic preparations and reference strains^{6, 7}. Our studies confirmed that, with the exception of the *Bacillus* strains for which high inhibitory activity was always found (*B. clausii*, *subtilis*, *firmus*, *pumilus*), antigenotoxicity in the other genera was strain dependent, and so both high active and moderate/inactive strains were found in *L. casei*, *plantarum*, *acidophilus* and *delbrueckii*, but also in *B. bifidum* and *S. thermophilus*. Evident modifications of spectroscopic profile of 4-NQO and MNNG have been observed after co-incubation with active strains, but not with killed cells of the same bacteria or with strains incapable of genotoxin deactivation. Different profiles, indicative of genotoxin bioconversion or its conjugation with bacterial metabolites, have been observed in relation to strains. Chemico-physical analyses (UV-vis and GC/MS spectra) confirmed indications obtained by biological short-term assays.

In conclusion, the evidence from different approaches, although not directly transferable to humans, is in line with the hypothesised efficacy of probiotics in providing a protective effect against genotoxins in the gut. Results from animal studies encourage further investigations about the validity of probiotics both in preventing carcinogen activation and protecting from risk due to exogenous and endogenous genotoxins. *In vitro* evidences suggest that research into the possibility of using probiotics as antigenotoxic agents is both promising and topical. Extensive fundamental studies oriented to probiotic strains suitable for this special application, by pharmaceutical industries, are needed. In particular, strain selection, their molecular characterisation and genetic stability on the one hand, and mechanisms involved in genotoxin inactivation together with toxicological evaluations on the other, are the major prerogatives in labeling probiotics for antigenotoxicity.

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Lactococcus lactis secreting IL10

Running title: Lactococcus lactis secreting IL10

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Summary

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, affects around 2 in every 1000 individuals in western countries. Incidence is increasing particularly amongst children. IBD shows extreme morbidity, having impact on all quality aspects of life. IBD can lead to death when left untreated.

Powerful immunosuppressive chemotherapies and surgical intervention make up conventional treatment for IBD. Because IBD is never cured, long-term anti-inflammatory medication is required and so, patients are often subject to a spectrum of disagreeable side effects. Interleukin-10 (IL-10) is a cytokine that acts to suppress inflammation. When administered by injection, IL-10 behaves no different from conventional immune suppressants and high levels of IL-10 that are dispersed throughout the body also lead to side effects.

Lactococcus lactis can be genetically modified (GM) to secrete biologically active cytokines. When applied to the mucosa, these *L. lactis* can actively deliver such cytokines. By use of this principle we developed a new therapeutic approach for IBD. Administration of *L. lactis* that secrete murine IL-10 both cure and prevent IBD in mice. Targeted delivery by use of this GM *L. lactis* allows dramatic reduction of the effective dose and may therefore lead to a more effective therapy.

Legitimate concerns exist about the implications of uncontrolled, deliberate release of genetically modified micro-organisms. This could occur following application in healthcare. We established adequate means for inheritable growth control of engineered *L. lactis*.

Inflammatory bowel disease

Inflammatory bowel disease (IBD) encompasses ulcerative colitis (UC), a disease of the large bowel, and Crohn's disease (CD), which can affect any part of the gastrointestinal tract and can even have extra-intestinal manifestations. IBD affects approximately 1-2 in every 1000 individuals in western countries and is increasing, especially amongst children. IBD is a life-long disease and most patients are diagnosed for the first time in adolescence or early adulthood. The clinical image is characterized by periodic inflammation, often resulting in a chronic, unpredictable course. The symptoms are extremely unpleasant and have a profound negative impact on the quality of life. They include diarrhea, abdominal pain, rectal bleeding, fever, nausea, weight loss, lethargy and loss of appetite. If left untreated, malnutrition, dehydration and anemia can follow, which, in extreme cases, can even lead to death.

IBD represents an indisputable problem for public healthcare because of the absence of etiologic treatment. At present, a number of immune suppressive chemicals can be used to revert to the non-inflamed state while others are administered to maintain remission. Corticosteroid drugs, such as the synthetic prednisone, are still the most effective treatment for active disease. These steroids have widespread actions on the immune system, especially on monocytes. 5-aminosalicylic acid (5-ASA) preparations such as mesalazine and analogues are widely used in remission maintenance therapy. Chemotherapy can be done by using azathioprine (AZA), its metabolite 6-mercaptopurine (6-MP), methotrexate and cyclosporin A. The importance of the intestinal bacterial load in the pathology of IBD allows for the use of antibiotics such as metronidazole or ciprofloxacin. Although many patients are managed successfully with conventional medical therapy most will have recurrent activity of disease and two-thirds will require surgery.

Crohn's disease is most likely a consequence of inappropriate recognition of antigen from the intestinal microflora by T-cells. In normal physiology, activated T-cells can produce both anti-inflammatory and pro-inflammatory cytokines. With this knowledge in hand, IBD can be counteracted in a rational manner. Modulation of the immune deregulations causal to IBD can now be achieved through novel anti-inflammatory therapies, which make use of neutralising monoclonal antibodies or anti-inflammatory cytokines. A highly prominent and effective new therapy is systemic treatment with anti-TNF monoclonal antibodies⁽¹⁾. This treatment blocks TNF, a powerful immune stimulator. Single intravenous doses, ranging from 5 to 20 mg.kg⁻¹, of the cA2 infliximab antibody resulted in a drastic clinical improvement in active Crohn's disease. Antisense oligonucleotide therapy blocking the expression of ICAM-1 – a cell surface molecule initiating immune response upon binding - seemed to be a promising new therapy for CD⁽²⁾. The large follow-up clinical trial however was negative⁽³⁾. Anti MadCAM/ α 4 β 7 integrin trials, blocking lymphocyte recruitment to the mucosa, showed to be promising⁽⁴⁾.

Interleukin-10 (IL-10) plays a central role in the establishment and maintenance of tolerance. Especially its involvement in the formation and in the activities of CD25+CD4+ regulatory T-cells – Tr1 cells that are central to tolerance and can actively suppress ongoing malignant inflammation - has recently received a lot of interest^(5,6,7). The physiology and pharmacological use of IL-10 have recently been reviewed. IL-10 can be used to suppress psoriasis, has limited effect in rheumatoid arthritis and is promising in hepatitis C treatment. Daily systemic administration of recombinant IL-10 during 7 days in doses ranging from 0.5 to 25 μ g.kg⁻¹ resulted in reduced Crohn's disease activity scores and increased remission⁽⁸⁾.

Cytokine delivery at mucosal surfaces

Cytokines – such as interleukins and interferons - are small soluble proteins that, together with numerous growth factors and chemokines, provide the means by which cells of the immune system communicate with each other and with most other tissues in the body. These molecules are the messengers in the regulation of many aspects in immune physiology in which numerous cells and tissues may be involved at any one time. Because of their activity as regulators of the immune system, cytokines and growth factors provide interesting tools for redirecting immunity. For any of such appliances they combine two potentially very interesting pharmacokinetic properties: they are effective at quite low concentrations and their half-life is relatively short. This means that, given good targeting, one here has a set of tools that allow precise bolstering of immune responses and intervention in malignant immunity. However, cytokine production is often complex and inappropriate targeting of these potent molecules may have devastating effects on the organism.

Bacterial expression systems offer an economical alternative for the production of various proteins from eukaryotic origin. Along the line of evaluating a range of microorganisms for heterologous gene expression, we came across *Lactococcus lactis*. We were struck by the apparent ease with which GM *L. lactis* can secrete cytokines without any apparent counterselection on its viability. Furthermore, the secreted proteins appeared correctly processed at their N-terminus and were fully biologically active. We have so demonstrated the potential of *L. lactis* cells to secrete murine IL-2, -6 and -10, human IL-2, -6 and -10 and murine soluble type 1 and type 2 TNF receptors (^{9,10} and unpublished data). This led to the idea of developing GM *L. lactis* strains for the delivery of these immune modulatory factors at mucosal surfaces.

It was reported earlier that *L. lactis* could be used as a vaccine delivery vehicle. Robinson and coworkers⁽¹¹⁾ used GM *L. lactis* that produce intracellular tetanus toxin fragment C (TTFC). Intranasal inoculation of the GM strain leads to protective immunization. To investigate whether the mucosal and systemic responses to TTFC, responsible for the above-mentioned protection, can be enhanced or modulated we constructed strains of *L. lactis* that produce TTFC intracellularly and secrete functional murine IL-2 or IL-6 ⁽¹²⁾. In general, both IL-2 and IL-6 act as potent stimulators in the onset and maintenance of immune reactions. Mice were immunised with these recombinant strains as well as with the relevant controls. Anti-TTFC serum antibody responses were up to 15 fold higher in mice immunised intra-nasally with those strains of *L. lactis* secreting IL-2 or IL-6. Also the concentration of serum IgA reactive with TTFC was considerably higher after immunisation with the IL-6 secreting strain. This is in good agreement with the fact that IL-6 acts as a B-cell growth and IgA secretion-stimulating factor.

Recently⁽¹³⁾, *L. lactis* have been constructed that secrete fully active IL-12, a potent Th1 stimulatory cytokine. Intranasal administration of IL-12-producing strains in mice resulted in enhanced IFN-gamma production from splenocytes. Immune responses elicited by GM *L. lactis* strain displaying a cell wall-anchored human papillomavirus type 16 E7 antigen was dramatically increased by coadministration with an *L. lactis* strain secreting IL-12. Splenocytes from these animals showed substantially enhanced antigen-specific secretion of Th1 cytokines IL-2 and IFN-gamma following in vitro challenge with E7 peptide.

Delivery of IL-10 for IBD therapy

As mentioned above, IL-10 fulfills a central role of in the establishment and maintenance of tolerance. It is a very potent anti-inflammatory mediator, both at the

level of antigen presentation and T-cell activity. Consecutive injected doses will substantially decrease the inflammation. From the literature it is however clear that IL-10 suffers from an important number of side effects that are induced upon systemic delivery, as through injection⁽¹⁴⁾. Recombinant IL-10 is very acid sensitive and is therefore rapidly degraded when given orally. We wanted to investigate whether in situ synthesis of this promising therapeutic protein could remedy these shortcomings. We investigated this in two different mouse models for IBD⁽¹⁵⁾.

The repeated addition of DSS to the drinking water of Balb/c mice leads to the induction of chronic colitis⁽¹⁶⁾ reminiscent of IBD. The histology is characterised by a gradual decrease of goblet cell presence in the epithelial layer, disappearance of crypt architecture, infiltration of lymphocytes in the lamina propria and submucosa, thickening of the mucosa, appearance of crypt abscesses, swollen lymph follicles and ulceration. On occasion we observed the formation of polyps, adenoma and squamous metaplasia. The colonic inflammation can be rated by interpreting blinded slides on the degree of the above-mentioned pathological histology. A mean score of 5 is the typically record. Intestinal inflammation is further associated with up regulation of several pro-inflammatory cytokines such as IFN γ , IL-12 and TNF and can be cured by the systemic administration of neutralising antibodies towards these proteins. The daily ingestion, for 2 weeks, GM *L. lactis* cells that produce mIL-10 results in the acquisition of a score of approximately 1 in 40% of the treated mice, which is a status equal to that of healthy control mice. Most other animals only showed minor patchy remnants of the inflammation. Killing of the IL-10 producing bacteria by UV irradiation abrogates the curative effect. Therefore the mechanism of delivery works through the active in vivo synthesis of IL-10. The level of healing is comparable to systemic treatment with prominent anti-inflammatory drugs such as dexamethasone, anti-IL-12 and recombinant IL-10. The amount of IL-10 required when given through *L. lactis* delivery is however 10000 fold lower, indicating considerably improved delivery.

IL-10 -/- mice spontaneously develop enterocolitis from week 3 on, resulting in a mean histological score of 6 at week 7⁽¹⁷⁾. When treated daily from week 3 on with IL-10 producing *L. lactis*, colitis could be prevented. In the treated animals the histological score stalled at approximately 1,5.

Design of biologically contained GM probiotics

Use in human or animal healthcare is obviously the final goal of any designer probiotic strain. Especially within the public a major - be it often overrated and even hyped - concern exists on the use of GM organisms (GMO). Medical use of GMO in fact almost inevitably represents deliberate release of the GMO in the environment. The design of recombinant functional microflora should therefore be such that subsequent to its use, environmental safety is guaranteed. This essentially relates to the prevention of lateral dissemination of the genetic modification and of antibiotic selection markers to other bacteria and precluding the GMO from accumulating in the environment. These concerns are addressed optimal and most elegant through a biological system that is inherited along generations. Such solutions are termed biological containment systems. Any biological containment strategy should meet all of the above-mentioned concerns. no reports have currently been made on the use of GM microflora in human or veterinary medicine. Likely candidate strategies will however be quite similar to the ones used in other disciplines of biotechnology in which proficient use is made of GMOs.

Biological containment systems can be subdivided in active and passive. The

former essentially control growth through conditional providing of a bacterial toxin. The toxin is either tightly controlled by an environmentally responsive element or suppressed by an immunity factor. Passive systems render growth dependent on complementation of an auxotrophy or other gene defect, by supplementing either the intact gene or the essential metabolite.

Gene exchange at thymidylate synthase gene

Active containment systems mostly provide effective killing of the host. An important number of shortcomings must however be mentioned. Firstly the suicide systems that are introduced often involve the incorporation of a large amount of foreign - often pathogen derived - DNA. From a regulatory perspective, this is highly undesirable for applications in humans. Secondly, many of these systems are plasmid borne. In any such system, functionality will depend on the relative expression levels of the different components. It remains to be demonstrated that, when integrated in the bacterial chromosome to reduce lateral dissemination, performance is maintained. Passive systems overcome these defects but are often bacteriostatic rather than bactericidal.

Disruption of the gene for thymidylate synthase, *thyA*, combines the advantages of both passive and active containment systems. "Thymine less death"⁽¹⁸⁾ was already described in 1954⁽¹⁹⁾ and involves activation of the SOS repair system and DNA fragmentation. It hereby essentially makes use of an indigenous suicide system. Thymine and thymidine growth dependence is intrinsically different from other auxotrophies⁽¹⁸⁾ because lack of the essential component, instead of being bacteriostatic, will rather kill the host. We recently reported targeted replacement of *L. lactis thyA* by *hIL-10* as a containment strategy⁽²⁰⁾.

The *thyA* gene from *L. lactis* MG1363 had been cloned⁹. The known flanking sequences are however too short to allow for efficient double homologous crossover. Therefore we isolated the wider *thyA* locus of MG1363 (GenBank AF462070). Comparison with the *thyA* locus of *L. lactis* IL1403¹¹ showed that both *thyA* genes share 88% homology. The sequences flanking *thyA* are, however, completely unrelated. Conditionally replicative plasmids in which *thyA* flanking sequences now are placed adjacent to *hIL-10* were used for targeted gene exchange by double homologous crossover. Erythromycin (Em) selection forces integration by homologous recombination at either one of the target regions. This was confirmed by PCR. Subsequent random loss of the Em resistance marker through the alternative cross over was verified by PCR.

Propagation and even survival of *thyA*-deficient *L. lactis* is critically dependent on the presence of thymidine or thymine in the growth medium. Moreover, thymidine auxotrophes are self-limiting because they inevitably use up the entire pool of the essential growth component. Depletion of this immediately induces cell death. Limited thymidine is present in the small intestine, yet *thyA* deficient strains show a substantial reduction in viability following passage through the porcine large intestine. We could never force the acquisition of foreign *thyA* genes from co-culturing with donor strains to complement *thyA* deficiency. Both in vitro and in vivo production of IL-10 was intact in all of the strains that were constructed by exchanging *thyA* for *hIL-10*. It is remarkable and providential that the strain carrying the absolute minimum of foreign DNA – Thy12 - showed the highest hIL-10 synthesis.

Thy12 *thyA* deficient *L. lactis* answer biosafety questions in a most adequate way. No resistance marker is required to guarantee stable inheritance of the transgene.

Accumulation of the GMO in the environment and the acquisition of *thyA* from other microorganisms are very unlikely. In the very doubtful event this were ever to happen, the most likely candidate donor for effective *thyA* reverse-in-recombination would be *L. lactis* subsp. *cremoris*. This incident would then automatically remove *hIL-10*, generating back the non modified state. The risk of lateral gene transfer is reduced to the minimum by integrating *hIL-10* in the *L. lactis* chromosome. This system of biological containment was subject to the scrutiny of the Dutch authorities and was corollary allowed for use in humans. This will be the first time ever that a GMO bacterium will be used as a therapeutic.

Conclusions and perspectives

It was not until fairly recently that reliable double blind experimentation has been introduced in the field of probiotics. Genuine scientific approach now allows for the establishment of solid knowledge on the potential of probiotic strains. Alongside, hypothesis on the mechanism of action have been developed and were tested through detailed microbiological, molecular biological, immunological and physiological analysis. From this we know that probiotic microorganisms can be effective in IBD therapy through a number of routes. Some strains can displace noxious bacteria and their toxins, some have defined influence on host immunity and some can influence epithelial and tissue integrity. Current probiotic treatment of IBD is however still largely limited to remission maintenance. To allow active intervention in “critical care” patients i.e. patients in need of acute immunosuppressive intervention, more powerful strains must be created through genetic engineering. In this way, the adaptation of existing or the establishment of novel mechanistic pathways may open new perspectives.

In order to create novel GM probiotic strains it is of great help to understand the different possible mechanisms of action. Knowledge of the mechanism by which defined probiotic strains exert their function allows for the rational design of probiotic microorganisms through genetic engineering. Such novel strains might utilise existing mechanisms that were strengthened. Mechanistic pathways present in different strains can also be combined to yield more potent strains. Further, completely novel concepts can be built and so one can now create additional mechanisms for probiotic activity.

With careful design and with all aspects of biological safety in mind, GM probiotics have the potential to become the next generation of functional microbiota. In view of the nearly unlimited knowledge emerging from contemporary molecular medicine, it is clear that within the concept of GM probiotics lies an enormous and virtually untapped potential.

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EXPERIENCE *WITH SACCHAROMYCES BOULARDII*, A "DIFFERENT" PROBIOTIC

Running Head: *Saccharomyces boulardii*

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INTRODUCTION

Saccharomyces boulardii is a non-pathogenic yeast, which grows at 37° C , survives gastric acid and bile after oral administration and is naturally resistant to antibacterial agents (1)

Its mechanisms of action on the intestinal tract include : a) An inhibitory effect on the growth of various microorganisms such as *Candida Albicans*, *Escherichia coli*, *Salmonella typhi*, *Shigella* species as well as the adhesion of *Entamoeba histolytica* to human red blood cells (2);

b) An antisecretory activity by counteracting the effect of the toxins by *Vibrio cholerae*, enterotoxic *Escherichia coli* and *Clostridium difficile* toxins (2,3); c) A trophic effect on enterocytes (4) which promotes intestinal adaptation after small-bowel resection (5) ; d) A stimulatory effect on intestinal IgA secretion (6) ; e) A possible reduction in intestinal nitric oxide production (7)

Clinical studies with *Saccharomyces boulardii* in both children and adults have shown that the probiotic is effective in the prevention and/or treatment of various types of diarrhea (antibiotic-associated, enteric feeding-induced, traveler's diarrhea) (2). In addition, due to its protective effect against the specific enterotoxins , *Saccharomyces boulardii* significantly reduces the number of relapses of *Clostridium difficile* infection.(8)

In view of the possible pathogenetic role of bacteria in inflammatory bowel disease (IBD), the rationale for employing a probiotic in this condition is to restore the unbalanced indigenous microflora, to inhibit the adverse effects of enteric pathogens and to counteract the inflammatory process by enhancing degradation of enteral antigens and reducing secretion of inflammatory mediators (9). In this respect *Saccharomyces boulardii* is, on theoretical grounds, an excellent candidate for a potential therapeutic role in IBD. We tested the efficacy of that probiotic agent in patients with active and inactive IBD (10,11)

PATIENTS AND METHODS

In the first study (10) 32 patients of both sexes, aged 23-49, with established diagnosis of Crohn's disease of ileum and/or colon in phase of remission (CDAI <150) for at least three months were randomly allocated to a six-month maintenance therapy either with mesalazine alone (500mg of sustained-release microgranules t.i.d.) or with the same mesalazine preparation 500mg b.i.d. plus *Saccharomyces boulardii* 500 mg o.d.. A clinical relapse was defined as CDAI >150 with an increase of 100 points over the baseline values for more than two weeks.

In the second, open-label study (11) 25 patients, aged 19-47, with a clinical flare-up of left-sided ulcerative colitis of mild to moderate degree, while on maintenance with mesalazine since at least three months, and with a history of poor tolerance to corticosteroids, were enrolled.

Saccharomyces boulardii 250 mg t.i.d. was added for four weeks to the ongoing mesalazine treatment. Clinical evaluation was performed before and after by means of Rachmilewitz's Activity Index, calculated on the basis of stool frequency, blood in the stools, general conditions, fever, abdominal pain, sedimentation rate and hemoglobin values. Only patients with a basal score

of 9 or higher were admitted to the study. Only a final score of 5 or less was considered a therapeutic success. In patients with clinical benefit sigmoidoscopy was performed to confirm the clinical remission.

RESULTS

In the first study, at the end of the six-month maintenance period, clinical relapses were observed in 37.5% of patients receiving mesalazine alone and in only 6.25% of subjects in the group treated with mesalazine plus *Saccharomyces boulardii* ($p = 0.04$ by Fisher's exact test).

In the second, pilot trial, the therapeutic success - endoscopically confirmed - was achieved in 17 patients (68% of cases on an intention-to-treat basis)

DISCUSSION

The rationale for employing probiotics in IBD treatment relies upon the postulated pathogenetic role of intestinal microflora. The mechanisms of action of *Saccharomyces boulardii*, a probiotic with a different taxonomic profile, being a yeast and not a bacterium, may explain the beneficial effects observed in the course of our studies. When combined with mesalazine the probiotic appears to significantly reduce relapses during maintenance treatment of inactive Crohn's disease. Whether *Saccharomyces boulardii* can be effective in this setting also if employed alone is still to be determined. As for its possible therapeutic role in selected cases of mild-to-moderate ulcerative colitis, our preliminary data are encouraging, but further, controlled studies are needed.

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Probiotici

Il termine probiotico è riservato a quei microrganismi che si dimostrano in grado, quando vengono ingeriti, di esercitare funzioni benefiche per l'uomo.

Da un punto di vista etimologico probiotico (pro bios) significa "a favore della vita" e quindi per definizione in antagonismo con gli antibiotici.

I microrganismi per essere considerati probiotici devono soddisfare i seguenti requisiti:

- essere di provenienza intestinale (normali componenti della microflora dell'intestino umano in condizioni di salute)
- essere attivi e vitali alle condizioni ambientali che sono presenti a livello intestinale
- essere sicuri per l'impiego dell'uomo senza causare effetti collaterali specialmente in pazienti debilitati o immunosoppressi
- essere resistenti ad un basso Ph, al succo gastrico, alla bile e al succo pancreatico (sopravvivere nel tratto digestivo)
- essere in grado di persistere almeno temporaneamente nell'intestino umano
- produrre sostanze antagonizzanti i patogeni

Per alimenti probiotici si intendono quegli alimenti, generalmente fermentati, che contengono, in numero sufficientemente elevato, microrganismi vivi ed attivi in grado di raggiungere l'intestino ed esercitare un'azione sulla microflora intestinale mediante colonizzazione diretta.

Sono quindi alimenti in grado di promuovere e migliorare le funzioni di equilibrio fisiologico dell'organismo, attraverso un insieme di effetti aggiuntivi alle normali attività nutrizionali.

I vantaggi di questi alimenti sono:

- stimolare la formazione di anticorpi e aumentare la risposta immunitaria
- alleviare i sintomi da malassorbimento del lattosio
- aumentare le resistenze naturali nei confronti delle infezioni intestinali
- migliorare i processi digestivi
- legare e disattivare agenti patogeni
- contrastare la stitichezza, la diarrea, la flatulenza

La quantità di batteri probiotici necessaria per ottenere una temporanea colonizzazione intestinale è non meno di 10^9 cellule vive per giorno per persona adulta.

Un latte fermentato probiotico deve avere alla scadenza una concentrazione di batteri lattici probiotici vivi non inferiore di 10^7 / g . Per mantenere equilibri microbici al livello intestinale e contribuire ad uno stato di benessere generale, occorre che il consumatore ingerisca con alimenti probiotici 10^9 cellule vive per giorno.

Concentrazioni di probiotici di 10^5 /g non sono sufficienti per ottenere una colonizzazione idonea.

Concentrazione di 10^6 /g hanno un effetto dubbio.

I probiotici sono costituiti essenzialmente dai cosiddetti " fermenti lattici" (Lactic Acid Bacteria), batteri anaerobi in grado di produrre acido lattico a partire da differenti substrati dietetici: Bacteroides, Bifidobacteria, Eubacteria, Peptostreptococcus e Fusobacteria.

I più frequentemente utilizzati sono i Lactobacilli, i Bifidobacteria ed alcuni ceppi di Streptococcus.

In commercio si trovano molti prodotti arricchiti con questi preziosi batteri ma quello che ha colpito di più l'attenzione del consumatore è sicuramente lo yogurt.

Oggi è possibile trovarlo in tutte le "forme": da bere, cremoso, naturale, alla frutta, ai cereali.

Che cos'è lo yogurt?

E' il prodotto che si ottiene mantenendo a 40-45 °C il latte, intero o scremato, addizionato con colture pure di Lactobacillus Bulgaricus e Streptococcus Thermophilus o generici fermenti lattici vivi.

L'errore più frequente è quello di confondere questi batteri con i probiotici.

I microrganismi contenuti nello yogurt normale infatti vengono aggrediti dai succhi gastrici e solo in piccola parte arrivano a destinazione, quelli probiotici invece hanno una maggiore resistenza e quindi riescono ad arrivare vivi nel tratto terminale dell'intestino dove svolgono le loro azioni benefiche.

I batteri lattici possono essere considerati batteri "buoni" in quanto sono coinvolti positivamente in diversi processi produttivi riguardanti soprattutto il settore agroalimentare e quello dietetico-farmaceutico. Hanno forma bastoncellare o coccoide. Fermentano gli zuccheri semplici (glucosio, lattosio, ecc..), dando origine ad acido lattico, acidificando quindi l'ambiente in cui si sono sviluppati.

Tra gli alimenti probiotici oltre lo yogurt ci sono lattici fermentati, lattici non fermentati, mousses, formule per l'infanzia, succhi, bevande, Cornflakes, formaggi freschi a pasta molle: crescenza, caciotta, ceddar, fiocchi di latte (il limite di tali prodotti è rappresentato dalla loro rapida deperibilità e che non possono essere consumati da coloro che sono portatori di deficit di lattasi).

Non sono ancora in commercio formaggi stagionati perchè il "bifido" riesce a sopravvivere solo ad una breve stagionatura a bassa temperatura.

Un'attenzione particolare va posta al momento dell'acquisto all'etichetta che deve riportare la quantità di batteri vivi, il genere di batterio presente, la scadenza (più è lontana maggiore è l'efficacia del prodotto), deve essere sincera e non ingannevole e deve comunicare al consumatore i benefici che derivano dall'acquisto del prodotto.

La confezione deve essere piccola, monodose. L'alimento deve essere completamente consumato perchè se lasciato aperto rischia di perdere rapidamente buona parte dei fermenti a causa dell'ossidazione prodotta dal contatto con l'aria.

Questi prodotti devono essere conservati in frigo a bassa temperatura. Dopo 24 ore a temperatura superiore a 4 °C il prodotto conterrà batteri buoni ma morti, innocui ma poco utili.

Devono essere consumati freddi poiché la cottura è nemica dei batteri e a digiuno in quanto se lo stomaco è pieno i probiotici saranno esposti per più

tempo all'attacco degli acidi gastrici e avranno minore possibilità di arrivare vivi nell'intestino.

E' sufficiente un vasetto di yogurt o un bicchiere di latte probiotico per soddisfare il fabbisogno giornaliero.

Tra lo yogurt, il latte o la mousse non esiste una differenza qualitativa come alimento probiotico, sono tutti efficaci, ciò che differisce è l'acidità e la fluidità del prodotto.

Nello yogurt, più acido, i batteri hanno vita più breve, mentre più l'alimento è liquido e più velocemente raggiunge l'intestino.

Sono oggi reperibili prodotti specificatamente designati per portare, in forma di cibo, dosi efficaci di specifici ceppi di probiotici con documentato effetto favorevole sulla salute:

Yakult, Actimel, GEFILUS, infant formula Nestlè, Halsofil, Stonyfield Farms, Cottage Cheese.

Esempio dieta per donna in menopausa, adulto normopeso.

Prebiotics e New-foods

Alessandra Mizzoni

I prebiotici e new foods

Nelle società occidentali la frequenza di affezioni croniche legate allo stile di vita, quali le malattie cardiovascolari, l'obesità, l'osteoporosi, il diabete e alcune forme di cancro, è aumentata in modo considerevole durante l'ultimo decennio:

Al fine di ridurre il grado di diffusione di questi disturbi si è ritenuto utile l'introduzione di misure preventive, in primis i cambiamenti di stile di vita, fra queste, particolare interesse, va riposto nell'adeguamento delle abitudini alimentari.

Durante l'ultimo decennio è stato riconosciuto che il tratto gastrointestinale, in particolare la composizione e il metabolismo della flora batterica, influisce notevolmente sul benessere degli individui. Di conseguenza si è manifestato un interesse crescente verso alimenti e nutrimenti specifici che migliorano la salute intestinale e il benessere, questi sono, infatti, i prebiotici e i probiotici.

Che cosa sono i prebiotici?

- ◆ I prebiotici sono glicidi selezionati non digeribili che sopravvivono alla digestione nell'intestino tenue e raggiungono immutati l'intestino crasso, dove esercitano la loro azione specifica.
- ◆ Nel colon stimolano la crescita selettiva e l'attività di un numero limitato di batteri con l'obiettivo di migliorare la salute ed il benessere dell'organismo ospite, diventano nutrimento per i batteri e impediscono la crescita di ceppi batterici dannosi per l'organismo.

Quali sono le sostanze in grado di soddisfare tali criteri?

- ◆ Zuccheri a basso peso molecolare già presenti in natura (carciofi, cicoria, aglio, porri, ed in misura minore nei cereali).

Vengono considerati i più efficaci

- **FOS** frutto-oligosaccaridi
- **GOS** galatto-oligosaccaridi

Alcuni tipi di prebiotici sono:

- oligosaccardi
- alcuni tipi di fibre dietetiche
- alcuni zuccheri come lattulosio disaccaridi sintetici
- derivati dal latte vaccino
- alcuni derivati della soia potrebbero possedere qualità prebiotiche, inoltre sono capaci di indurre anche una riduzione della lipemia e di migliorare l'assorbimento del calcio magnesio e ferro.

L'aspetto più interessante dei prebiotici ed in modo particolare del frutto-oligosaccaride è di contribuire a ridurre l'assorbimento al livello intestinale dei grassi e degli zuccheri e quello di stimolare in modo specifico la crescita dei bifidobatteri, aumentando pertanto la funzione protettiva e depurativa delle mucose intestinali.

Che cosa sono i bifidobatteri?

- ◆ Sono specie batteriche con effetto benefico sulla flora intestinale: hanno un impatto positivo sul processo digestivo e hanno attraverso la loro azione metabolica, effetti immunostimolanti e anticancerogeni

EFFETTI FISIOLÓGICI MAGGIORMENTE EVIDENZIATI E VANTAGGI:

1. Attivazione del metabolismo glucidico da parte della flora intestinale, aumento delle resistenze contro i patogeni
2. Assenza di idrolisi da parte dei microrganismi del cavo orale, protezione della carie
3. Assenza di effetti sul livello della glicemia, potenzialmente utile nei diabetici
4. Stimolo aspecifico del sistema immunitario, resistenza alle infezioni
5. Modulazione del metabolismo carcinogenico, diminuito rischio di cancro
6. Riduzione della sintesi epatica di colesterolo-VLDL e trigliceridi plasmatici, diminuito rischio di cardiopatia
7. Aumento dell'assorbimento del calcio e magnesio, diminuito rischio di osteoporosi

Vista l'importanza, gli effetti maggiormente evidenziati ed i vantaggi dei prebiotici sul nostro organismo andiamo ora ad analizzare in pratica un esempio di dieta normocalorica

di un adulto e vediamo quali tipi di alimenti sono necessari per apportare una quantità discreta di prebiotici:

Colazione.	
Yogurt parz screm arricchito con bifidobatteri	g 160
Fiocchi d'avena o biscotti ricchi in fruttosio	g 40
Miele	g 15
Spuntino matt.	
Frutta ricca in fibre (mele, pere, banane, uva) Oppure bevande di frutta arricchite con fibre	g 150 o un bicchiere
Pranzo	
Pasta o riso condite con pomodoro fresco con Aglio, cipolla, carote	g 110
Pollo alla piastra	g 160
Pane	g 70
Verdure (broccoli, spinaci, cicoria cavolfiori)	g 200
Olio di oliva	g 10
Frutta	g 150
Merenda	
Frutta o un bicchiere di una bevanda arricchita con fibre	g 150
Cena	
Pane	g 100
Minestrone con verdure (broccoli spinaci fagiolini ecc.)	g 150
Mozzarella o altri tipi di formaggi con fermenti lattici vivi	g 90
Verdure (asparagi, carciofi)	g 200
Olio	g 10
Spuntino serale	
Frutta o un bicchiere di una bevanda arricchita con fibre	g 150

Calorie	2245
Proteine	g 106
Lipidi	g 71
Glicidi	g 315
Oligosaccaridi	g 95
Fibra	g 38

Molta importanza hanno assunto in questi ultimi anni i New-foods o Alimenti funzionali.

Che cosa sono gli Alimenti funzionali?

- Gli alimenti funzionali sono quegli alimenti che oltre al loro valore nutrizionale di base, contengono ingredienti naturali in grado di recare benefici fisiologici a chi li consuma. Hanno un effetto positivo sulla salute e sul benessere della persona e contribuiscono a ridurre il rischio di sviluppo di numerose malattie croniche.

Esempi di Functional foods innovativi	
Alimento	Beneficio funzionale
Latti fermentati e yogurt con colture prebiotiche	Miglioramento della flora microbica intestinale
Margarina, yogurt, formaggio da spalmare a base di grassi vegetali	Riduzione dell'assunzione di colesterolo
Latte e uova arricchiti in acidi grassi omega-3	3 – 4 uova la settimana possono fornire la stessa quantità di acidi grassi omega-3, secondo i livelli raccomandati per la riduzione del rischio di infarto
Cereali per la colazione arricchiti in acido folico	Riduzione del rischio della nascita di bambini con spina bifida
Pane, barrette di Muesli arricchiti in isoflavoni	Riduzione del rischio di cancro e malattie cardiovascolari
Succhi di frutta vitaminizzati	Arricchiti di vitamine idrosolubili e di sali minerali possono essere utili nel caso che la dieta sia povera di vegetali naturalmente ricchi di queste sostanze

Quindi per il consumatore deve avere molta importanza l'etichettatura di un prodotto alimentare

- un importante funzione di tutela, che lo informa sul prodotto che sta acquistando e che gli consente di scegliere quello che è maggiormente rispondente alle proprie esigenze

Sopra l'etichetta di un qualsiasi prodotto alimentare deve essere riportato:

- **ELENCO DEGLI INGREDIENTI**

Elenco degli ingredienti in ordine di quantità decrescente
Quest'elenco fornisce informazioni utili per individuare la presenza di sostanze più o meno gradite e per effettuare un confronto fra prodotti analoghi.

YOGURT E LATTI FERMENTATI - PROBIOTICI E PREBIOTICI

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Gli yogurt e i lattici fermentati occupano un ruolo importante nella nostra alimentazione non solo per le proprietà nutritive ma anche per gli effetti benefici che possono esercitare sull'organismo. Questi effetti sono attribuiti alla presenza di specifici microrganismi vivi che interagiscono con la flora batterica intestinale.

Il primo a porre l'attenzione sugli effetti dei microrganismi dei lattici fermentati fu Elia Metchnikoff, quasi un secolo fa. Molte sono le ricerche che si sono sviluppate nel tempo sui lattici fermentati e se alcune proprietà non hanno trovato conferma, altre sono state dimostrate in maniera ineccepibile, mentre per taluni effetti si cerca ancora una verifica convincente. Le informazioni ottenute sono suddivisibili in due sezioni una sull'effetto probiotico e l'altra sulla dipendenza dai prebiotici affinché alcuni microrganismi possano mantenersi vivi e vitali su di un substrato lattico, in entrambe valido è il consenso sui risultati ottenuti.

1. **Attività probiotiche certe** I lattici fermentati danno la possibilità agli individui intolleranti al lattosio di non avere quegli effetti indesiderati che provoca, invece, l'ingestione del latte come tale. Questo aspetto è stato il primo ad essere dimostrato scientificamente. L'intolleranza al lattosio consiste nell'incapacità di scindere il disaccaride nei due monosaccaridi che lo compongono, cioè glucosio e galattosio, a causa della carenza della lattasi. Ciò provoca un transito veloce dello zucchero nel piccolo intestino, dove avviene anche accumulo di acqua che determina sensazioni di malessere. Una volta giunto nel colon il lattosio viene metabolizzato dai batteri anaerobi, i cui prodotti di fermentazione risultano irritanti per la mucosa intestinale, originando crampi, flatulenza e diarrea. Nel bambino l'intolleranza al lattosio è dovuta molto spesso a deficit di lattasi intestinale secondario ad enteropatia; nell'adulto è più che altro un problema primario. Infatti, nell'uomo, l'attività lattasica intestinale è massima alla nascita e comincia a diminuire dopo lo svezzamento, fino a giungere nell'adulto ad un tasso residuo del 10%. La mancanza di lattosio è anche variabile a seconda delle diverse etnie. Lo yogurt, i lattici fermentati e il latte non fermentato

ma con aggiunta di colture batteriche vive e vitali consentono di inserire nell'alimentazione degli intolleranti i prodotti derivati del latte. I prodotti fermentati hanno un tempo di transito oro-cecale più lento: ciò consente alla lattasi intestinale (presente in percentuale minore) un tempo d'azione maggiore. Questo permette anche agli stessi batteri lattici di metabolizzare parte del lattosio, alleviando i sintomi della maldigestione. E' bene ricordare che ciò si verifica solo se il prodotto in questione contiene cellule batteriche vive e attive, poiché i trattamenti termici possono ridurre considerevolmente l'attività lattasica batterica. La colonizzazione dei batteri lattici nel tratto intestinale dipende dalla dose effettiva, definita come la quantità di cellule batteriche vive e vitali che devono essere somministrate all'individuo affinché si compia l'azione probiotica. La capacità di permanere nell'intestino è detta invece capacità di colonizzazione. La colonizzazione nell'intestino dei ceppi batterici ingeriti è possibile solo se questi riescono a superare le varie barriere dell'apparato gastrointestinale, tra cui il succo gastrico e i sali biliari. I diversi ceppi hanno differenti comportamenti, comunque anche quelli che superano la barriera gastrica non riescono a colonizzare se ingeriti in basse dosi, infatti la flora intestinale di un individuo sano è un ambiente ecologicamente stabile, che si oppone all'insediamento di nuovi batteri (cosiddetta "resistenza alla colonizzazione"). Studi realizzati finora *in vitro* e *in vivo* hanno permesso di stilare speciali tabelle con le dosi giornaliere raccomandate.

2. **Proprietà prebiotiche** Le ricerche nel settore dei carboidrati prebiotici si sono molto ampliate negli ultimi dieci anni. Alcuni carboidrati non digeribili (in particolare l'inulina e i fruttoligosaccaridi: FOS) hanno dimostrato possedere azione prebiotica. Cioè questi composti - estratti da fonti vegetali particolarmente ricche, quali cicoria e topinambur - una volta ingeriti non vengono digeriti e sono totalmente e rapidamente fermentati dalla microflora intestinale. L'effetto positivo più rilevante provocato dai prebiotici è l'aumento delle cariche di bifidobatteri nella microflora fecale. E' stato visto che la somministrazione di 15 grammi al giorno di inulina in soggetti sani per 15 giorni è in grado di modificare l'ecosistema intestinale. I bifidobatteri passano dal 20 al 70% della flora microbica, a scapito dei comuni ceppi patogeni. Tuttavia persistono ancora dei dubbi riguardo all'azione dei prebiotici sul

metabolismo lipidico (riduzione di colesterolo e trigliceridi) e sulla modifica dei markers di cancerogenesi del colon. Un gruppo di esperti nel 1999 ha stilato un documento sull'effetto dei probiotici, il quale evidenziava, tra l'altro, che l'inulina e i FOS hanno la capacità di regolarizzare la funzionalità intestinale e che l'inulina sembra poter migliorare l'assorbimento di minerali. Comunque gli stessi studiosi specificano che sono necessari ulteriori studi sull'uomo per una completa dimostrazione di questi effetti.

Se il mondo scientifico si mostra prudente nell'attribuire virtù ai prebiotici e ai probiotici, il settore farmaceutico ha già lanciato sul mercato un'ampia varietà di integratori contenenti microrganismi associati a sostanze prebiotiche. A tale proposito sarebbe opportuna un'accurata informazione dei consumatori circa la reale efficacia di tali prodotti.

Come si evince dalle tabelle di seguito riportate, oggi in commercio si trova una vasta gamma di derivati del latte, in particolare yogurt e latti fermentati. Basta osservare il banco frigo di un supermercato per rendersi conto di quanto sia ampio l'assortimento di tali prodotti. Ognuno vanta le proprie differenti caratteristiche (microrganismo probiotico, composto prebiotico, aggiunta di frutta, etc.). Le confezioni sono di vario tipo, dal tradizionale vasetto-bicchierino alla più moderna bottiglietta, le denominazioni di vendita sono frutto di accurate strategie di marketing e il prodotto offerto ha consistenze diverse, dal liquido al compatto. Ma il consumatore come può regolarsi nella scelta? Può preferire una marca conosciuta, essere attirato da una confezione accattivante, ma molto spesso è la pubblicità che lo guida.

Appena dieci anni fa l'assortimento si limitava a tre o quattro varianti di prodotti (yogurt intero o magro, o aromatizzato con qualche gusto di frutta), ed anche le marche erano ben poche. Con il passare degli anni il settore dell'alimentazione ha acquisito una grande importanza, sia dal punto di vista dello sviluppo sul mercato, sia sulla scienza dell'alimentazione. Le ricerche scientifiche si sono concentrate sugli effetti degli alimenti, ma molto spesso i risultati ottenuti da queste ricerche vengono strumentalizzati per indirizzare i consumatori verso determinati prodotti, attribuendo degli effetti salutistici non ancora dimostrati sull'uomo.

La stessa cosa sta succedendo per i lattici fermentati probiotici e prebiotici. Molti consumatori si affidano a questi prodotti senza sapere che i microrganismi probiotici riescono ad esplicare i loro effetti solo se ingeriti in determinate dosi e con regolarità, e che molti presunti effetti benefici necessitano di ulteriori studi scientifici sull'uomo per essere dimostrati, in quanto la loro azione è nota solo *in vitro*. Le uniche attività probiotiche finora provate scientificamente sono quella di aiutare la digestione a chi è intollerante al lattosio, e quella di riuscire a colonizzare l'intestino (sempre se assunti in dosi sufficienti e con regolarità, e che resistano alle condizioni che si realizzano nel tubo digerente). Riguardo all'azione prebiotica insita in alcuni di questi lattici fermentati, si è visto che certi oligosaccaridi sono realmente substrati per i batteri probiotici, ma anche qui si deve ricordare che le quantità da assumere rivestono una certa importanza. Pertanto è doveroso dire che sono ancora necessari studi approfonditi sull'uomo per attribuire effetti salutistici a tali prodotti.

I consumatori si ritrovano così davanti ad una ricchissima offerta di nuovi prodotti dei quali non conoscono né l'esatta definizione, né il corretto utilizzo. Una completa informazione del consumatore è quindi essenziale per evitare che egli si affidi a questi prodotti con eccessive aspettative. L'ingestione di consistenti quantità di microrganismi probiotici può provocare modificazioni nell'assetto della flora intestinale dell'organismo.

Un buono stato di salute è strettamente legato a corrette abitudini alimentari, che rischiano oggi di essere modificate, in modo non del tutto prevedibile, dalla varietà di prodotti presenti sul mercato. Scelte sbagliate possono alterare le abitudini, e quindi riflettersi sullo stato di nutrizione di alcune fasce di consumatori, con particolare attenzione per quelli che sembrano preferire questi alimenti: le donne (soprattutto le adolescenti) e gli anziani.

Yogurt e Latti fermentati in commercio

Azienda produttrice	Denominazione di vendita	Denominazione commerciale	Confezione	Lista ingredienti	Tabella nutrizionale			
					Valore energetico	Proteine	Carboidrati	Grassi
BOTTIGLIA								
Yomo	Latte parzialmente scremato fermentato con 8 fermenti vivi e attivi	ABC bianco latte fermentato con 8 fermenti Vivi e Attivi	6 x 90g	Latte parzialmente scremato 90%, zucchero, oligofruztosio, amido di tapioca, St. thermophilus, B. breve, B. infantis, B. longum, Lb. acidophilus, Lb. plantarum, Lb. casei, Lb. bulgaricus	79 kcal/331 kJ	2,7 g	12 g	1,9 g
	Latte parzialmente scremato fermentato con 8 fermenti vivi e attivi con succo di mirtilli e ribes	ABC mirtilli e ribes latte fermentato con 8 fermenti Vivi e Attivi	6 x 90g	Latte parzialmente scremato 86,6%, zucchero, succo concentrato di mirtilli 3,2%, oligofruztosio, succo concentrato di ribes 0,8%, amido di tapioca, St. thermophilus, B. breve, B. infantis, B. longum, Lb. acidophilus, Lb. plantarum, Lb. casei, Lb. bulgaricus.	83 kcal/352 kJ	2,7 g	13 g	1,9 g
	Latte parzialmente scremato fermentato con 8 fermenti vivi e attivi con succo di agrumi di Sicilia	ABC agrumi di Sicilia latte fermentato con 8 fermenti Vivi e Attivi	6 x 90g	Latte parzialmente scremato 83%, succo concentrato di agrumi di Sicilia 8%, zucchero, oligofruztosio, amido di tapioca, St. thermophilus, B. breve, B. infantis, B. longum, Lb. acidophilus, Lb. plantarum, Lb. casei, Lb. bulgaricus.	89 kcal/375 kJ	2,7 g	14,5 g	1,8 g
	Latte parzialmente scremato fermentato con 8 fermenti vivi e attivi con estratto naturale di bacche di vaniglia	ABC vaniglia latte fermentato con 8 fermenti Vivi e Attivi	6 x 90g	Latte parzialmente scremato 90%, zucchero, oligofruztosio, amido di tapioca, estratto naturale di bacche di vaniglia 0,2%, St. thermophilus, B. breve, B. infantis, B. longum, Lb. acidophilus, Lb. plantarum, Lb. casei, Lb. bulgaricus.	79 kcal/331 kJ	2,7 g	12 g	1,9 g
	Latte scremato allo 0,1% di grassi fermentato con 8 fermenti vivi e attivi, con ananas	ABC 0,1% di grassi ananas latte fermentato con 8 fermenti Vivi e Attivi	6 x 90g	Latte magro 85,6%, zucchero, preparazione di frutta 6% (di cui succo d'ananas 50% pari a 3% sul prodotto finito, estratti di ananas gambo e frutto 8% pari a 0,48% sul prodotto finito, aromi), oligofruztosio, amido di tapioca, St. thermophilus, B. breve, B. infantis, B. longum, Lb. acidophilus, Lb. plantarum, Lb. casei, Lb. bulgaricus	69 kcal/293 kJ	2,6 g	13,7 g	0,1 g
	Latte scremato allo 0,1% di grassi fermentato con 8 fermenti vivi e attivi, con arancia, carota, limone e vitamine	ABC 0,1% di grassi ACE latte fermentato con 8 fermenti Vivi e Attivi	6 x 90g	Latte magro 81,9%, zucchero, preparazione di frutta 10% (di cui succo di arancia 9,3% pari a 0,9% sul prodotto finito, succo di carota 5,2% pari a 0,5% sul prodotto finito, succo di limone 0,1% pari a 0,01% sul prodotto finito, vitamina C, provitamina A (β-carotene), vitamina E, aromi), oligofruztosio, amido di tapioca, St. thermophilus, B. breve, B. infantis, B. longum, Lb. acidophilus, Lb. plantarum, Lb. casei, Lb. bulgaricus	71 kcal/301 kJ	2,5 g	14,3 g	0,1 g
Danone	Bevanda a base di latte scremato zuccherato fermentato (fermenti dello yogurt e Lb. casei) dolcificato con edulcoranti	Actimel L. casei imunitass 0,1% di grassi	6x100g	Latte scremato, latte scremato concentrato ricostituito, fibre solubili (inulina), destrosio, stabilizzante: pectina; fermenti dello yogurt e Lactobacillus casei; edulcoranti: aspartame, acelsulfame k; aromi	33 kcal/140 kJ	2,5 g	14,3 g	0,1 g
	Bevanda a base di latte zuccherato fermentato (fermenti dello yogurt e Lb. casei)	Actimel L. casei imunitass	6x100g	Latte intero, latte scremato, zucchero liquido 14,5%, destrosio, fermenti lattici specifici dello Yogurt e Lb. Casei.	83 kcal/350 kJ	2,8 g	4,9 g	1,6 g
	Bevanda a base di latte fermentato zuccherato, preparazione di frutta e Lactobacillus casei	Actimel L. casei imunitass	6x100g	Latte intero, latte scremato, zucchero liquido 13,8%, preparazione di frutta 5% (arancia 43%, zucchero, sciroppo di glucosio, addensanti: gomma di guar, farina di semi di carrube, pectina; aromi), destrosio, fermenti lattici specifici dello Yogurt e Lb. Casei.	88 kcal/373 kJ	2,7 g	16 g	1,5 g

	Bevanda a base di latte fermentato zuccherato, preparazione di frutta e Lactobacillus casei	Actimel L. casei imunitass	6x100g	Latte intero, latte scremato, zucchero liquido 13,8%, preparazione di frutta 5% (fragole 42%, zucchero, addensante: E1442, aromi), destrosio, fermenti lattici specifici dello yogurt, Lactobacillus casei.	88 kcal/373 kJ	2,7 g	15,9 g	1,5 g
Danone	Bevanda a base di latte fermentato zuccherato, preparazione dolciaria ai succhi di frutta e Lactobacillus casei	Actimel L. casei imunitass MultiFrutti	6x100g	Latte intero, latte scremato, zucchero liquido 13,8%, preparazione dolciaria ai succhi di frutta 5% (zucchero, succhi di frutta a base di succhi concentrati 42% (ananas, pesca, arancia, fragola); addensante: E1442, pectina; aromi), destrosio, fermenti lattici specifici dello yogurt, Lactobacillus casei.	88 kcal/373 kJ	2,7 g	16,0 g	1,5 g
Fattoria Scaldasole	Bevanda a base di latte fermentato magro con Lactobacillus casei bioVitalitis	BioVitalitis magro	6x90g	Latte scremato* e latte parzialmente scremato* fermentati con Lactobacillus casei bioVitalitis, fermenti lattici vivi, zucchero di canna* * da agricoltura biologica	64 kcal/273 kJ	2,98 g	11,65 g	0,65 g
Parmalat	Latte scremato zuccherato fermentato con S. thermophilus, L. bulgaricus, L. acidophilus, L. paracasei, Bifidobacterium Bb12, con pappa reale e vitamine	KYR Principia Bianco e Pappa Reale	4x110g	Latte scremato, acqua, zucchero, fibra alimentare solubile (fruttoligosaccaridi), maltodestrine, stabilizzante: pectina, pappa reale (0,2%), vitamine (B1, B2, B6, PP), aromi, S. thermophilus, L. bulgaricus, L. acidophilus, L. paracasei, Bifidobacterium Bb12	59kcal/245 kJ	2,4 g	12,0 g	0,1 g
	Latte scremato fermentato con S. thermophilus, L. bulgaricus, L. acidophilus, L. paracasei, Bifidobacterium Bb12, con agrumi, ginseng, fibra e vitamine	KYR Principia Agrumi e Ginseng	4x110g	Latte scremato, acqua, zucchero, succo di agrumi (arancia, limone, pompelmo) (5%), fibra alimentare solubile (fruttoligosaccaridi), maltodestrine, stabilizzante: pectina, estratto di ginseng (0,1%), vitamine (B1, B2, B6, PP), aromi, S. thermophilus, L. bulgaricus, L. acidophilus, L. paracasei, Bifidobacterium Bb12	59kcal/245 kJ	2,4 g	12,0 g	0,1 g
Latteria Sociale Merano	Yogurt parzialmente scremato alla frutta	Bella vita carota&arancia probiotic drink	200g	Yogurt parzialmente scremato con fermenti probiotici (Lactobacillus acidophilus LA5), zucchero, arancia 5%, carota 2%, aromi	77 kcal/324 kJ	2,9 g	13,3 g	1,3 g
	Yogurt parzialmente scremato alla frutta	Bella vita banana (o fragola) probiotic drink	200g	Yogurt parzialmente scremato con fermenti probiotici (Lactobacillus acidophilus LA5), zucchero, banana (o fragola) 6%, aromi	77 kcal/324 kJ	2,9 g	13,3 g	1,3 g
Mila	Bevanda a base di latte fermentato	Benessere Rinforzo Bianco	6x90g	Latte parzialmente scremato fermentato con Streptococcus termophilus, Bifidobacterium BB12 e Lactobacillus bulgaricus, zucchero, estratti naturali 0,03% (Foeniculum vulgare, Achillea millefolium, Taraxacum officinale), aromi	72 kcal/305 kJ	3,0 g	12,1 g	1,3 g
	Bevanda a base di latte fermentato	Benessere Rinforzo arancia	6x90g	Latte parzialmente scremato fermentato con Streptococcus termophilus, Bifidobacterium BB12 e Lactobacillus bulgaricus, zucchero, arancia 2,6%, limone 0,16%, estratti naturali 0,03% (Foeniculum vulgare, Achillea millefolium, Taraxacum officinale), aromi, colorante: β -carotene	76 kcal/320 kJ	3,0 g	13,0 g	1,3 g
Muller	Latte fermentato con Bifidobacterium Lactis (BB12) e Lactobacillus Acidophilus (La-5)	Crema Actidrink con Bifidobacterium Lactis (BB12) e Lactobacillus Acidophilus (La-5) Bianco	6x100g	Latte scremato, latte intero, preparazione dolciaria (acqua, sciroppo di glucosio, amido modificato, aroma, correttore di acidità: citrati di sodio), zucchero, fibra solubile (inulina), fermenti lattici, Lb. acidophilus (La-5), Bifidobacterium lactis (BB12).	66 kcal/281 kJ	2,9 g	9,8 g	1,4 g
	Latte fermentato al gusto di vaniglia con Bifidobacterium Lactis (BB12) e Lactobacillus Acidophilus (La-5)	Crema Actidrink con Bifidobacterium Lactis (BB12) e Lactobacillus Acidophilus (La-5) al gusto di vaniglia	6x100g	Latte scremato, latte intero, preparazione gusto vaniglia 18% (con colorante: beta carotene e con aroma), zucchero, fibra solubile (inulina), fermenti lattici, Lb. acidophilus (La-5), Bifidobacterium lactis (BB12).	77 kcal/325 kJ	2,5 g	13 g	1,3 g

	Latte fermentato al gusto di fragola con Bifidobacterium Lactis (BB12) e Lactobacillus Acidophilus (La-5)	Crema Actidrink con Bifidobacterium Lactis (BB12) e Lactobacillus Acidophilus (La-5) al gusto di fragola	6x100g	Latte scremato, latte intero, preparazione alla fragola 15% (di cui fragole 36%, aroma), zucchero, fibra solubile (inulina), fermenti lattici, Lb. acidophilus (La-5), Bifidobacterium lactis (BB12).	84 kcal/357 kJ	2,6 g	14,6 g	1,4 g
Nestlé	Latte fermentato zuccherato	Lc1 go	6x90g	Latte (1,1% di grasso), zucchero (9,2%), fermenti lattici vivi (fra cui Lb. Johnsonii La1), stabilizzante pectine, aromi, antiossidante ascorbato di calcio, coloranti (E150d, E110).	70 kcal/297 kJ	2,7 g	12,8 g	0,9 g
	Latte fermentato con frutta	Lc1 go multifruit	6x90g	Latte (1,1% di grasso), zucchero, fermenti lattici vivi (fra cui Lb. Johnsonii La1), polpa e succo di frutta 1,8% (mango, arancia, pesca, maracuja), succo di carota (1,8%), destrosio, stabilizzante pectine, antiossidante ascorbato di calcio, aromi.	70 kcal/297 kJ	2,6 g	12,9 g	0,9 g
	Bevanda a base di latte fermentato a ridotto contenuto calorico all'ananas, con fruttosio ed edulcoranti	Lc1 go 0,1% di grassi ananas	6x88g (85ml)	Latte scremato, polpa e succo di ananas 2,7%, fruttosio, succo d'uva, proteine del latte e lattosio, stabilizzante pectine, edulcoranti aspartame ed acelsulfame K, fermenti lattici vivi (fra cui Lactobacillus johnsonii La 1), antiossidante ascorbato di calcio, aromi, coloranti caroteni.	40 kcal/169 kJ	3,0 g	6,7 g	0,1 g
	Bevanda a base di latte fermentato a ridotto contenuto calorico alla fragola, con fruttosio ed edulcoranti	Lc1 go 0,1% di grassi fragola	6x88g (85ml)	Latte scremato, polpa e succo di fragola 2,2%, fruttosio, succo d'uva, proteine del latte e lattosio, stabilizzante pectine, edulcoranti aspartame ed acelsulfame K, fermenti lattici vivi (fra cui Lactobacillus johnsonii La 1), antiossidante ascorbato di calcio, aromi, coloranti cocciniglia.	40 kcal/169 kJ	3,0 g	6,7 g	0,1 g

VASETTO

Danone	Latte fermentato con Lactobacillus bulgaricus, Streptococcus thermophilus e Bifidobacterium	Activia con Bifidus Attivo Essensis Naturale	2x125g	Latte fermentato con Lb. bulgaricus, St. thermophilus e Bifidobacterium.	72 kcal/304 kJ	4,2 g	5,1 g	3,5 g
	Latte fermentato zuccherato e preparazione di frutta	Activia con Bifidus Attivo Essensis Fragola	2x125g	Latte fermentato con Lb. bulgaricus, St. thermophilus e Bifidobacterium, preparazione di frutta 17% (fragole 65%, zucchero, sciroppo di glucosio, aromi), zucchero 3,9%.	102 kcal/431 kJ	3,5 g	13,9 g	3,2 g
	Latte fermentato zuccherato e preparazione di frutta	Activia con Bifidus Attivo Essensis Prugna	2x125g	Latte fermentato con Lb. bulgaricus, St. thermophilus e Bifidobacterium, preparazione di frutta 17% (prugne secche reidratate 65%, zucchero, aromi), zucchero 3,9%.	104 kcal/440 kJ	3,5 g	14,5 g	3,2 g
	Latte scremato fermentato con Lactobacillus bulgaricus, Streptococcus thermophilus e Bifidobacterium	Activia con Bifidus Attivo Essensis 0,1% di grassi Naturale	2x125g	Latte scremato fermentato con Lb. bulgaricus, St. thermophilus e Bifidobacterium.	49 kcal/206 kJ	5 g	6,1 g	0,1 g
	Latte scremato fermentato e preparazione di frutta dolcificata con fruttosio e edulcoranti	Activia con Bifidus Attivo Essensis 0,1% di grassi Ananas	2x125g	Latte scremato fermentato con Lb. bulgaricus, St. thermophilus e bifidobacterium, preparazione di frutta 13% (ananas 65%, sciroppo di fruttosio, oligofruttosio, addensanti: E1422, pectina, E412; edulcoranti: aspartame 0,111%, acelsulfame K 0,057%; aromi).	53 kcal/227 kJ	4,3 g	7,9 g	0,1 g
	Latte fermentato zuccherato e preparazione di frutta e cereali	Activia con Bifidus Attivo Essensis Fibre BiancoCereali	2x125g	Latte fermentato con Lb. bulgaricus, St. thermophilus e Bifidobacterium, preparazione di frutta e cereali 17% (zucchero, mele 15%, succo d'uva concentrato, oligofruttosio, fiocchi di grano, orzo e avena 5,5%, crusca 1%, addensanti: pectina, aromi), zucchero 3,9%.	109 kcal/461 kJ fibra: 1,7 g	3,6 g	15,5 g	3,3 g

	Latte fermentato zuccherato e preparazione di frutta e cereali	Activia con Bifidus Attivo Essensis Fibre kiwiCereali	2x125g	Latte fermentato con Lb. bulgaricus, St. thermophilus e Bifidobacterium, preparazione di frutta e cereali 17% (kiwi 50%, zucchero, oligofruttosio, sciroppo di isoglucosio, fiocchi di grano, avena e orzo 4,5%, addensanti: E412, E415; aromi), zucchero 3,9%.	103 kcal/435 kJ fibra alimentare: 1,7 g	3,5 g	13,8 g	3,3 g
Fattoria Scaldasole	Yogurt magro e latte fermentato con preparato di albicocche e maracuja	Lactobacillus casei Biovitalitis albicocche e maracuja	500g	Yogurt magro grassi inferiori 1%* (latte, fermenti lattici vivi e vitali: Lactobacillus bulgaricus e Streptococcus termophilus), zucchero*, albicocche* e maracuja* (7,4%), latte fermentato con Lactobacillus casei (4%), amido di mais ceroso, aromi naturali * da agricoltura biologica	80 kcal/341 kJ	3,80 g	14,60 g	0,75 g
	Yogurt magro e latte fermentato con preparato di arance bionde e limoni	Lactobacillus casei Biovitalitis arance bionde e limoni	500g	Yogurt magro grassi inferiori 1%* (latte, fermenti lattici vivi e vitali: Lactobacillus bulgaricus e Streptococcus termophilus), zucchero*, succo di arance bionde* e limoni* (6,3%), latte fermentato* con Lactobacillus casei (4%), amido di mais ceroso, aromi naturali * da agricoltura biologica	82 kcal/347 kJ	3,80 g	15,00 g	0,75 g
	Yogurt magro e latte fermentato con preparato di mirtilli neri	Lactobacillus casei Biovitalitis mirtilli neri	500g	Yogurt magro grassi inferiori 1%* (latte, fermenti lattici vivi e vitali: Lactobacillus bulgaricus e Streptococcus termophilus), zucchero*, mirtilli neri* (7,3%), latte fermentato con Lactobacillus casei (4%), amido di mais ceroso, aromi naturali * da agricoltura biologica	81 kcal/344 kJ	3,80 g	14,80 g	0,75 g
	Yogurt magro e latte fermentato con preparato di uva, prugna, crusca e germe di grano	Lactobacillus casei Biovitalitis uva, prugna, crusca e germe di grano	500g	Yogurt magro grassi inferiori 1%* (latte, fermenti lattici vivi e vitali: Lactobacillus bulgaricus e Streptococcus termophilus), miele*, latte fermentato* con Lactobacillus casei (4%), succo concentrato d'uva* (3,9%), succo di mela*, prugne secche* (1,35%), amido di mais ceroso, crusca di frumento* (0,4%), germe di grano* (0,4%), fiocchi d'orzo*, aromi naturali * da agricoltura biologica	81 kcal/344 kJ	4,10 g	14,40 g	0,80 g
Granarolo	Yogurt magro alle fragoline di bosco con fermento probiotico LGG	Vivi vivo alle fragoline di bosco	2x125g	Yogurt magro (latte scremato, Str. thermophilus, Lb. bulgaricus), preparazione di frutta 17% (di cui fragoline di bosco 6,2% e fragole 5,8% nel prodotto finito), fibra alimentare solubile (frutto-oligosaccaridi) 2,4%, aromi, edulcorante: aspartame, Lactobacillus rhamnosus GG	46 kcal/194 kJ	4,2 g	6,2 g	0,1 g
Mila	Latte fermentato con fermenti lattici vivi	Benessere Regularis bianco	2x125g	Latte intero fermentato con fermenti lattici vivi: Streptococcus termophilus, Lactobacillus rhamnosus e Lactobacillus bulgaricus	72 kcal/299 kJ	3,9 g	5,0 g	4,0 g
	Latte fermentato con fermenti lattici vivi e preparazione di frutta	Benessere Regularis ananas (o fragola)	2x125g	Latte intero fermentato con fermenti lattici vivi: Streptococcus termophilus, Lactobacillus rhamnosus e Lactobacillus bulgaricus, preparazione di frutta 18% (ananas o fragola in media 48%, zuccheri, aromi)	94 kcal/396 kJ	3,2 g	12,9 g	3,3 g
Nestlè	Latte parzialmente scremato fermentato (con fermenti lattici vivi)	LC1 Bianco	2x125g	Latte parzialmente scremato, proteine del latte, fermenti lattici vivi: Lb. johnsonii (La1) e St. thermophilus.	56 kcal/237 kJ	5,4 g	5,3 g	1,5 g
	Latte parzialmente scremato fermentato (con fermenti lattici vivi) con preparazione ala fragola	LC1 Fragola	2x125g	Latte parzialmente scremato, preparazione alla fragola 15% (di cui fragola 10,5% sul prodotto finito, amido modificato, colorante cocciniglia), latte concentrato scremato, zucchero, crema di latte, proteine del latte, fermenti lattici vivi: Lb. johnsonii (La1) e St. thermophilus.	91 kcal/381 kJ	4,5 g	14,9 g	1,5 g
	Latte parzialmente scremato fermentato (con fermenti lattici vivi) con preparazione ai frutti di bosco	LC1 Frutti di bosco	2x125g	Latte parzialmente scremato, preparazione ai frutti di bosco 15% (frutti di bosco 9,6% sul prodotto finito, amido modificato), latte concentrato scremato, zucchero, crema di latte, proteine del latte, fermenti lattici vivi: Lb. johnsonii (La1) e St. thermophilus.	100 kcal/423 kJ	4,6 g	17 g	1,5 g

	Latte parzialmente scremato fermentato (con fermenti lattici vivi) con preparazione alla pesca e al gusto di vaniglia	LC1 Vaniglia pesca	2x125g	Latte parzialmente scremato, preparazione alla pesca e al gusto di vaniglia 15% (pesca 9,6% sul prodotto finito, amido modificato, colorante caroteni), latte concentrato scremato, zucchero, crema di latte, proteine del latte, fermenti lattici vivi: Lb. johnsonii (La1) e St. thermophilus.	100 kcal/421 kJ	4,6 g	16,9 g	1,5 g
Parmalat	Yogurt con Bifidobacterium Bp 12, L. acidophilus e fibra, arricchito con vitamine	Kyr Bianco	2x150g	Yogurt, latte fermentato con Bifidobacterium Bp12 e L. acidophilus, fibra alimentare solubile (fruttooligosaccaridi), vitamine (C, E, D3).	61 kcal/255 kJ	3,7 g	3,7 g	3,5 g
	Yogurt con Bifidobacterium Bp 12, L. acidophilus e fibra, arricchito con vitamine, con pappa reale	Kyr pappa reale	2x125g	Yogurt, zucchero, fibra alimentare solubile (fruttooligosaccaridi), pappa reale (0,2%), vitamine (C, E, D3), Bifidobacterium Bp12 e L. acidophilus.	95 kcal/397 kJ	3,5 g	9,6 g	4,7 g
	Yogurt con Bifidobacterium Bp 12, L. acidophilus e fibra, arricchito con vitamine, con ananas e the verde	Kyr Ananas & the verde	2x150g	Yogurt, preparazione di frutta (ananas 60% pari a 8% sul prodotto finito), zucchero, latte fermentato con Bifidobacterium Bp12 e L. acidophilus, fibra alimentare solubile (fruttooligosaccaridi), estratto di the verde (0,1%), vitamine (C, E, D3), aromi.	106 kcal/446 kJ	3,1 g	14 g	4,2 g
	Yogurt con Bifidobacterium Bp 12, L. acidophilus e fibra, arricchito con vitamine, con banana (o fragola)	Kyr Banana (o Fragola)	2x150g	Yogurt, preparazione di frutta (banana o fragola 60% pari a 8% sul prodotto finito), zucchero, latte fermentato con Bifidobacterium Bp12 e L. acidophilus, fibra alimentare solubile (fruttooligosaccaridi), vitamine (C, E, D3), aromi.	106 kcal/446 kJ	3,1 g	14 g	4,2 g
	Yogurt con Bifidobacterium Bp 12, L. acidophilus e fibra, arricchito con vitamine, con frutti di bosco e biancospino	Kyr Frutti di bosco e biancospino	2x150g	Yogurt, preparazione di frutta (more-mirtilli-fragole-lamponi 60% pari a 8% sul prodotto finito), zucchero, latte fermentato con Bifidobacterium Bp12 e L. acidophilus, fibra alimentare solubile (fruttooligosaccaridi), estratto di biancospino (0,1%), vitamine (C, E, D3), aromi.	106 kcal/446 kJ	3,1 g	14 g	4,2 g
	Yogurt magro con Bifidobacterium Bp 12, L. acidophilus e fibra, arricchito con vitamine, con ananas e the verde	Kyr 0,1% di grassi Ananas & the verde	2x125g	Yogurt magro, preparazione di frutta (ananas 60% pari a 8% sul prodotto finito), zucchero, fibra alimentare solubile (fruttooligosaccaridi), estratto di the verde (0,1%), vitamine (C, E, D3), aromi, Bifidobacterium Bp12 e L. acidophilus	81 kcal/345 kJ	4,1 g	16 g	0,1 g
	Yogurt magro con Bifidobacterium Bp 12, L. acidophilus e fibra, arricchito con vitamine, con mela verde e camomilla	Kyr 0,1% di grassi Mela verde & camomilla	2x125g	Yogurt magro, preparazione di frutta (mela 60% pari a 8% sul prodotto finito), zucchero, fibra alimentare solubile (fruttooligosaccaridi), estratto di camomilla (0,1%), vitamine (C, E, D3), aromi, Bifidobacterium Bp12 e L. acidophilus	81 kcal/345 kJ	4,1 g	16 g	0,1 g
	Yogurt magro con Bifidobacterium Bp 12, L. acidophilus e fibra, arricchito con vitamine, con pompelmo rosa e guaranà	Kyr 0,1% di grassi Pompelmo rosa & guaranà	2x125g	Yogurt magro, preparazione di frutta (pompelmo rosa 60% pari a 8% sul prodotto finito), zucchero, fibra alimentare solubile (fruttooligosaccaridi), estratto di guaranà (0,1%), vitamine (C, E, D3), aromi, Bifidobacterium Bp12 e L. acidophilus	81 kcal/345 kJ	4,1 g	16 g	0,1 g
Zen	Yogurt cremoso alla fragola	Linea Salute Yogurt cremoso alla fragola	2x125g	Yogurt di latte Jersey con fermenti lattici vivi (Lactobacillus bulgaricus e Streptococcus thermophilus), zucchero, fragole 5%, succo di sambuco, aromi.	114kcal/480 kJ	3,3 g	14,5 g	4,8 g

Nutrition and Well-being: the role of the food industry

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The close link between nutrition and health, already guessed at by Hippocrates in 480 b.C. (“*Good health requires knowledge of the basic constitution of man and the power of various foods, be they natural or derived from man’s abilities*”), then taken up by Leonardo da Vinci (“*The life of man is based on what he eats*”) and by other great figures from the past, is now confirmed by the results of numerous clinical and epidemiological trials.

The fundamental importance of a correct diet for the individual and for civilized Society has led the *Health Authorities* to draw up and make public “guide lines” for a balanced diet, with the correct intake of all nutrients that are necessary for the human organism.

These guide lines, in their most modern expression, are closer to the type of diet found in the late Palaeolithic period rather than current dietary habitsⁱ.

Lean meat, fish, greens, fruit, nuts, berries, honey, milk, cheeses and yoghurt guaranteed *ancient man*, in addition to “energy-providing” nutrients, calcium (1500/2000 mg/day), fibre (100/150g/day) omega 3 fatty acids and antioxidants in a quantity significantly higher than that which is taken in with the current diet of industrialized Western Society. Our modern diet is also characterized by a level of caloric intake in excess of requirements, by a reduction in complex carbohydrates and fibre, and by a lower consumption of fruit and vegetables. In addition, the ratio between omega six/three fatty acids, fundamental for the correct functioning of *all vital organs*, has passed from 0.79 to 16, whereas the recommended value is 5.

These changes have led to an increase in the incidence of degenerative diseases such as atherosclerosis, hypertension, diabetes, osteoporosis, obesity.

This situation, which might appear to be surprising, indicates that the human organism has still not had time to adapt to current alimentary habits, which are very different from those for which it was programmed. It is therefore necessary to move towards that kind of diet.

Also the nutritional situation of the Italians is today characterized by some imbalances and deficienciesⁱⁱ: in particular, it is clear that there is a high percentage of overweight people, 47.7% of men and 32.3% of women, with 7.3% of the adult population identified as obese. Then, as concerns the balance between the nutrients, the intake of proteins and simple sugars is too high and there is an insufficient consumption of starch, fibre and above all omega3 fatty acids. If the population is divided into age groups, one can see some deficiencies among the older people (calcium, zinc, magnesium, folates, fibre, vitamin B6), in adolescents (calcium, folates, vitamins A and E, and fibre) and in certain age groups of women (Calcium, iron).

Nowadays we know that maintaining the well-being of the organism and the prevention of many pathologies are the expression, not only of a possible genetic predisposition, but also of a “healthy lifestyle” which starts in early childhood. The creation of this lifestyle depends on the will of the individual and the degree of acquired knowledge, and is achieved through physical activity and diet. According to the modern concept of programming^{iii iv}, an adequate and correct diet starting from early childhood is advantageous for good health not only day by day, but also in the prevention of future problems. In other words, the type of diet when one is young, is able to programme a series of long-term responses which can impede or favour the development of chronic degenerative diseases such as, atherosclerosis, hypertension, diabetes, osteoporosis, obesity. An inadequate intake of critical “micronutrients” such as calcium when one is young, leads to an increased bone fragility and a greater predisposition to fractures in adulthood, when bone has a natural tendency to

lose the mineral component. The degree of this fragility is directly related to a poor calcium intake in adolescence which conditions the peak value, from which the demineralization process starts^v.

The food industry, aware that food substances can be thought of as those “natural agents” that man consumes three times a day, has the task of “making available to all” high quality products that are able to contribute to the creation of that “healthy lifestyle” which starts in early childhood. The food industry carefully chooses food substances, which already contain high nutritional value, and treats them with modern and “mild” technologies, if necessary integrating them with “active principles” such as vitamins, mineral, polyunsaturated fatty acids, prebiotics and probiotics, in order to meet any deficiencies in the diet, and the requirements of the different age groups and physiological states. The integration and the enrichment must take into consideration the bio-availability of the added element and these operations must be performed on food substances which allow optimum solubility and absorption, the latter being influenced by the presence of other components in the food substance or in the diet.

The natural calcium found in milk, or that which is added to the milk in the form of caseinate, is easily absorbed in the intestine because milk contains elements which favour its absorption as simple sugars, free amino acids, a correct calcium/phosphorous ratio, and does not contain inhibitory factors such as phytates, oxalates and uronic acid, which are present in vegetables.

Milk, a food substance of great intrinsic value, which is easy to assume, cheap, and consumed by wide sections of the population, is an ideal vehicle for the intake of many nutrients such as vitamins, mineral salts and polyunsaturated fatty acids, because of its lipophilic and hydrophilic characteristics. A clinical trial has demonstrated that, by replacing the daily diet of 1/2 litre of milk with the same quantity of milk containing added omega-3 fatty acids, there is a significant improvement in the lipidic profile after only three weeks, with a reduction in triglycerides and an increase in HDL cholesterol^{vi}.

Nutrition is therefore a strategic element for maintaining the health of the individual and for tackling the problems of a society which aims to increase the lifespan, living actively, independently and with awareness. It is necessary to prepare and execute a real Social Programme, which is able to satisfy the nutritional and health needs of the population, in particular in those groups most at risk. The more aware Food Companies are already operating in close cooperation with the Institutions and Scientific Research, because only a “multidisciplinary prevention project” makes possible to ensure that, in the not too distant future, Western Society does not reach a burdensome and unacceptable level of disability. As Voltaire said some centuries ago: ”He who is not conscious of his era, has all the problems of his era”.

ⁱ Berra B., Bellia G., Montorfano G., “Acidi grassi ω-3: nutrienti, alimenti funzionali o farmaci?”, Progress in Nutrition, vol.5, num.2/2003, 149-159.

ⁱⁱ D’Amicis A., “Alimenti arricchiti con nutrienti”, Congresso Fo.S.A.N., Bologna 18 febbraio 2000.

ⁱⁱⁱ Lucas A., “Influence of nutrition on long term outcome”. Nestlé Nutrition workshop series, 32 Raven Press 1993: 183-196.

^{iv} Lucas A., “Programming by early nutrition: an experimental approach”. J. Nutr. 1998, 128 (2suppl.): 401-406S.

^v Matkovic et al., Am. J. Clin. Nutr., 1979 : 32 : 540-549.

^{vi} Visioli F., Risè P., Plasmati E., Pazzucconi F., Sirtori C.R. e Galli C., “Very low intakes of n-3 fatty acids incorporated bovine milk reduce plasma triacylglycerol and increase HDL-cholesterol concentration in healthy subjects”. Pharmacological Research 2000;41: 571-576.

“NEW GENERATION OF SYMBIOTIC FERMENTED MILKS”

Gian Luigi Maiocchi

The rapid development of Functional Foods derives from the ever increasing awareness of the close link between diet and health and from the greater scientific knowledge of the mechanisms through which foods modulate the metabolism and the psychological/physical health of the human organism, contributing to prevent and reduce the risk of certain important diseases. Numerous scientific research projects have confirmed the existence of health-protecting properties in addition to the nutritional properties of functional foods, causing the science of nutrition to pass from the concept of “adequate nutrition” to that of “optimal nutrition” (1).

During the past two years the market trend in functional products, especially prebiotics and probiotics fermented milks, has been in constant growth and evolution (**Fig. 1 –Nielsen data**); in particular there has been a move from the concept of “functional” food with generic beneficial properties for the organism, to the concept of “functional food with a more specific action”, that is to say, with a specific beneficial effect on certain physiological functions or biological activities, naturally without forgetting the distinction that lies between what is a food and what is a drug.

In this particular and interesting segment of the market in rapid evolution, there are already numerous products, mainly in the area of milk and dairy products claiming probiotic function (drinkable or in pots fermented milks, fresh cheeses, etc.).

However, since these are foods specifically intended for the protection of the consumer’s health, it is essential, in order not to create false hopes, that they be adequately supported and their value confirmed through serious scientific testing.

In this context YOMO, which has always been involved in Scientific Research in the sector of lactic acid bacteria, yoghurt and fermented milks, launched in 2003 the new symbiotic line ABC, in collaboration with the “Centro Sperimentale del latte” and with some of the top Italian Universities specialized in the field of Microbiology, Technology and Nutrition. This innovative symbiotic line consist of 6 different fermented milks (**Figure 2 – ABC products**) characterized by the presence of an exclusive combination of 8 different types of probiotic bacteria in a lyophilized form, highly concentrated, capable of a synergic relationship which determines an increased capacity to protect human health from the gastrointestinal point of view. This combination consists of probiotic species that are among the most resistant and active and the most representative in the human intestine:

- *S. thermophilus*
- *L. delbrueckii* subsp. *bulgaricus*,
which have a technological function in the preparation of fermented milks and form the health-promoting properties on which the product is based;
- *Lactobacillus acidophilus*
- *Lactobacillus casei*
- *Lactobacillus plantarum*
- *Bifidobacterium infantis*
- *Bifidobacterium longum*
- *Bifidobacterium breve*,
which are intestinal colonizers, very active during the first few months of life, and which are always there to intervene with beneficial effects when the intestinal microbiota becomes complex and over abundant (2).

The selection of the probiotic strains was carried out in accordance with the guide lines, the criteria and the methods established by the FAO and by the World Health Organisation, which made up a work group to demonstrate the nutritional and functional effects of the probiotic bacteria. The guide lines are as follows: (3):

- ❑ Identification of the strains by means of phenotypic and genotypic characterization.
- ❑ Functional characterization and evaluation of safety of the strains used in foods by means of *in vitro* and *in vivo* testing.
- ❑ Study of the probiotic effects *in vivo* using difference methods that make it possible to evaluate how effective the probiotic microorganisms are on the organism.

The careful selection of the probiotic strains present in ABC fermented milk, the exclusive combination of live cells present in the product in large quantities, the controlled accumulation of fermentation metabolites, make these ABC fermented milks particularly suitable for people of all ages since, after ingestion, they have a rapid biological action with a variety of beneficial effects on the organism (2).

In order to obtain the best probiotic effects, ABC fermented milk has been integrated with prebiotics, in this case with soluble non-digestible fibres (oligofructose), which have a beneficial effect on the individual subsequent to a selective action stimulating the development of both the probiotic bacteria resident in the colon and those ingested thanks to the ABC product, which can therefore be considered to all intents and purposes a **new generation functional symbiotic food**.

The nutritional and health-promoting properties and the quality of YOMO's innovative ABC symbiotic specialities, do not only depend on the synergy of action among the 8 probiotic bacteria and between the latter and the prebiotic fibres, but also on all the ingredients that make up these functional fermented milks as a whole. For the formulation of ABC fermented milk, in fact, ingredients of a completely natural composition have been used, derived in particular from fruit, without colorants, preservatives or other additives, so as not to interfere with the growth and vitality of the selected probiotic strains. This has made it possible to offer the consumer foods that are safe, genuine, and of high nutritional and functional value, thus ensuring an adequate level of protection for the health of the consumer, in accordance with the indications recently issued by the European Parliament (Reg. 178/2002 - Food Law).

To this end, fruit and other ingredients have been selected that are especially rich in anti-oxidant components (carotenoids, flavonoids, polyphenols, anthocyanins), as well as vitamins and mineral salts of particular importance (vitamins A,C,E, potassium, magnesium), such as the juice of blueberries/red currants, Sicily citrus fruits, carrots, lemons; fibres naturally present in the ingredients or added (fructo-oligosaccharides), which perform their prebiotic and synergic activity with the selected probiotic bacteria.

In relation to what has been said regarding the formulation of the ABC fermented milks, there are also important technological aspects that influence the quality of these new generation symbiotic foods. In order to valorize to the full the nutritional, probiotic and prebiotic characteristics of the intermediate and finished products, it is fundamental that the entire manufacturing process be realised using "mild technologies" both in the preliminary fermentation phases and in the subsequent phases. The principal process parameters have to be systematically monitored on-line with the adoption of the latest plant and equipment, in order to guarantee the constituent, organoleptic and functional requisites necessary, as well as a high level of production standardisation. Another essential requisite, especially for functional dairy products aimed at protecting the health of the consumer, is the respect of high standards of hygiene throughout the whole of the manufacturing process, which can be obtained, for example, through the disinfection of primary packaging with hydrogen peroxide or ozonated water and by operating in areas with air filtering systems and by using assembly lines with a high level of hygiene. Lastly, a further instrument to guarantee the alimentary safety of functional foods is the application of Traceability and the HACCP regulations for all the phases of the manufacturing process, the raw materials, the semi-finished product and the packaging materials.

In the continual research and development of ever more innovative milk and dairy product specialities with specific functional activities, there are four main steps to be followed (1):

1. Identification of the type of functionality that is sought after
2. Identification of the source that can provide this specific functionality
3. Identification of the molecule or biological complex with this functional property
4. Application of the know-how acquired and the formulation of the new functional food as well as the definition of the manufacturing process.

In addition to the other scientific aspects mentioned above, the realization of new functional foods must take into account further fundamental market aspects, such as (4):

- a thorough understanding of the motivation for consuming functional foods;
- understanding as to whether there are differences in the background and in the modalities/occasions for the consumption of probiotic functional foods related to: the type of consumer in the various age ranges (high-average-low consumption); the various differences within the category (e.g. drinkable fermented milks versus the product in pots).

In this specific case, it may be useful to carry out a consumer survey regarding the occasions for consumption; the logic of consumption (as a meal replacement rather than an addition to normal dietary habits); the reasons underlying consumption (enhancement, integration, prevention, health-protection, etc.) and possible barriers to the consumption of this type of product (price, doubts about the efficacy of the product, etc.).

Another essential element in the development of a Functional Food, is the perception on part of the consumer of the real nutritional and functional benefits of the food and its added value. While, in fact, the organoleptic properties of a food, such as the structure and the flavour, can be easily appreciated by the consumer, a beneficial effect on health is not “visible or tangible” and it will therefore be successful only if the consumer knows and believes in the scientific validity of the marketing claims. This can be obtained thanks to clear, simple and accurate information and communication through, for example, detailed labelling as well as scientific information leaflets regarding the specific functional properties.

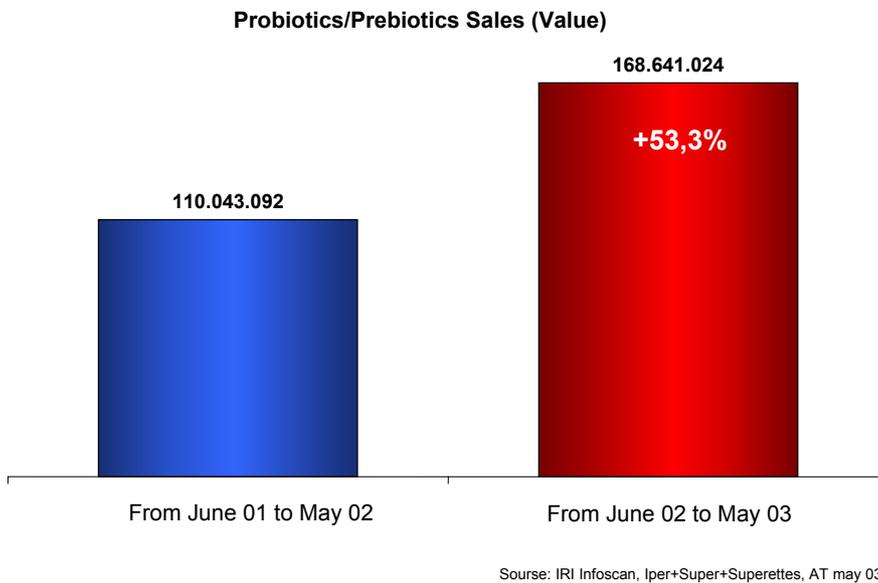
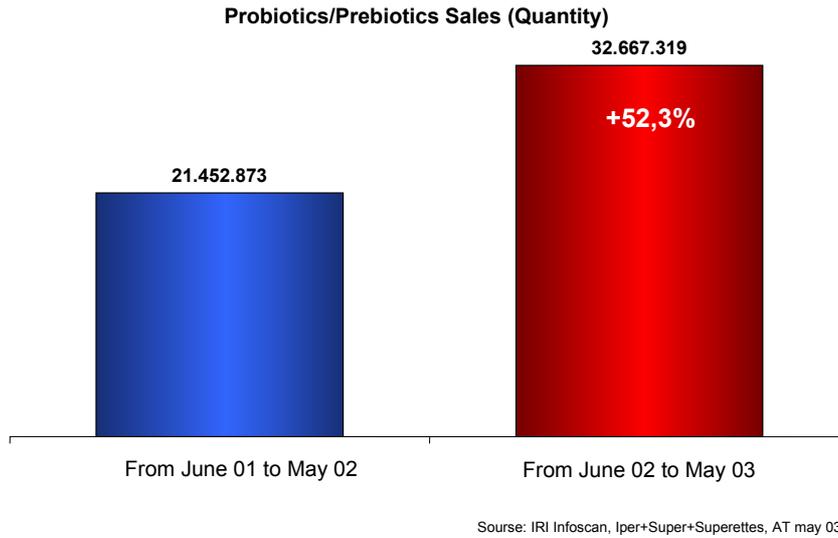
It is in any case essential, in the face of the present market situation, which is becoming increasingly crowded and more popular, that the value of these functional foods be controlled in

relation to their specific probiotic and prebiotic properties, through *in vitro* and *in vivo* tests on human beings with a statistically significant number of individuals, and that they contain in their formulation top quality ingredients and probiotic bacteria in high concentrations and of proven efficacy for human beings as far as gastrointestinal health and immune function are concerned.

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Fig. 1 Nielsen Data



Market Players from June '01 to May '02

YOMO:	ABC
Danone:	Activia Actimel
Granarolo:	Vivi Vivo
Merano:	Bellavita AC Active
Nestlé:	Lc1 Lc1 go
Parmalat:	Kyr

Market Players from June '01 to May '02

YOMO:	ABC	Nestlé:	Lc1 Lc1 go
Danone:	Activia Actimel	Parmalat:	Kyr Kyr Principia
Merano:	Bellavita AC Active	Scaldasole:	Biovitalitis
Mila:	Benessere Rinforzo Benessere Regularis	Vipiteno:	Fitline
Muller:	Actidrink	Granarolo:	Vivi Vivo



MORE AND MORE CROWDED MARKET

Fig. 2 - ABC products



WHITE

Partly skimmed fermented milk with 8 live and viable lactic acid bacteria

AVERAGE NUTRITIONAL VALUE (PER 100g):

ENERGY	PROTEIN	CARBOHYDRATE	FAT
kcal 79 / kJ 331	2,7g	12g	1,9g



BLUEBERRY REDCURRANT

Partly skimmed fermented milk with 8 live and viable lactic acid bacteria, with blueberry and redcurrant juice

AVERAGE NUTRITIONAL VALUE (PER 100g):

ENERGY	PROTEIN	CARBOHYDRATE	FAT
kcal 83 / kJ 352	2,7g	13g	1,9g



VANILLA

Partly skimmed fermented milk with 8 live and viable lactic acid bacteria, with vanilla beans natural extract

AVERAGE NUTRITIONAL VALUE (PER 100g):

ENERGY	PROTEIN	CARBOHYDRATE	FAT
kcal 79 / kJ 331	2,7g	12g	1,9g



PINEAPPLE 0,1

Skimmed fermented milk (0,1% fat) with 8 live and viable lactic acid bacteria, with pineapple

AVERAGE NUTRITIONAL VALUE (PER 100g):

ENERGY	68 kcal / 289 kJ	FAT	0,1g
		of which saturates	0,06g
PROTEIN	2,6g	FIBRE	0,8g
CARBOHYDRATE	13,5g	SODIUM	0,05g
of which sugars	12,8g		



SICILY CITRUS FRUIT

Partly skimmed fermented milk with 8 live and viable lactic acid bacteria, with Sicily citrus fruit

AVERAGE NUTRITIONAL VALUE (PER 100g):

ENERGY	PROTEIN	CARBOHYDRATE	FAT
kcal 89 / kJ 375	2,7g	14,5g	1,8g



ACE 0,1

Skimmed fermented milk (0,1% fat) with 8 live and viable lactic acid bacteria, with orange, carrot, lemon and vitamins

AVERAGE NUTRITIONAL VALUE (PER 100g):

ENERGY	70 kcal / 289 kJ	FIBRE	0,8g
PROTEIN	2,5g	SODIUM	0,05g
CARBOHYDRATE	14,1g	Vitamin C	9mg
of which sugars	13,5g	Vitamin E	1,5mg
FAT	0,1g	Provitamin A (betacarotene)	0,9mg
of which saturates	0,06g		

**TOXICITY-BASED SAFETY ASSESSMENT OF A MIXTURE OF PROBIOTIC
BACTERIA IN RATS**

Running head: Safety assessment of a probiotic mix

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SUMMARY

Eptavis[®]/Yovis[®] is a freeze-dried pharmaceutical probiotic preparation containing lactic acid bacteria and bifidobacteria. To verify its tolerance the product was administered by gavage to 80 rats for 14 and 30 days at the recommended dosage and tenfold the maximum recommended dosage. The results were compared with a control group (time 0) and placebo treated groups.

Daily observations of subjects did not reveal any negative effect of treatments. No differences in growth and aspect of faeces, and no morbidity and mortality. During necropsies, no macroscopic anomalies of the splanchnic organs were observed. In particular, stomach, foregut and hindgut did not reveal any alteration.

As regards haematological and haematochemical analyses it was observed that, red cell parameters, compared to placebo, were significantly higher in 14-day normal-dosage treated subjects and lower in 30-day subjects, with a slight reduction in RBC count and Hb-Ht, but higher MCH and MCHC. Urea levels were significantly higher in normal-dosage treated subjects, but within range.

In high-dosage treated subjects, treatment did not influence the red parameters, except for a higher MCH and MCHC, but within range, a significant reduction in plasma calcium and in 14-day subjects a higher plasma TP and lower cholesterol.

As a conclusion Eptavis[®]/Yovis[®] is well-tolerated in rats since no side effects were observed, also at high dosages.

Key Words: Probiotics, safety, tolerance, assessment

INTRODUCTION

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (1). Probiotic strains must colonize the host so as to affect microbial intestinal balance. However, because probiotics do not permanently colonize the intestine, they must be taken in “adequate” quantities ($>10^{10}$ CFU/d) (2) to arrive in sufficient amounts in the GI tract. Microorganisms that are principally used as probiotics include various species of lactic acid bacteria and bifidobacteria used individually or in combination. Amongst lactic bacteria, the more used species are *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus* and others. Amongst bifidobacteria, *Bifidobacterium bifidum*, *B. breve*, *B. longum*, *B. infantis*, *B. adolescentis*. Also some strains of *Enterococcus faecium* are regarded as probiotics for humans or animals (3,4).

Probiotics are beneficial to human health because they improve the intestinal microbial balance, reduce risk of constipation, prevent diarrhoea and produce an immunostimulating and antiallergic effect (2).

Safety of lactic acid bacteria has been widely established by centuries of daily intake of dairy products, that are an important component of human diet. Many lactic acid bacteria have been used in several food processing over a very long period of time in human history and there is ample experience of ingestion of the viable bacteria and their metabolites without negative effects on health. In addition, ecologically speaking, the bifidobacteria are amongst the predominant bacteria in the human colon and they contribute greatly to the intestinal health. Both lactic acid bacteria and bifidobacteria are classified as “GRAS” (Generally Recognized as Safe) by FDA of USA. Safety of probiotics was recently reviewed and assessed by several scientific reports (5, 6, 7; 8). Factors related to safety include absence of

pathogenicity, infectivity, and toxicity. Historically, LAB and bifidobacteria have been considered as bacteria that do not possess pathogenicity and infectivity under normal condition. Toxicity tests are effective methods for proving safety (8, 9).

Probiotics are administered *per os* as single or mixed strains in foods or pharmaceutical products (10, 4). Eptavis[®]/Yovis[®] (CSL Italy, 1995) is a novel, high bacteria-concentration, pharmaceutical preparation containing 300 billion CFU/g of freeze-dried bacteria. Bacterial species include three strains of lactobacilli, three strains of bifidobacteria, and yoghurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*). These bacterial strains promote positive effects on humans . Efficacy of this probiotic was verified through clinical trials, with satisfactory results on the remission of several pathologies and lack of negative effects (10, 11, 12). In order to better emphasize the absence of side effects and therefore the safety of this probiotic mix, the preparation has been tested on rats treated *per os* with the maximum recommended dosage and ten times the recommended dosage, in order to verify its tolerance (toxicity) according to European guidelines for the assessment of microorganisms in feedingstuffs (9).

MATERIALS AND METHODS

Product characteristics

EPTAVIS®/YOVIS® is the brand name (registered in Italy and the USA) of the commercial probiotic pharmaceutical product originated from VIS-01 (active principle), which has been reported in many studies since 1997 under the scientific acronym VSL#3 .

Eptavis®/Yovis® appears as a powder containing freeze-dried bacteria with the following microbial content and cell concentration:

<i>Streptococcus thermophilus</i>	200 billion cells g ⁻¹
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	300 million cells g ⁻¹
<i>Lactobacillus plantarum</i>	220 million cells g ⁻¹
<i>Lactobacillus casei</i>	220 million cells g ⁻¹
<i>Lactobacillus acidophilus</i>	Two billion cells g ⁻¹
<i>Enterococcus faecium</i>	30 million cells g ⁻¹
Bifidobacteria (<i>B. breve</i> , <i>B. infantis</i> , <i>B. longum</i>)	90 billion cells g ⁻¹

Subjects and treatments

The trial was conducted on a total number of 80 Sprague-Dawley female rats, weighing 150-180 grams each (Charles River Italia). Animals were housed for 10 days without any type of treatment in order for them to adapt to the new feed regimen and environment. Rats were split into 7 treatment groups as follows:

Group A (n=12)- Control (time zero)

Group B (n=12)- Treatment with “normal” dosage (190 mg Eptavis®/Yovis®/kg lw/day) for 14 days

Group C (n=14)-Treatment with “normal” dosage (190 mg Eptavis®/Yovis®/kg lw/day) for 30 days

Group D (n=10)-Treatment with placebo (tap water) for 14 days

Group E (n=10)-Treatment with placebo (tap water) for 30 days

Group F (n=10)-Treatment with “normal” dosage x 10 (1900 mg Eptavis®/Yovis®/kg lw/day) for 14 days

Group G (n=10)-Treatment with “normal” dosage x 10 (1900 mg Eptavis®/Yovis®/kg lw/day) for 30 days

Normal dosage was calculated on the basis of the maximum recommended dosage in human (mg/Kg live weight per day) and the metabolic weight of subjects (MW=live weight^{0.75}), that is 190 mg/kg live weight/day per rat. The product was suspended in tap water (1.9 g/100mL for the “normal” dosage, and 19g/100mL for the high dosage) and administered to rats by gavage (gastrooesophageal catheter). Every day a fresh suspension was prepared. Placebo subjects were treated with a calculated volume of tap water. Each rat was weighed immediately before treatment. During weighing procedures, subjects were observed to survey eventual anomalous reactions or disease symptoms.

All rats were housed in cages (5-7 units each) and fed commercial gamma-ray sterilized (2 Mrad, that is 20 kGy) feed (Mucedola SRL, Settimo Milanese, Milano, Italia). Water was administered *ad libitum*.

Haematological and haematochemical analyses

After each treatment period (14 or 30 days), rats were anesthetized with diethylether: a linea alba laparotomy was performed, and a sample of blood (1-3 mL) was taken from abdominal artery by a 5 mL polypropylene disposable syringe. Specimens were immediately transferred

into lithium heparin-containing glass tubes. All samples were analyzed for haematology, then centrifuged (3000 r.p.m. for 15') and plasma was recovered within 1 hour.

Haematological parameters were performed with an impedance semiautomated apparatus (Sysmex F-800, Toa Electronics Inc., Tokyo, Japan). On whole blood the following analyses were determined:

Total leukocyte count (WBC, $10^3/\text{mm}^3$)

Red blood cell count (RBC, $10^6/\text{mm}^3$)

Haemoglobin (Hb, g/100 mL)

Haematocrit (Ht, %)

Mean corpuscular volume (MCV, fL)

Mean corpuscular haemoglobin (MCH, pg)

Mean corpuscular haemoglobin concentration (MCHC, %)

Red cell volume dispersion (RDW, %)

Platelet count (PLT, $10^3/\text{mm}^3$)

On plasma the following parameters were determined:

Total proteins (TP, g/100 mL)

Total cholesterol (Chol, mg/100 mL)

Triglycerides (Trig, mg/100 mL)

Urea (mg/100 mL)

Total calcium (Ca, mg/100 mL)

Inorganic phosphorus (Phos, mg/100 mL)

All plasma analyses were performed with an automated haematochemistry apparatus (Kone Specific, Ivry, France). All kits were purchased from DASIT S.p.A., Cornaredo, Milano Italy. Variables were analyzed by end-point analyses.

The methods and the intra-assay coefficient of variations (by taking into account 10 values) are reported in table I.

In order to assess the effect of treatments on systemic organs, after blood drawing each rat was submitted to a complete necropsy. Stomach, intestine, liver, spleen and kidneys were observed on their surface and in their mucosa or parenchyma.

Statistical analyses

Data were summarized as mean±standard deviation; weight values in each group were analyzed by randomized blocks with one-way analysis of variance (ANOVA) on days 0, 7, 14, 21, 30.

Haematological and haematochemical data on days 14 and 30 were analyzed with the ANOVA test following these comparisons:

Group B *versus* D

Group C *versus* E

Group F *versus* D

Group G *versus* E

Group A results (Time zero), expressed as mean ± standard deviation and min-max range, were taken as control values. Differences between group A and the other experimental groups were assessed by the Tukey test for multiple comparisons: the method assesses the significance between mean values of treatment groups. All analyses were performed with the SPSS Statistical package (13)

RESULTS

General observation and post-mortem examination

Daily observations of subjects did not reveal any abnormal effect of treatment. A rat from group B (190 mg/Kg for 14 days) deceased for accidental cause. During the whole observation time, no cases of diarrhoea were observed; feces of all animals resulted normal for all groups. No noticeable changes in activity were observed; hair lustre was considered normal. During necropsies, no macroscopic anomalies of the splanchnic organs were found: in particular, stomach, small and large intestine did not reveal alterations due to probiotic consumption. Macroscopic alterations on the gut mucosa (bleeding, erosions, mucus overproduction) were not observed.

Results concerning the weight of subjects during the 30-day trial are reported in graphs 1 and 2: for each point, the groups did not differ in weight ($p>0.05$).

Haematological and haematochemical analyses

Table II summarises the ranges and mean values of haematological and haematochemical variables, used as control data (group A).

Rats of group A showed a great variability in some parameters (e.g. Trig, Urea, Phos), while red cell variables showed a very narrow dispersion.

Results concerning haematological and haematochemical analyses in normal dosage rats compared with placebo during the first 14-day trial are reported in table III. Subjects differ mainly in red cell parameters, with a slight reduction in RBC count and Hb-Ht in treated subjects, that showed anyway higher values in MCH and MCHC ($p<0.01$). Treated rats had higher urea concentrations in plasma, but with mean values within the established ranges.

Table IV summarises the mean values and statistical analyses for the 30-day trial with normal dosage. Red cell direct parameters (RBC, Hb, Ht) were higher in placebo group ($p < 0.05$), but MCH and MCHC remained higher in treated group ($p < 0.05$). Urea levels was higher in treated rats ($p < 0.05$), but within reference limits.

Tables V and VI summarise the results of haematological and haematochemical variables in high dosage rats (1900 mg Eptavis®/Yovis®/kg lw/day) during the 14 and 30-day trials, respectively. The high dosage did not influence the red parameters, except for MCH and MCHC, that remained high in treated subjects. In the 14-day trial rats, a higher plasma TP, and lower cholesterol ($p < 0.01$ for both variables) in treated subjects was noted. A significant, remarkable reduction in plasma calcium was found in high-dosage treated groups.

Table VII summarises the differences between time zero group (group A) and treatment/placebo groups. In E group occurred a reduction in the direct red cell parameters. To be noticed also the significant lower cholesterol levels in rats fed high dosage of Eptavis®/Yovis® for 14 days (group F) compared with group A.

DISCUSSION

On the basis of the scientific background and the results of the present study, Eptavis®/Yovis® is a well-tolerated probiotic mix, also at high daily dosage (1900 mg/kg bw) for a prolonged period (30 days). During the whole observation period, growth of subjects was identical in all groups, and the external appearance of rats and feces, as well as fecal consistency were normal in all subjects.

Paired placebo/treated comparisons showed, in treated subjects, a significant reduction in the red cell parameters, mainly in the 14 and 30-day trials with normal dosage (groups B and C). However, these variations were not different from control group A (time zero).

It is remarkable that significant variations among red cell patterns were also observed by Depta et al. (14) on conventional piglets and Bomba et al (15) on gnotobiotic piglets. These authors found a reduction in Hb, RBC and Ht in the first period of administration of *Lactobacillus* spp. and *L. casei*. Mohan et al. (16) also noticed a significant diminution in the hemoglobin content in broiler chickens after administration of a probiotic constituted by *L. acidophilus*, *L. casei*, *B. bifidum*, *Aspergillus oryzae* and *Torulopsis*, but without observing alterations to the hematocrit. The whole mechanism appears unclear, but Miller et al., (17) showed that microorganisms cause an alteration in iron requirements, and Bruyneel et al. (18) observed strong iron complexation by lactobacilli with ecological advantage over clostridia. As reported by Bezkorovainy and Kot (19), both bifidobacteria and lactobacilli (*B. thermophilum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*) can bind ferric iron, and *B. breve* can also extract iron from lactoferrin.

Perhaps this mechanism, although favouring a better equilibrium of the microbial gastrointestinal ecosystem, could be the cause of the reduction in direct erythrocyte parameters in treated groups, when compared to placebo.

However, although reduction in erythrocyte parameters was significant in placebo/treated compared groups, an anaemic status must be excluded: rats in group A, in fact, did not differ from treated groups, and red cell parameters in such groups were within acceptable limits.

Among haematochemical variables, a slight variation in plasma protein concentration in group G was observed, when compared with group A.

A significant, remarkable reduction in plasma calcium was found in high-dosage treated groups. This feature was also described by Tortuero and Fernandez (20) in hens: the subjects fed *S. faecium* showed a (non significant) better calcium retention, with a decrease in plasma calcium and phosphorus levels, and a greater calcium content in egg shell. Therefore,

although this parameter has not been verified, a better calcium deposition in rat bones can be hypothesized.

Nahashon et al. (21) hypothesize that the retention of calcium due to supplementation with lactobacilli could be the consequence of the diminution in intestinal pH.

Perhaps a more acid intestinal environment, due to the metabolic activity of probiotic bacteria, would support the ionization and, accordingly, the absorption, utilization, and consequently, calcium deposition in the bones.

A significant reduction in inorganic phosphorus was found in all groups when compared with group A. This phenomenon does not seem to be related to treatment: paired comparisons between treatment and placebo did not evidence differences, and this reduction was common in all groups.

In normal dosage groups (B and C) we remark a slight increase in plasma urea with respect to placebo, while rats in group F showed a reduction in urea concentration when compared to group A.

Treatment with lactobacilli can lead to a reduction in plasma cholesterol in rabbits and pigs (15, 22, 23) and also in broilers (16) and laying hens (24). Haddadin et al. (25) found a reduction in egg yolk cholesterol (18.8%) after administration of *L. acidophilus*. It is suggested that the reduction in yolk cholesterol is a reflection of lower serum cholesterol concentration in treated birds. Such reduction could be ascribed to an inhibitory effect exerted by some strains of lactobacilli (26), or by direct assimilation of cholesterol by lactobacilli (27). In our trial, reduction of cholesterol after administration of Eptavis®/Yovis® appears controversial: only group F (high-dosage, 14 days) showed a significant reduction in plasma cholesterol with respect to placebo and control group (A).

To conclude, on the basis of our results, we can affirm that the probiotic Eptavis®/Yovis® is well-tolerated in rats as a model: no side effects were observed among rats, also in high-dosage group (10 times the maximum dosage). Rats fed the probiotic preparation had no adverse effects on the general health status, growth, haematology, blood biochemistry and other macroscopic parameters examined in the present study.

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Table I. Results of intra-assay variations (Coefficient of variation) of analytical plasma biochemistry methods.

Parameter	Method	CV%
TP	Biuret-EDTA	0.88
Trig	GPO-Trinder	5.0
Chol	CHOD	1.01
Urea	Urease/UV	1.50
Phos	Molybdate	0.98
Ca	Arsenazo III	4.80

Table II. Ranges (n=12) of examined rats at time zero (group A).

Variable	Mean \pm sd	Range	Variable	Mean \pm sd	Range
WBC ($10^3/\text{mm}^3$)	5.4 \pm 1.9	2.6-8.8	TP (g/100 mL)	6.1 \pm 0.4	5.6-6.7
RBC ($10^6/\text{mm}^3$)	5.7 \pm 1.2	4.25-7.76	Chol (mg/100 mL)	37.3 \pm 8.3	29-55
Hb (g/100mL)	14.1 \pm 1.2	13-17.4	Trig (mg/100 mL)	58.4 \pm 30.2	15-112
Ht (%)	31.0 \pm 6.1	22.8-41.6	Urea (mg/100 mL)	37.7 \pm 5.2	29.2-45.8
MCV (fL)	53.9 \pm 1.2	52.1-55.6	Ca (mg/100 mL)	9.2 \pm 1.0	7.6-10.7
MCH (%)	25.3 \pm 4.2	20.7-32.2	Phos (mg/100 mL)	10.9 \pm 4.2	6.4-18
MCHC (pg)	46.9 \pm 7.7	38.2-60.1			
RDW (%)	14.8 \pm 0.3	14.4-15.2			
PLT ($10^3/\text{mm}^3$)	527.9 \pm 119. 4	294-747			

Table III. Haematological and haematochemical analyses of normal dosage group (B) compared with placebo group (D). Group B -Treatment with normal dosage (190 mg Eptavis®/Yovis®/kg lw/day) for 14 days. Group D -Treatment with placebo for 14 days.

Parameter	Group B mean±sd n=11	Group D mean±sd n=10	Parameter	Group B mean±sd n=11	Group D mean±sd n=10
WBC (10 ³ /mm ³)	7.4±1.8	7.7±1.5	TP (g/100 mL)	6.5±0.4	6.4±0.5
RBC (10 ⁶ /mm ³)	5.7±0.6**	6.6±0.8	Chol (mg/100 mL)	38.4±7.1	33.1±4.8
Hb (g/100mL)	13.1±0.6	13.5±1.4	Trig (mg/100 mL)	59.2±26.9	54.8±20.2
Ht (%)	30±3.0**	35.8±4.6	Urea (mg/100 mL)	42±4.6*	37.8±7.2
MCV (fL)	52.4±1.5	54.1±2.2	Ca (mg/100 mL)	9.4±0.7	8.6±1.9
MCH (%)	23.2±2.4**	20.5±2.1#	Phos (mg/100 mL)	6.8±2.3	5.8±0.4#
MCHC (pg)	44.1±4.1**	37.9±3.9#			
RDW (%)	15±0.4*	15.3±0.42			
PLT (10 ³ /mm ³)	572.2±209*	750.2±148.5 #			

*- $p < 0.05$ difference between groups; **- $p < 0.01$ difference between groups;

#- Mean value out of reference limits

Table IV. Haematological and haematochemical analyses of normal dosage group (C) compared with placebo group (E). Group C -Treatment with normal dosage (190 mg Eptavis®/Yovis®/kg lw/day) for 30 days. Group E -Treatment with placebo for 30 days.

Parameter	Group C mean±sd n=14	Group E mean±sd n=10	Parameter	Group C mean±sd n=14	Group E Mean±sd n=10
WBC (10 ³ /mm ³)	6.7±1.9	8.0±2.5	TP (g/100 mL)	6.4±0.4	6.6±0.6
RBC (10 ⁶ /mm ³)	6.3±0.5**	8.3±2.7#	Chol (mg/100 mL)	39.6±7.7	35.5±5.2
Hb (g/100mL)	13.6±0.8*	14.3±0.8	Trig (mg/100 mL)	73.3±41.0	87.8±55.0
Ht (%)	32.8±2.4*	42.2±13.4#	Urea (mg/100 mL)	38.9±7.1*	34.5±7.1
MCV (fL)	52.3±0.8	51.0±2.3	Ca (mg/100 mL)	10.2±0.9	9.7±0.7
MCH (%)	21.8±1.8*	18.5±4.4#	Phos (mg/100 mL)	6.2±0.9#	5.6±0.9#
MCHC (pg)	41.6±3.2*	36.2±8.5#			
RDW (%)	15.3±0.5#	15.5±0.6#			
PLT (10 ³ /mm ³)	628.4±70.8	811.0±371.4 #			

*- $p < 0.05$ difference between groups; **- $p < 0.01$ difference between groups;

#- Mean value out of reference limits

Table V. Haematological and haematochemical analyses of high dosage (x10) group (F) compared with placebo group (D). Group F -Treatment with tenfold normal dosage (1900 mg Eptavis®/Yovis®/kg lw/day) for 14 days. Group D -Treatment with placebo for 14 days.

Parameter	Group F mean±sd n=10	Group D mean±sd n=10	Parameter	Group F mean±sd n=10	Group D mean±sd n=10
WBC (10 ³ /mm ³)	6.0±0.9	7.7±1.5	TP (g/100 mL)	7.1±0.5#	6.4±0.5**
RBC (10 ⁶ /mm ³)	6.5±0.4	6.6±0.8	Chol (mg/100 mL)	25.8±6.5#	33.1±4.8**
Hb (g/100mL)	14.4±0.8	13.5±1.4	Trig (mg/100 mL)	53.2±19.9	54.8±20.2
Ht (%)	33.9±2.2	35.8±4.6	Urea (mg/100 mL)	28.8±4.4#	33.2±6.8
MCV (fL)	51.7±1.4#	54.1±2.2*	Ca (mg/100 mL)	5.1±1.1	8.6±1.9*
MCH (%)	22.0±1.2	20.5±2.1#	Phos (mg/100 mL)	5.83±0.9#	5.84±0.4#
MCHC (pg)	42.6±2.3	37.9±3.9*#			
RDW (%)	15.5±0.4	15.3±0.42			
PLT (10 ³ /mm ³)	674.1±147.4	750.2±148.5 #			

*- $p < 0.05$ difference between groups; **- $p < 0.01$ difference between groups;

#- Mean value out of reference limits

Table VI. Haematological and haematochemical analyses of high dosage (x10) group (G) compared with placebo (E). Group G -Treatment with tenfold normal dosage (1900 mg Eptavis®/Yovis®/kg lw/day) for 30 days. Group E -Treatment with placebo for 30 days.

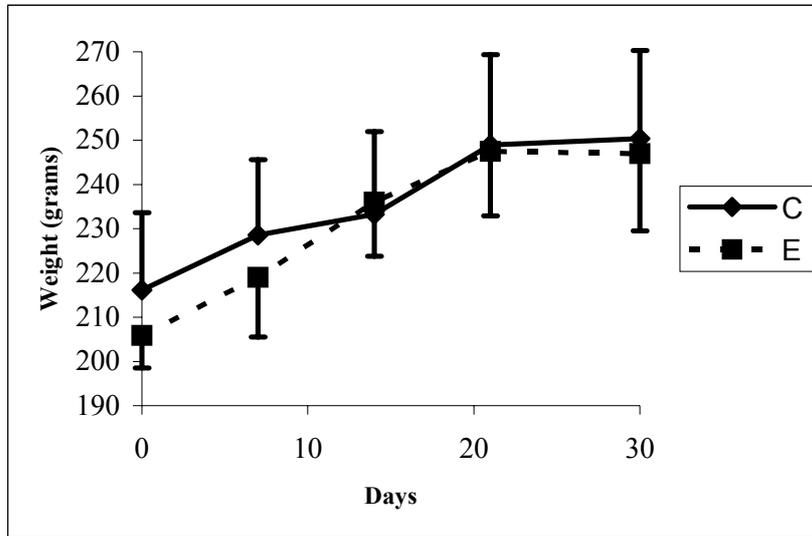
Parameter	Group G mean±sd n=10	Group E mean±sd n=10	Parameter	Group G mean±sd n=10	Group E mean±sd n=10
WBC (10 ³ /mm ³)	7.5±2.5	8.0±2.5	TP (g/100 mL)	6.9±0.5#	6.6±0.6
RBC (10 ⁶ /mm ³)	6.7±0.4	8.3±2.7#	Chol (mg/100 mL)	33.8±4.9	35.5±5.2
Hb (g/100mL)	14±0.5	14.3±0.8	Trig (mg/100 mL)	90.6±60.3	87.8±55.0
Ht (%)	35.6±3.2	42.2±13.4#	Urea (mg/100 mL)	37.2±5.0	34.5±7.1
MCV (fL)	52.8±1.9#	51.0±2.3	Ca (mg/100 mL)	6.9±2.0	9.7±0.7**
MCH (%)	20.8±0.8	18.5±4.4#	Phos (mg/100 mL)	6.3±0.8#	5.6±0.9#
MCHC (pg)	39.5±2.4	36.2±8.5#			
RDW (%)	15.4±0.5#	15.5±0.6 #			
PLT (10 ³ /mm ³)	660.5±131.8	811.0±371.4 #			

*- $p < 0.05$ difference between groups; **- $p < 0.01$ difference between groups;

#- Mean value out of reference limits

Table VII. Multiple comparisons of control group A (time zero) with treatment groups (Tukey test). Means±sd . Only significant differences are reported *-P<0.05; **-p<0.01; ***-p<0.001.

Group A	A vs. group	P	Group A	A vs. group	P
WBC ($10^3/\text{mm}^3$) 5.4±1.9	E (8.0±2.5)	***	TP (g/100 mL) 6.1±0.4	E (6.6±0.6) G (6.9±0.5)	*** **
RBC ($10^6/\text{mm}^3$) 5.7±1.2	E (8.3±2.7)	***	Chol (mg/100 mL) 37.3±8.3	F (25.8±6.5)	**
Hb (g/100mL)14.1±1.2	-	-	Trig (mg/100 mL) 58.4±30.2	-	-
Ht (%) 31.0±6.1	E (42.2±13.4)	*	Urea (mg/100 mL) 37.7±5.2	F (28.8±4.4)	*
MCV (fL) 53.9±1.2	E (51.0±2.3) F (51.7±1.4)	* *	Ca (mg/100 mL) 9.2±1.0	F (5.1±1.1) G (6.9±2.0)	*** **
MCH (%) 25.3±4.2	C (21.8±1.8) D (20.5±2.1) E (18.5±4.4) G (20.8±0.8)	* ** *** *	Phos (mg/100 mL) 10.9±4.2	B (6.8±2.3) C (6.2±0.9) D (5.8±0.4) E (5.6±0.9) F (5.8±0.9)	** ** ** ** **
MCHC (Pg) 46.9±7.7	D (37.9±3.9) E (36.2±8.5) G (39.5±2.4)	** *** *			
RDW (%) 14.8±0.3	E (15.5±0.6) F (15.5±0.4) G (15.4±0.5)	** * *			
PLT ($10^3/\text{mm}^3$) 527.9±119.4	E (811±371.4)	*			



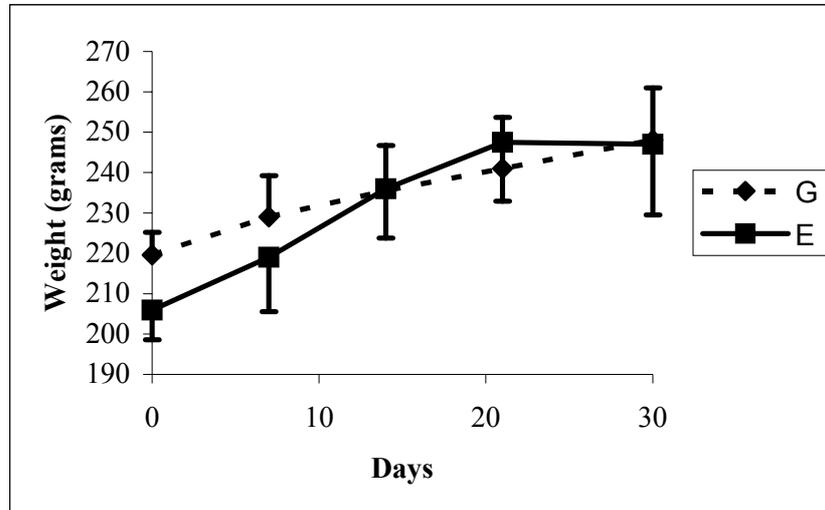


FIGURE CAPTIONS

Figure 1. Weight of subjects during the 14-day trial. Group C – Normal dosage; Group E – Placebo

Figure 2. Weight of subjects during the 30-day trial. Group G – 10 x Normal dosage; Group E - Placebo

Olive oil as a functional food?

Running title: Olive oil as a functional food

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Introduction

A large body of epidemiological studies shows how the incidence of coronary heart disease (CHD) and certain cancers, *e.g.* breast and colon cancers, is lowest in the Mediterranean basin (Keys, 1995). It has been suggested that this is largely due to protective dietary habits of this area (Keys, 1995, Hertog et al, 1995). The traditional Mediterranean diet, rich in fruit, vegetables, fish, and whole grain, is thought to promote good health and longevity. Olive oil, the primary source of fat of this diet, differs significantly in composition from dietary lipids that are consumed by other populations. The formulation of an antioxidant/atherosclerosis hypothesis stimulated experimental and epidemiological studies on the possible role of antioxidants, including olive oil phenolics, in the protection from CHD observed in the Mediterranean area. In fact, among the several minor constituents of virgin olive oil, there are vitamins such as alpha- and gamma-tocopherols (around 200 ppm) and beta-carotene, phytosterols, pigments, terpenic acids, flavonoids, squalene, and a number of

phenolic compounds, usually grouped under the rubric "polyphenols" (Boskou, 2000).

A large body of credible scientific research is needed to confirm the benefit of a particular food or of its components. The question is: how to establish the scientific bases to support claims for functional components or for the foods that contain them? In our opinion, requisites for a functional component/food must answers to four main questions:

- i) does the food contain any possible active component(s)? It will be fundamental to know the composition of the food. In this way, reliable analytical methods for the characterization of the food composition will be set up.
- ii) Do this/these component(s) exert *in vitro* activity(ies)? It is necessary to choose appropriate and representative models to verify such biological activity(ies)
- iii) Are the component(s) absorbed/metabolized and do they show biological activity in humans?
- iv) Is there any epidemiological evidence concerning the beneficial effect of the nutrient?

In this paper we will describe the reasons why, in our opinion, olive oil can be considered as a functional food.

i) Characterization of the phenolic fraction of olive oil

Finger print of phenolic fraction

Olive oil, as opposed to seed oil, contains a series of phenolic “minor components” that grants its peculiar aroma and taste. Among them, hydroxytyrosol and oleuropein contain catecholic moiety that contribute to the antioxidant properties of this nutrient.

As already pointed out, the concentration of antioxidants such as OHT in olive and oil depends on a number of factors, including processing of olives, crushing and separation of the oil, as well as the time and the method of storage of olive and oils (Ryan and Robards, 1998). The total phenols content is not representative of the antioxidant capacity of the oil. For this reason, it is important to determine the antioxidant content in the oils used in metabolic studies.

When oil extracts were analyzed by APCI-MS without LC separation, the ions detected were those reported in Fig. 1. Main ions were originated from aglycons derived from Oleuropein (m/z 377) and from Ligstroside (m/z 361), the latter containing a phenolic residue instead of the catecholic residue of oleuropein (Cortesi et al., 1995). Other phenolic components known to occur in oil extracts were identified (see legend to Fig. 1). Among those compounds, only hydroxytyrosol, deacetoxy- and 10-hydroxy-oleuropein contain a catecholic moiety and may therefore contribute to the antioxidant properties of virgin

olive oil (VOO). For this reason, we are specifically focusing on these compounds.

Quantitative determination of oleuropein and hydroxytyrosol

Oleuropein in oils is present as an aglycon, due to the action of hydrolytic enzyme released during the preparation of the oil. This hydrolysis also causes partial modification of the aglycon due to keto-enolic tautomeric equilibrium that involves the ring opening of secoiridoids (Gariboldi et al., 1986; Angerosa et al., 1996) (Scheme 1). LC-MS injection of the aglycon (Fig. 2a), obtained in our laboratory by enzymatic hydrolysis of oleuropein, shows indeed a number of peaks in the trace of the ion at m/z 377, corresponding to the $[M-H]^-$ of OleA (fig.1). Consequently, it is very difficult to obtain a reliable quantitative LC-analysis. For this reason, an MS/MS analysis of an crude extract by colliding of the ion at m/z 377 (fig. 2b) which in turn originates the ion at m/z 307 (Fig. 2c) was performed and the corresponding product ion generated from all aglycon isomers was then used for quantification of OleA (Caruso et al, 1999).

The quantitative determination of OHT in oils was performed by means of GC-MS technique, using deuterated OHT as internal standard.

The analyses were performed on oils from different *cultivars* and in particular on these used for the described experiments.

ii) In vitro studies

The lower incidence of CHD observed in the Mediterranean area (Keys, 1995) lead to the hypothesis that olive oil phenolics exert a protective effect with respect to chemically-induced oxidation of human LDL, which is one of the initial steps in the onset of atherosclerosis (Steinberg et al, 1989).

Oxysterols derived from both free and esterified cholesterol are an important consequence of the peroxidation of lipids occurring during the oxidative modification of LDL, involved in the development of atherosclerotic lesions (Steinberg et al 1989). Reports identifying oxLDL and oxysterols in atherosclerotic lesions of rabbit and man in atherosclerotic lesions suggest that inhibition of oxysterol formation by dietary antioxidant supplements can be important for reversing or limiting the complications of atherosclerosis *in vivo* (Ylä-Herttuala et al, 1989; Carpenter et al, 1995).

Results obtained on human oxLDL (Caruso et al, 2000), demonstrate that catecholic compounds present in the hydroalcoholic extract of virgin olive oil inhibit the formation of oxysterols in a dose dependent manner and are effective at a concentration lower than that of pure tyrosol, a phenolic component of the oil, and of probucol, used as reference compounds. This effect is probably due to the synergistic action of hydroxytyrosol, oleuropein aglycones, and of some flavonoids, such as quercetin (Bertulli et al, 1995), luteolin, and apigenin present in the virgin olive oil extract in minute amount

(Cortesi et al, 1995a and b). In addition, changes in electrophoretic mobility of apo B are also prevented by the phenols.

Hydroxytyrosol (HT) and oleuropein (OE) both potently and dose-dependently inhibit copper sulphate-induced oxidation of LDL at concentrations of 10^{-6} to 10^{-4} M (Visioli et al, 1994; Visioli et al, 1995). The inhibition of LDL oxidation by HT and OE can be demonstrated through the assessment of several markers, such as a reduced formation of short-chain aldehydes (evaluated as thiobarbituric acid-reacting substances, TBARS) and of lipid peroxides, by a higher vitamin E content in the residual LDL (indicating sparing of endogenous antioxidants), and by reduced formation of malondialdehyde-lysine and 4-hydroxynonenal-lysine adducts, indicating protection of the apoprotein layer (Visioli et al, 1995).

The free radical scavenging activities of hydroxytyrosol and oleuropein were further confirmed (Visioli et al, 1995, Aruoma et al, 1998), by the use of metal-independent oxidative systems and stable free radicals, such as DPPH (Visioli and Galli, 1998), in a series of experiments that demonstrated both a strong metal-chelation and a free-radical scavenging action.

As far as the mechanism of action of olive oil phenolics is concerned, it is well-known that the antioxidant properties of *o*-diphenols are related to hydrogen-donation, which is their ability to improve radical stability by forming

an intramolecular hydrogen bond between the free hydrogens of their hydroxyl group and their phenoxy radicals (Visioli and Galli, 1998). Although specific investigations of olive oil phenols are yet to be carried out, studies performed on the structure-activity relationship of flavonoids indicated that the degree of antioxidant activity is strictly related with the number of hydroxyl substitutions (Rice-Evans et al, 1996).

The mutagenic properties of oxidatively-damaged DNA suggest that antioxidants might have protective activity toward tumor formation. Low concentrations of hydroxytyrosol, *i.e.* 50 μM , are able to scavenge peroxynitrite and therefore to prevent ONOO^- -dependent DNA damage and tyrosine nitration, as demonstrated by Aruoma and Deiana (Arouma, 1987, Deiana et al, 1999); also, in a model of copper-induced DNA damage, the prooxidant activities of hydroxytyrosol (which are due to its copper-reducing properties) become evident at non-physiological concentrations ($>500 \mu\text{M}$) and are 40-fold weaker than those of the widely-employed reducing agent ascorbate (Deiana et al, 1999).

Oleuropein increases the functional activity of immune-competent cells (macrophages), as demonstrated by a significant increase ($+ 58.7 \pm 4.6\%$) in the lipopolysaccharide (LPS)-induced production of nitric oxide, a bactericidal and cytostatic agent (Visioli et al, 1998). This increase is consequent to a direct tonic effect of oleuropein on the inducible form of the enzyme nitric oxide

synthase (iNOS), as demonstrated by Western blot analysis of cell homogenates and by coincubation of LPS-challenged cells with the iNOS inhibitor L-nitromethylarginine methylester (Visioli et al, 1998).

iii) *In vivo* studies

Experimental evidence that phenolic compounds of different origin are absorbed from the diet is accumulating. Animal studies in rats and rabbits demonstrated that LDL isolated from animals fed virgin olive oil exhibit a higher resistance to oxidation when compared to animals given a triglyceride preparation with an equivalent amount of oleic acid, *i.e.* triolein (Scaccini et al, 1992), or «plain» olive oil (Wiseman et al, 1996).

In collaboration with the group of Prof. Pagnan of the University of Padua, we demonstrated the post-prandial absorption of olive oil phenolics and their incorporation into human lipoproteins (Bonanome et al, 2000). The findings of the study suggest that phenolics compound in olive oil are absorbed from intestine through a pathway independent of chylomicron formation and exert a significant antioxidant effect *in vivo*, in the postprandial phase.

With respect to the excretion, we demonstrated that olive oil phenolics are dose-dependently absorbed in humans and that they are excreted in the urine mainly as glucuronide conjugates; it is noteworthy that increasing amounts of phenolics administered with olive oil stimulated the rate of conjugation with

glucuronide (Visioli et al, 2000). These data add to the growing experimental evidence that indicates absorption and urinary disposition of flavonoids in humans (Wiseman et al, 1996).

It is noteworthy that HT exists in the brain as an endogenous catabolite of catecholic neurotransmitters such as dopamine and norepinephrine (Lamensdorf et al, 2000), but its presence in urine has never, until now, been described. On the other hand, the formation of homovanillic alcohol (HVAIc), i.e. the O-methylated derivative of HT, was reported by Manna (Manna et al, 2000) in human Caco-2 cell incubated with HT. In view of this report, we analysed the human urinary samples described by Visioli et al. (2000) for the presence of *catechol-O-methyl transferase* (COMT) derivatives and/or additional HT metabolites. In particular, we reported for the first time the urinary excretion of HVAIc, in large excess over its basal excretion ($57 \pm 3 \mu\text{g}$ excreted in 24 hours, means \pm SD, n= 6). We also described the substrate-induced enhancement of HVA formation, also a product of catecholamines metabolism, in addition to its basal urinary excretion ($1660 \pm 350 \mu\text{g}$ excreted in 24 hours, means \pm SD, n= 6). Indeed, the results reported there suggest that HT increases the basal excretion of HVA even at low doses of phenols administered. A major limitation of the study is that it employed oil samples artificially enriched with a phenolic extract, and thus extrapolation of these results to a typical Mediterranean diet pattern should be exerted with care. However, the

correlation between the metabolites and the total amount of HT provided with the oil samples, which is maintained over the range of doses we adopted, *i.e.* from 7.00 to 23.15 mg, suggests that the proportion of the different metabolites is also retained at much lower intakes. Future investigations will adopt commercially available virgin olive oils, thus allowing to further elucidate the *in vivo* kinetics of olive oil phenolics in habitual consumption quantities.

Recently we demonstrated that hydroxytyrosol, administered to rats as the only bioactive component of an olive mill waste water extract, is able to increase plasma antioxidant capacity (Visioli et al, 2001). Also, low doses of HT, *i.e.* 414 $\mu\text{g}/\text{rat}$, are able to blunt the side stream smoke-induced increase in urinary excretion of F₂-isoprostanes, the non-enzymatic product of arachidonic acid oxidation that is considered the most reliable biomarker of *in vivo* oxidative stress. A human experiment was performed and demonstrated an inverse correlation between olive oil phenolics ingestion and urinary concentrations of F₂-isoprostanes. These *in vivo* data strongly suggest that olive oil catechols, namely hydroxytyrosol, retain their antioxidant activity after oral administration.

iv) Epidemiological studies

Epidemiological studies continue to link Mediterranean dietary habits with a lower occurrence of cardiovascular events and cancers. From a nutritional

point of view, choice of a phenol-rich olive oil contributes to the dietary intake of biologically-active compounds, in estimated quantities that have been correlated with a reduced risk of developing CHD (Hertog et al, 1993). Indeed, the use of extra-virgin olive oil as the principle source of dietary fat - and therefore in substitution for animal fat - in addition to providing a considerable amount of oleic acid, provides intake of bioactive compounds with potential healthful effects, as described above. It then appears that the intake and the interaction of several "micronutrients" provided by a healthful diet, such as that in use in the Mediterranean area during the mid-1940s, is likely the link that affords protection from such pathologies. In turn, the answer to the current debate on the efficacy of antioxidant supplements is likely to be found in the adoption of a Mediterranean-style diet, in which the abundance of bioactive, functional compounds provided by fruits, vegetables, wine, and olive oil grants a higher protection toward ROS-induced diseases.

In conclusion, the biologically relevant properties of olive phenolics described in this article, although still to be fully investigated in controlled clinical trials, provide evidence to support the hypothesis that virgin olive oil consumption may contribute to human health. In this respect, being endowed with health-promoting activities, we think it proper to propose virgin olive oil as an example of functional food.

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Figure legends

Scheme 1 Chemical structure of isomers formed during oleuropein hydrolysis.

The structure above the dotted lines indicates the hydroxytyrosol-containing fragment at m/z 307, lost by collision of the ion at m/z 377 in APCI-MS/MS analysis [see Fig. 2, panels B and C].

Fig. 1 - APCI-MS spectrum obtained by loop injection of a virgin olive oil extract.

Ion at m/z 137 = tyrosol, m/z 153 = hydroxytyrosol, m/z 241 = elenolic acid, m/z 303 = deacetoxy ligstroside aglycon, m/z 319 = deacetoxy oleuropein aglycon, m/z 361 = ligstroside aglycon, m/z 377 = oleuropein aglycon, m/z 393 = 10-hydroxy-oleuropein.

Fig. 2 - Mass spectrometric analysis of oleuropein aglycon

Panel A: LC-APCI-MS of oleuropein aglycons: ion at m/z 377 (see scheme 1)

Panel B: APCI-MS spectrum obtained by loop injection of oleuropein aglycon

Panel C: APCI-MS/MS spectrum obtained by collision of ion at m/z 377

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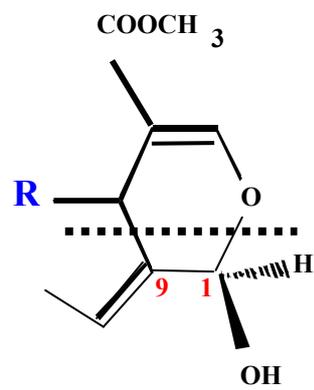
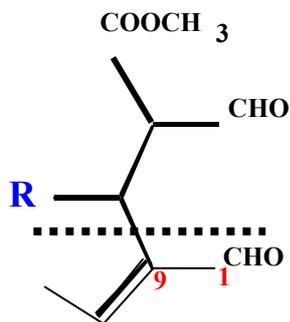
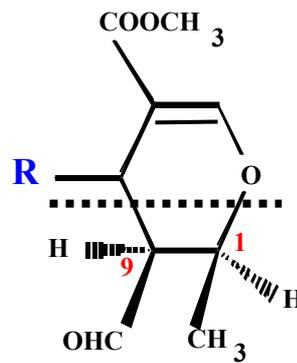
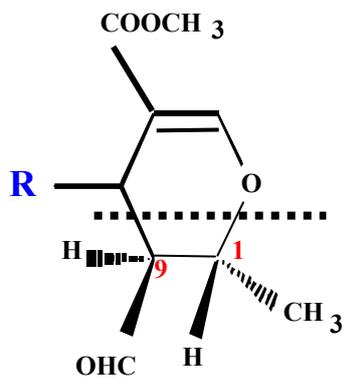
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Fig. 2 - Mass spectrometric analysis of oleuropein aglycon

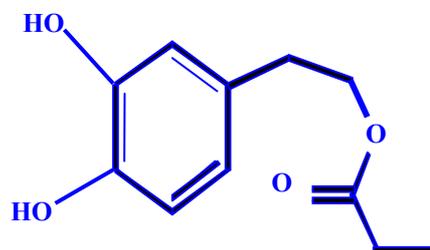
Panel A: LC-APCI-MS of oleuropein aglycons: ion at m/z 377 (see scheme 1)

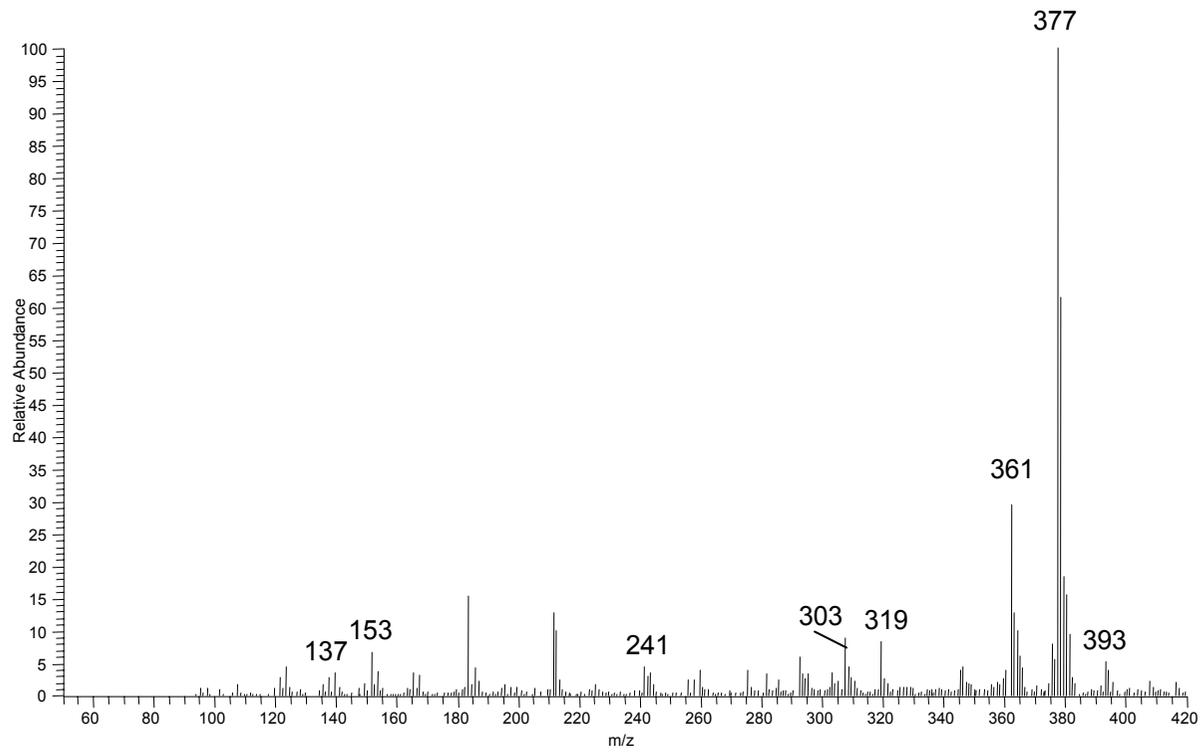
Panel B: APCI-MS spectrum obtained by loop injection of oleuropein aglycon

Panel C: APCI-MS/MS spectrum obtained by collision of ion at m/z 377.

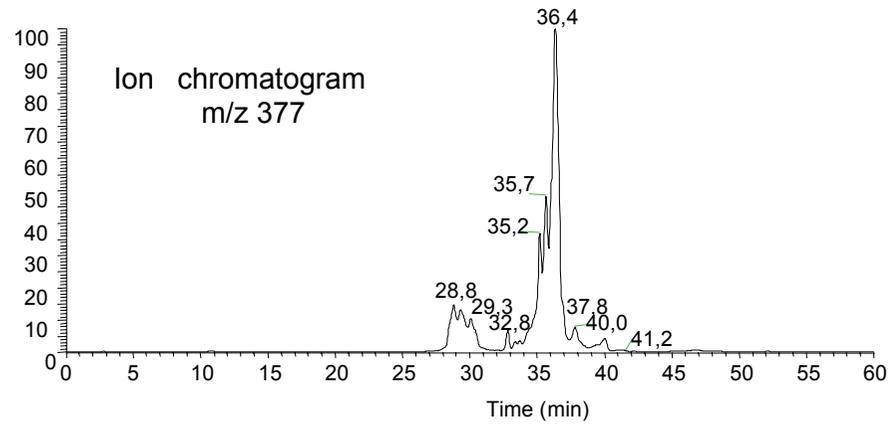


R = Hydroxytyrosol residue

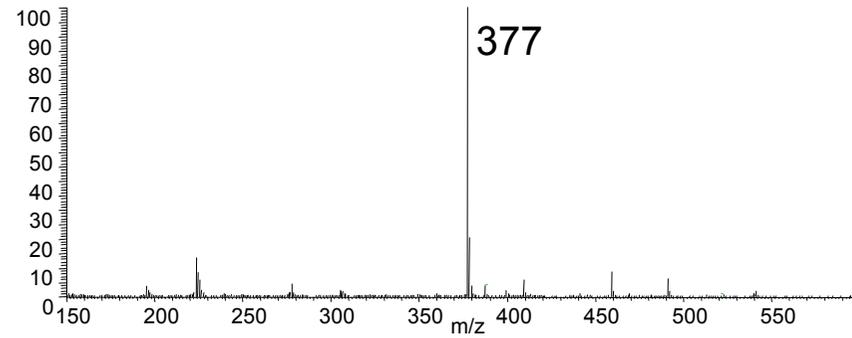




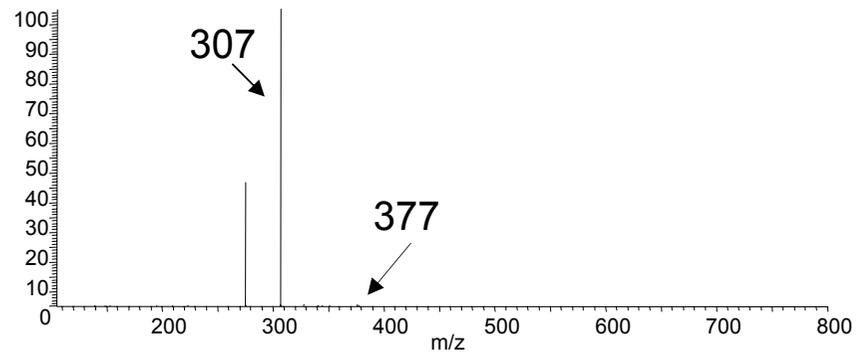
Panel A



Panel B



Panel C



TOMATO FUNCTIONAL FOODS AS INTERFERON ADJUVANT IN HCV ERADICATION THERAPY

Short title: Functional Food for HCV infection

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Summary

Background: Oxidative stress plays a major role in the physiopathology of hemolytic anemia during ribavirin therapy. The efficacy of antioxidant supplementation (vitamin C and E as pure compounds) is still controversial. **Aim:** we conducted a study to verify if the supplementation with an antioxidant-rich tomato-based functional food (FF) reduces the anemia during peginterferon (PEG-IFN) and ribavirin (RBV) therapy for chronic hepatitis C (CHC). **Patients and methods:** A functional food with high content of natural antioxidants and with high carotenoid bioavailability was developed. We enrolled 92 patients (70M/22F) with CHC, treated with standard combination therapy. 46 of them received a daily dose (100g.) of FF (group 1), and 46 did not (group 2). At baseline, the groups were similar for demographics (M/F, body weight, age); mean hemoglobin (Hb) levels, and both groups received similar mean dose of RBV. The effect of antioxidant activity was assessed comparing the compliance to the full dose of RBV and the Hb levels during the first three months of treatment. **Results:** Only 8.7% patients of group 1 had to reduce daily RBV dose, while RBV reduction was necessary for 30.4% of group 2 ($p=0.09$). Hb levels showed significant differences at 15, 30, and 90 days during the observation time. **Conclusions:** Results demonstrated that our FF reduces the severity of ribavirin-related anemia and it improves the tolerance to the full dose of ribavirin in patients with chronic hepatitis C.

Introduction

It is well known that Ribavirin, the first line therapy for chronic hepatitis C in association with interferon-alpha, has a reversible anemia as a typical side effect (1-2). The impact of this adverse effect on the management of patients is high and in most cases the severity of the anemia leads to dosage reduction or discontinuation of the drug (2). This issue is a major concern if we consider that the success of antiviral therapy is well related to the adherence to the full dose of the therapy. Reduction or discontinuation of ribavirin potentially causes a reduction in efficacy, and every modification has to be avoided.

The mechanism of ribavirin-induced anemia is not clearly understood, but it seems related to a phenomenon of oxidative stress that induces hemolysis after significant alterations of the erythrocyte membrane. The lack of adequate amount of antioxidant compounds induces membrane oxidative damage, more rapid senescence of the erythrocytes and extravascular hemolysis by reticuloendothelial system (3).

This study was designed to evaluate whether the supplementation with an antioxidant-rich carotenoids-based functional food (FF) was able to prevent and /or to reduce the ribavirin-induced anemia during peginterferon (PEG-IFN) and ribavirin (RBV) therapy in patients with chronic hepatitis C (CHC).

Patients and Methods

Production of functional food and bioavailability study

A functional food with high content of natural antioxidants compounds has been designed and produced at the Department of Food Science University of Naples "Federico II". The FF is characterized by a high content of carotenoids and in particular by the presence of different cis-isomers of lycopene.

The plasma concentrations of carotenoid components has been determined in 20 patients with chronic hepatitis C candidates to antiviral therapy. The patients were randomly assigned to intake or not 100 g/die of FF for a period of 30 consecutive days. In patients with food supplementation a significant increase of plasma concentration of the various species of the carotenoids was documented and the plasma levels showed a direct relation to the carotenoids concentration in the food. In particular the levels of beta-carotene and lycopene showed an increase of about 1/3 compared to basal levels.

Clinical study design

We admitted 92 patients (70M/22F) with CHC, all treated with Peginterferon and Ribavirin with a standard schedule therapy. Forty six of them received a daily dose (100g) of FF for 3 months (group 1), and 46 did not (group 2). At baseline, the two groups of patients were similar for the principal demographic characteristics (M/F, body weight, age); mean hemoglobin (Hb) levels, and both group received similar mean dose of RBV.

The efficacy of FF was assessed at 12 weeks comparing the proportion of patients with:

1. improvement of the oxidative status (assessed by D-ROM test , Diacron Italy);
2. discontinuation of ribavirin therapy due to anemia (usually < 8.5 g/dl);
3. reduction of ribavirin dose due to decrease in hemoglobin levels (usually for hemoglobin between 10 and 8.5 g/dl)

with median hemoglobin levels during the three months of therapy .

Results

Baseline values of serum hydroperoxides did not differ significantly between the two groups.

During treatment, an improvement of the total oxidative status was observed in both groups even if the improvement was higher in patients with FF supplementation.

No patients in both groups have discontinued ribavirin. On the other hand, only 8.7% patients of group 1 had to reduce daily RBV dose, while RBV reduction was for 30.4% of group 2 ($p=0.09$). Finally, the comparison of mean hemoglobin levels between groups showed significant differences at 15, 30, and 90 days of treatment with the benefit for the group with food supplementation.

Discussion

The combination of ribavirin and interferon- α has been the standard treatment for chronic hepatitis C (4). The most frequent effects of the treatment is anemia that leads to dose reduction or discontinuation of the therapy. Both interferon and ribavirin contribute to anemia by inhibiting erythropoiesis, but it has also been demonstrated that ribavirin induces reversible hemolytic anemia by oxidative damage of the erythrocyte membrane (3).

The involvement of oxidative stress in red cell damage supports the utilization of antioxidant molecules even if the efficacy of supplementation with vitamin E and vitamin C as pure compounds is still controversial. The lack of efficacy is probably due to the low dose used, which in return leads to low bioavailability (5).

The studies using 800 IU daily of vitamin E seems to show more benefits than 400 IU, which is not effective, but in general the therapeutic trials give inconclusive results.

Carotenoids have been proposed as a good source of antioxidants and recent studies have been suggested that the consumption as fruits and vegetables are more efficacious than the consumption as pure compounds for a particular interaction and synergism that improves the bioavailability (6).

Tomato and tomato products, have been inversely related to a development of some types of cancer, and lycopene has been hypothesized as being the principal source responsible for this effect for its high antioxidant activity (7-8).

Indeed, the use of carotenoid-based FF can be considered for its antioxidant properties a promising treatment in preventing and limiting ribavirin-induced anemia.

In patients with FF supplementation we have observed an improvement in mean hemoglobin levels of 1g/dl compared to the levels of patients managed without FF. Moreover, this FF was well accepted, without side effects, cost effective and particularly suitable for large scale studies. This therapy may be key to further improvement of the treatment.

In conclusion, this study shows that the regular use of our carotenoid-based functional food minimizes the severity of ribavirin- induced anemia in patients with chronic hepatitis C and improves the tolerance to the full dose of antiviral therapy.

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SOY ISOFLAVON BASED FUNCTIONAL FOOD

Short Running Title: SOY ISOFLAVON

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Epidemiological studies (1-3) clearly establish a connection between diet and many of major causes of morbidity and mortality in Western nations. According to the World Health Organization (4) in 1996 over 10,000,000 individuals worldwide were affected by tumors of which 30-40% are preventable with appropriate diet modification.

A link between disease and Western diets appears particularly well established for some of the more important hormone-dependant pathologies, the frequency of which is much lower in Asian nations (5). These include cardiovascular diseases, breast and prostate cancer, osteoporosis, and even post-menopausal climateric syndrome. The specific components of the diet responsible for the protective effects

are difficult to identify with certainty and a broad range of non-nutrient bioactive compounds have been investigated. Of these phytoprotectants, the phytoestrogens have received perhaps the most attention.

The principle classes of phytoestrogens are represented by the isoflavones, lignans and coumestans (Fig. 1).

The isoflavones (comprising mainly genistein and the daidzein and their glycoside conjugates) are present in legumes, with the highest concentration being in soy. Lignans, on the other hand are ubiquitous to the plant kingdom and are found in whole-grains, cereals, fruits and vegetables, but the highest concentrations are found in seeds of flax (Table I) behave as natural selective estrogen receptor modulators (6-7) having many of the beneficial properties of estrogen but apparently without the deleterious effects. They are partial agonists, by having a high affinity for estrogen receptors (particularly for ER β) and a low estrogenic activity (10^{-2} to 10^{-3} as compared to estradiol or estrone). The estrogens instead have a high affinity and a high activity for the same receptors (6-7). It is important to note that there are two main receptor subtypes for the estrogens: a) ER α b) ER β . The classical estrogens and tamoxifen bind preferentially to ER α . Instead, the phytoestrogens and the isoflavones preferentially bind to ER β (the ability to bind to the receptor of the estrogens is 100 to 10,000 times greater than for the estradiol). These two populations of receptors are differentiated by their distribution in the organs, diversity in affinity for their ligands and their different biological effects (8).

The estrogen' receptors have a different distribution in the organs with ER β being expressed in the prostate gland, breast, vascular endothelium, brain, bone, and bladder and thymus sites that are specific targets of substitutive hormonal therapy.

There is a strict structural similarity between isoflavones and estrogens, especially in the two diphenolic rings (Fig. 2). There are innumerable beneficial effects in the tumoral, inflammatory, cardiovascular, post-menopausal, cognitive, immune and alcoholic pathologies. Schematically we can focus on the most important biological properties connected to the hormonal modulation of the isoflavones:

- a) **cardiovascular system:** ↓ LDL-cholesterol and ↑ HDL-cholesterol and associated risk factors for cardiovascular disease;
- b) **post-menopausal syndrome:** ↓ hot flashes; ↓vaginal mucosa dryness
- c) **osteoporosis:** regulation of the activity of osteoclasts and osteoblasts
- d) **anticarcinogenic:** Breast, prostate, lung and colon-rectal cancers

Additionally, there are many other non-hormonal actions of isoflavones that are of relevance to chronic disease prevention and/or treatment. These include:

- a) **antioxidant effect:** permits the neutralization of free radicals (substances implicated in the peroxidation of cellular membrane lipids and in mutagenesis)
- b) **angiogenesis inhibition:** (control tumoral growth and metastasis)
- c) **control of blood vessel elasticity and vasomotor tone**

Action on the cardiovascular apparatus: the United States of America, Food and Drug Administration (1999) and recently the JCHI in the United Kingdom (2002) approved a health claim for soy protein in reducing risk of heart disease. This claim allowed producers of soy based foods containing the mandatory 6.25 grams of soy protein per serving to label soy foods as follows: “Soy protein lowers the risk of heart disease by its beneficial effects on blood lipids”(9, 10, 11, 12). Crouse JR et al. (13) showed that the reduction of LDL-cholesterol was in a dose-dependent manner related to the presence of isoflavones by determining the lipid-lowering effects of 25 g/d of soy protein containing varying levels of isoflavones. The hypocholesterolemic effect (Fig. 3), appeared substantially in the group of patients with highest levels of LDL cholesterol (> 4.29 mmol/L) as compared to patients with intermediate values or to the control group that consuming 25 g of casein instead soy proteins. In another study Anthony MS in 1998 (14) demonstrated a significant decrease of LDL and VLDL cholesterol levels in monkeys and a reduction of atheromatous lesions in the aorta of rabbits feed a diet rich of isoflavones, as compared to the control groups. Tikkanen et al. (1998) moreover demonstrated a reduction in the susceptibility of LDL to the oxidative modification in subjects treated with isoflavones (57 mg/day) for two weeks (15).

Activity on osteoporosis and post-menopausal syndrome: It has been hypothesized that there may be an important role for phytoestrogens for bone tissue; this idea originates from the observation that Asiatic nations have a

lower incidence of osteoporotic fractures in respect to Western nations. Pansini et al. (1997) revealed a beneficial effect by phytoestrogens in osteoporosis. In their study it was shown that a diet rich in phytoestrogens (60 mg/day) caused a reduction of osteoclastic activity and an increase in bone formation, indicating a respective reduction, in 4 and 12 weeks, of blood levels of N-telopeptide (osteoclastic activity marker) and an increase of osteocalcin (osteosynthesis activity marker) (16). Moreover Lydeking et al (17) in the first two year study 201 postmenopausal women were randomized to consume two glasses of soymilk each day either with or without isoflavones, isoflavone rich soymilk prevented bone loss as measured by changes in lumbar spine BMD. Lumbar spine BMD decreased 4.3% respectively over 2 year period in the group consuming soymilk with negligible amounts of isoflavones. By contrast, those consuming soymilk that contained 50 mg isoflavones showed an increase of 0.5% in lumbar spine BMD, thus confirming the preventive effect of isoflavones on endocrine-related bone loss.

There is evidence that isoflavones have a positive effect on postmenopausal syndrome, by reducing hot flashes and vaginal mucosa dryness. This appears to be particularly the case in those women with the most severe symptoms of hot flashes (18). Murkies et al studied a sample of 28 postmenopausal women to whom they administered 45 g daily of soy for a period of 12 weeks. They reported a reduction of 40% in the frequency of hot flashes (19).

Anticarcinogen action: In the last years it has been shown that isoflavones may have a protective role against some types of hormone-dependent tumors, such as breast, prostate and endometrium cancers.

Epidemiological studies have revealed that in Asian countries such as Japan, the incidence of these neoplasias is much lower than in the Western countries (5). This has led to the proposal that isoflavones in soy foods may have chemopreventive effects. This hypothesis is supported by the observations that consumption of soy foods is inversely correlated with the risk of hormone-dependant tumors. A case control study found that women with breast cancer had the lowest urinary concentration of isoflavones and lignans. A study conducted by Barnes et al. in rats showed that a diet rich in isoflavones reduced the formation of breast tumors induced by carcinogen N-methyl-nitrosourea by 50% (20). These animals studies have been confirmed by many other workers and it has been additionally shown that the earlier that the animal is exposed to soy isoflavones the greater is the reduction in breast tumors. This seems to be applicable to humans because a recent Chinese study found that women who ate the most soy foods as adolescents were at the lowest risk for developing breast cancer (21).

Other important results have been found for prostate and colon-rectal cancers, even though they need to be confirmed with other studies. Adlercreutz et al. (22) in 1997 found that Japanese individuals with high levels of isoflavones in their prostatic liquid also have a low level of PSA and a minor

increase in the prostate dimension in the elderly. Other studies conducted by Kuo et al. have indicated an inhibition of the cellular line of colon cancer (CaCo-2; HT-29) after exposure to isoflavones (23) while Hu J. et al. have revealed a reduction in rectal tumors in individuals that have a high consumption of soy (24). The anticarcinogenic activity of isoflavones seems to be established by its control over the enzyme activity of thyroxine-kinase, DNA topoisomerase I-II, S6 kinase, important enzymes in the control of mitogenesis, the cellular cycle, cellular survival and cellular transformation. Moreover the isoflavones seem to inhibit the angiogenesis of the tumoral vessels (25) (Fig. 4). Even though the beneficial effects have clearly been demonstrated, the traditional Asian foods based on soy (tofu, miso and soymilk) are not particularly liked by Western populations. This creates a difficulty in stimulating a generalized increase in the consumption of the daily quota of isoflavones. We believe that this quota could be easily reached, instead, with a commonly recognized food enriched with isoflavones, which would permit the consumption adequate amounts of these substances with good compliance and without upsetting the dietetic habits of individuals. What is needed then is to transform an everyday food, generally appreciated for its good taste, in to a so called “functional food”, in which there would be ingredients with biological effects clearly healthy. We have therefore developed a pasta with a natural extract of soy riched in isoflavones. We have evaluated the acceptability, the tolerability and the health effects in patients

with hyperlipidemia and alteration in endothelial function. Preliminary data in 30 patients has shown after 5 weeks of soy-enriched pasta a significant decrement in cholesterol levels (-6%), followed by an increment on cholesterol after 4 weeks from the last introduction of pasta (-1.8) (Fig. 5). Moreover the introduction of soy-enriched pasta showed a decrement in C-reactive protein an important marker of inflammation. Functional food can be useful for the prevention and treatment of hormonal-dependent diseases.

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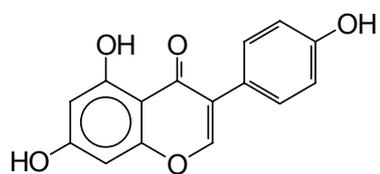
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Where we can find Isoflavones and Lignans ($\mu\text{g/g}$)

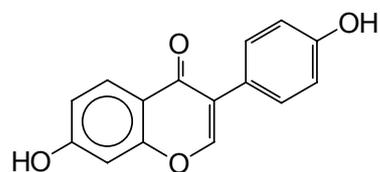
	ISOFLAVONES		
	Daidzein	Genistein	
•Soy bean	105-850	268-1025	<i>Mazur et al (1998)</i>
•Lentils	0,03-0,10	0,07-0,19	“
•Beans	0,07-0,40	0,18-5,18	“
•Peas	0,11-1,92	0,69-2,14	“
	LIGNANS		
	Total contents		
•Linseed	526,8		<i>Thompson et al (1991)</i>
•Lentils	17,9		“
•Soy bean	8,6		“
•Wheat	4,9		“
•Sunflowers	4,0		“
•Asparagus	3,7		“
•Carrots	3,5		“
•Sweet potato	3,0		“
•Broccoli	2,3		“
•Pear	1,8		“

Table I.

Isoflavones

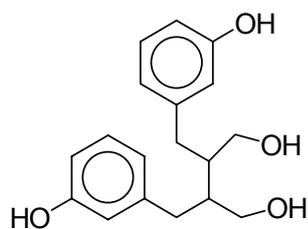


Genistein

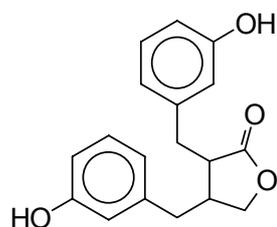


Daidzein

Lignans



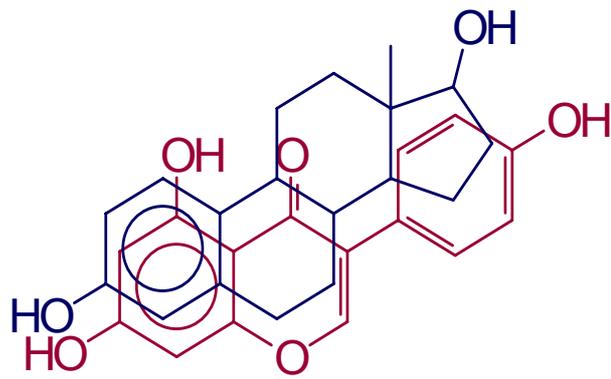
Enterodiol



Enterolactone

Fig. 1

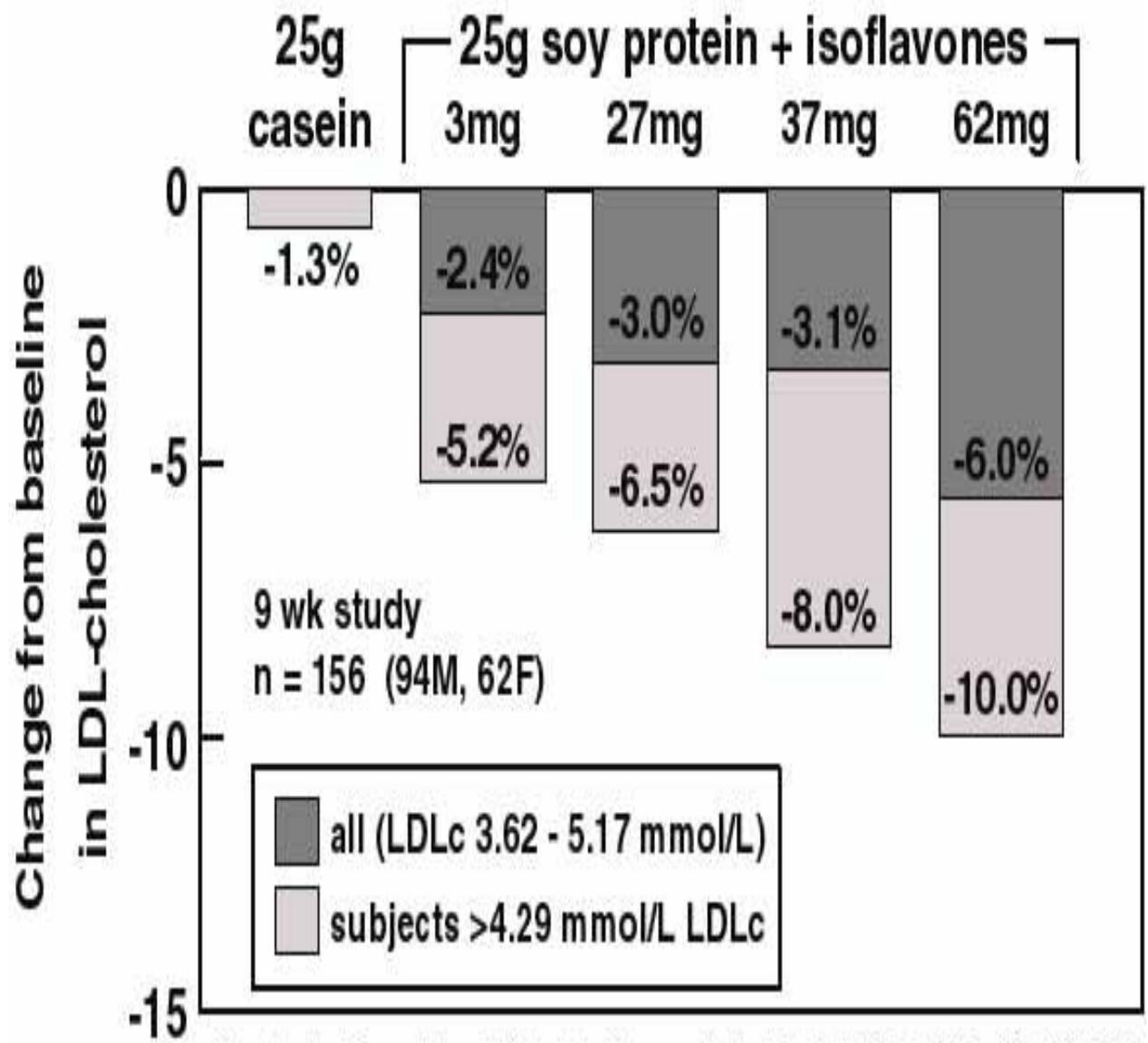
Similarity of Isoflavones to Estrogens



Estradiol

Genistein

Fig. 2



Constructed from data published by Crouse JR et al, Arch Int Med, 1999; 159:2070-2076

Fig. 3

ANTICARCINOGENIC ACTIVITY OF ISOFLAVONES

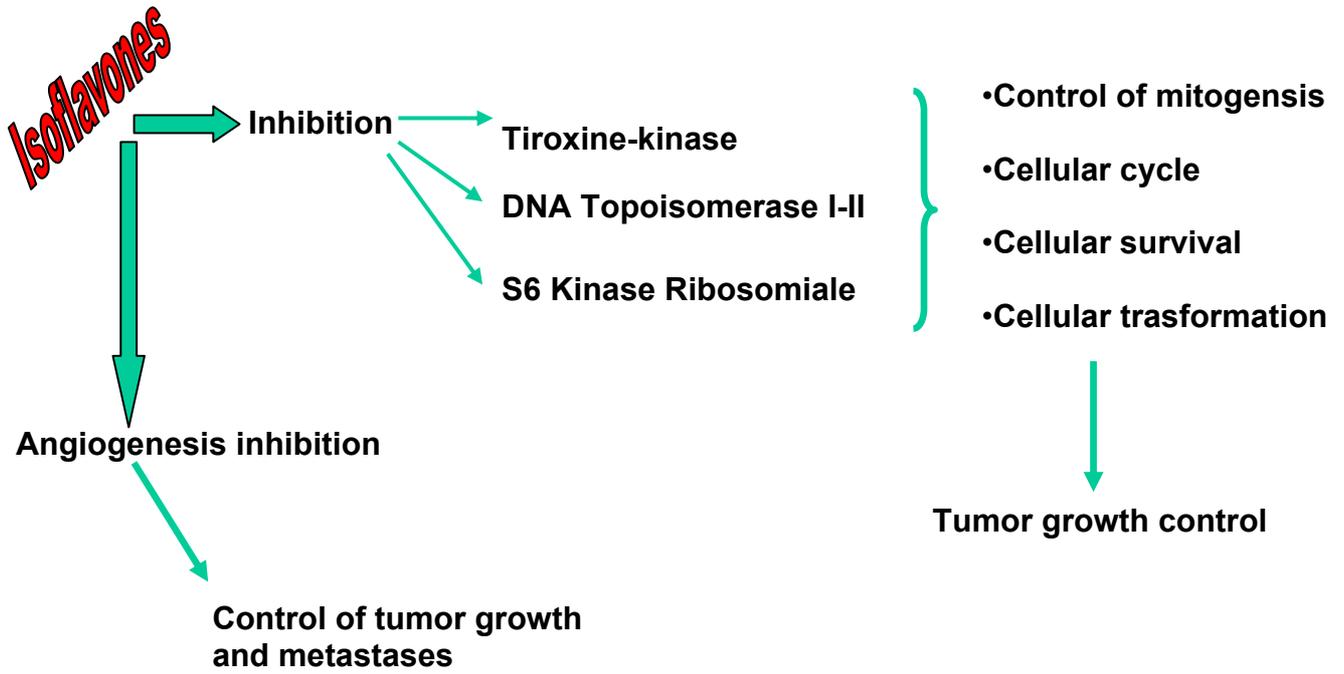


Fig. 4

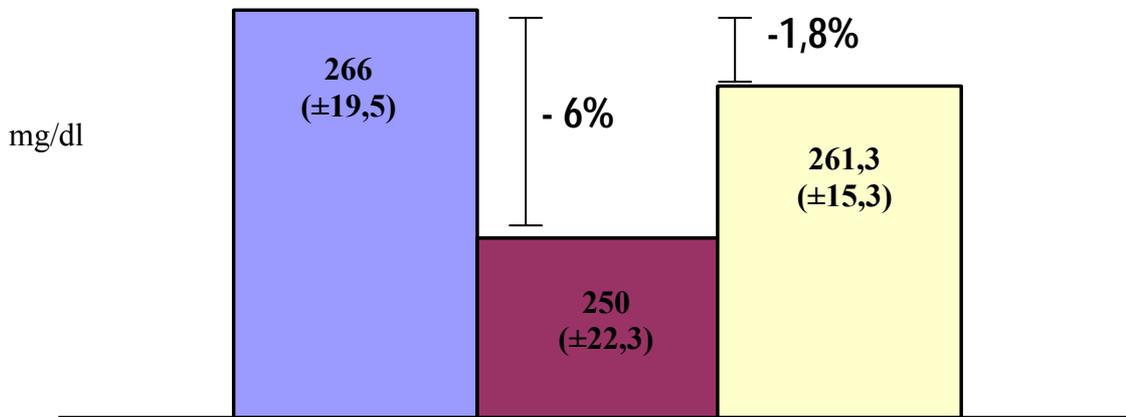


Fig. 5. Comparison of average levels of total cholesterol (T-Ch). ■ T-Ch before pasta (after 2 weeks from the beginning of study); ■ T-Ch after pasta (after 5 weeks of soy enriched pasta); ■ T-Ch after 4 weeks of only diet (without soy enriched pasta).

Gut Microbe Diversity

- the human commensal intestinal microbiota revisited: a phylogenetic perspective -

J Doré

INRA, Jouy-en-Josas FRANCE

Over the past century, the recognition of anaerobiosis and the design and application of anaerobic culture methods has allowed the isolation and characterization of a large diversity of human faecal bacteria. It is commonly considered that over 400 bacterial species constitute the dominant faecal flora of Man. Yet as it became clear that only a minor fraction of the intestinal microbiota was culturable *in vitro*, emerging technologies allowed the reassessment of its species diversity without the bias of culturability. Comparative sequencing of cloned 16S rRNAs proved especially instrumental in that respect. We will review the information derived from the rRNA approach in comparison to culture based knowledge.

Based on molecular estimates, over 80% of the phylotypes observed in the human faecal flora belong to four main phylogenetic lineages : Gram negatives of the *Bacteroides-Porphyromonas-Prevotella* cluster, high G+C gram positives of the *Bifidobacterium* genus and low G+C gram positives within the phylum of “*Clostridium* and relatives” belonging to the *Eubacterium rectale-Clostridium coccooides* group (cluster XIV) or the *Fusobacterium prausnitzii-Clostridium leptum* sub-group (cluster IV). Hybridization studies have confirmed that all human adults bear these groups in dominance in their faecal flora.

In the fecal flora of adults, more than 80% of the molecular species observed (more than 60% of cloned sequences) derive from microorganisms that have no representative in current culture collections, and hence may be non-yet cultured. This is especially true for gram positives of the *Clostridium* clusters IV and XIV. The proportion of yet unrecognized species is even greater in the elderly fecal flora where we have observed an increase in the overall diversity and recorded the presence of totally novel, deep branching, phylogenetic groups. The specificities of the fecal flora of patients suffering from inflammatory bowel disease was also investigated.

Cultured representatives of the 20% known molecular species observed so far in the adult human fecal flora all originated from gastro-intestinal contents of man or animals. Very few of the known molecular species and overall very few of the molecular species observed were common to the faecal flora of several individuals. In other words, in spite of the functional homogeneity of human faecal contents based on metabolites or enzymatic activities, it clearly appears that at the species level, each dominant human faecal flora is unique. This is consistent with denaturing gradient gel electrophoresis data which also showed a great stability over time in any given human faecal flora.

A complete reassessment of the human faecal flora is hence underway and application of culture-independent methods over the past few years has sharply increased our understanding of this ecosystem. It becomes clear that its biodiversity is far more complex than anticipated, and this should orient future research towards an essential goal of the gut microbial ecologist - understanding the role microorganism play in their ecosystem. It will require a return to the ecosystem to sort out and characterize microorganisms of dominant functional groups that have so far escaped cultivation.

Tolerance of probiotics and prebiotics

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Probiotics have been defined as non pathogenic micro-organisms which, when ingested, exert a positive influence on host health or physiology (1). Some strains have a high survival capacity till faeces, and some can colonise the digestive tracts for some time while other are rapidly killed by acid and bile (2). Prebiotics have been defined as non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve host health (3). They are not digested in the small bowel but fermented in the colon. At the present time they essentially consist in non digestible oligosaccharides (NDOs) which stimulate the growth of bifidobacteria. There is a growing interest in the field of both probiotics and prebiotics as randomised controlled trials have shown that they may have clinical benefits in various physiological or pathological situations (4,5). They are thus more and more used in drugs, food supplements or even functional foods. The tolerance of the available products is usually excellent, however, they may have side effects which are reviewed here. Potential side effects are more acceptable for probiotic or prebiotic drugs (for which a risk to benefit ratio can be estimated and which can be more easily monitored using the classical pharmacovigilance methods) than for food products which have to be "very safe" as they are consumed by the general population without restriction.

Probiotics

The safety of the current products is excellent, however, probiotics as living micro-organisms may theoretically be responsible for four types of side effects in susceptible individuals: infections, deleterious metabolic activities, excessive immune stimulation, and gene transfer.

Infections

Probiotics are not selected among pathogens, and the theoretical risk of infections is thus very low. However, the zero risk does not exist and one may imagine that they could invade the organism and cause infection either via translocation from the intestine or via other routes (for example external colonisation of catheter devices). Translocation is defined as the passage of micro-organisms from the gastrointestinal tract to extraintestinal sites, such as the mesenteric lymph nodes, liver, spleen, and bloodstream. Indigenous bacteria are continuously translocating in low numbers but are rapidly killed in the lymphoid organs. Bacterial translocation is a major cause of severe infections in immunosuppressed, trauma, and postsurgical patients. It may result from 4 mechanisms which may act simultaneously: aggressiveness of certain micro-organisms (especially pathogens), intestinal bacterial overgrowth, increased permeability or damage of the intestinal mucosal barrier, and immunodeficiency. Whether some probiotics physiologically translocate in man has never been studied until now ; trials are ongoing in this field. One may also imagine that translocation of probiotics would enhance their immunological effects or that it could influence the translocation of deleterious micro-organisms.

Infections due to endogenous micro-organisms close to probiotics

Rare cases of infections including septicaemia and endocarditis due to lactobacilli, bifidobacteria or other lactic acid bacteria have been reported [6]. Most Lactobacillus strains isolated from clinical cases

belong to the species *L. rhamnosus*, *L. casei* or *paracasei*, and *L. plantarum*. *Enterococcus faecium* and *E. faecalis* are more frequently involved in clinical infections, and there is a special concern over vancomycin resistant strains. Nearly all patients have had serious underlying conditions which predisposed them to infection, particularly abnormal heart valves in the case of endocarditis, and the presence of a catheter in cases of septicaemia (7). Vancomycin treatment may also be a risk factor in immunocompromised patients, as lactobacilli are naturally resistant to vancomycin (7). In most cases of infection, the organism appeared to have come from the patient's own microflora (8).

Infections with probiotic microorganisms

A few cases of clinical infections due to probiotics have been observed : about 30 cases of fungaemia in sick patients treated with *S. boulardii* [8-12], and two case of infection with probiotic *L. rhamnosus* [13,14].

All subjects who had a fungaemia with *S. boulardii* were very ill patients who had an indwelling vascular catheter, and were treated in intensive care units (ICU) [9-12]. The yeast was used in these patients as randomised controlled trials demonstrated its efficacy in various clinical situations including antibiotic associated diarrhoea, *Clostridium difficile* infection, diarrhoea associated with enteral nutrition, and (with a lower evidence) in other situations such as Crohn's disease or HIV diarrhoea (15-19). It is available in closed capsule but was also developed in sachets which had to be open and poured in enteral nutrition formulae or drinks. The majority of infections occurred with the sachet form or when capsules were opened. Translocation of the yeast was not demonstrated (even in patients with intestinal ulceration (19,20) and a clinical study showed that the route of entry was probably an external contamination of the catheter (9). Indeed, Henequin et al. observed a contamination of the air, environmental surfaces, and hands of the nurses following the opening of the probiotic packets. It was therefore recommended for hospitalised patients that packets or capsules of *S. boulardii* (and this should be extended to all probiotics) should be opened with gloves and outside the patient's room [9]. Noticeably, several cases were reported in the same ICU while others did not observe any case which could suggest local hazards. The risk for immunosuppressed patients is unclear ; indeed *S. boulardii* has been shown to significantly protect immunodeficient mice against pathogens but yeast infection was also reported in immunosuppressed patients [10-12, 21]. It is likely that the risk would be similar if other probiotics were used in the same conditions in the same kind of patients. One should therefore learn from that, that probiotics should not be used in powder forms in ICU, that strict hygiene rules concerning their use should be followed, and that they should not be used in severely ill patients unless they have a medical indication and their tolerance is properly monitored.

One case of endocarditis due to a probiotic *L. rhamnosus* occurred in a 67 year old man with a mild mitral valve regurgitation who chewed a probiotic mixture after a dental extraction and antibiotic prophylaxis. *L. rhamnosus* was isolated from his blood and detailed analysis showed that the clinical strain was indistinguishable from one of the organisms present in the probiotic preparation that he used to consume [14]. Several "unusual" risk factors were thus associated in this case. However, a severe infection due to *L. rhamnosus* similar to the probiotic GG strain was also observed in a 74 year old woman whose only "risk factor" was non insulin dependent diabetes [13]. This woman suffered from a liver abscess and pleuropulmonary infection. No cause for the infection was found but the woman reported a regular consumption of dairy drinks containing *L. rhamnosus* GG. The clinical strain isolated from the abscess appeared to be indistinguishable from the GG strain. Searching for other potential cases, Saxelin et al. [22,23] studied the bacteraemia due to *Lactobacillus* species in Southern Finland over long periods and compared the characteristics of the blood culture isolates and of probiotic dairy strains. *L. rhamnosus* GG is largely used in dairy products in Finland. In their first study, lactobacilli were identified in eight among 3,317 blood culture isolates and none of the isolates corresponded to a

dairy strain. In a second study, 5,912 blood cultures were analysed, and none of the 12 lactobacilli isolated was identical to any of the commercial *Lactobacillus* strains.

Metabolic effects

If one admits that probiotics can vehiculate or promote metabolic activities in the gastrointestinal tract that may have positive effects for health, one may also admit that they may induce metabolic activities which may be detrimental to the host. High concentrations of micro-organisms are physiologically unusual in the small bowel. During bacterial colonisation of the small bowel, the deleterious micro-organisms present in high numbers can induce diarrhoea and intestinal lesions, especially through deconjugation and dehydroxylation of bile salts [24]. A study drew attention to the potential risk of excessive deconjugation or dehydroxylation of bile salts in the small bowel by some probiotics. Indeed, it showed in patients with ileostomy that ingested *L. acidophilus* and *Bifidobacterium sp.* could transform conjugated primary bile salts into free secondary bile salts (25). However, in this study this effect was very limited and did not have relevant dangers for the host. The development of bile salt hydrolysing probiotics which aimed to decrease blood cholesterol but may theoretically expose to this side effect has apparently been stopped. Excessive degradation of the intestinal mucus layer by probiotics may theoretically be detrimental. Some endogenous bacteria including lactobacilli and some strains of bifidobacteria have the ability to degrade mucus. Ruseler-van Embden et al. studied the mucus degrading properties of three probiotic strains contained in fermented milks (*L. acidophilus*, *Bifidobacterium sp.*, *L. rhamnosus* GG). No mucus degradation was observed *in vitro* nor in gnotobiotic rats monoassociated with these strains [26].

Immunological adverse events

When administered parenterally, bacterial cell wall components such as peptide-glycan-polysaccharides from different gram-positive bacteria including lactobacilli can induce side effects such as fever, arthritis or auto-immune diseases [27]. These side effects are mediated by cytokines, and it is now well established that cytokine secretion is elicited by some probiotics (28). Oral administration of high doses of lactic acid bacteria did not induce immunological side effects in mice [29]. However, a systemic uptake of cell wall polymers from the intestinal lumen, hence the immunological side effects, have been observed in rats with colonic injury [30]. The consequences of probiotic consumption in patients with autoimmune diseases should be studied. Indeed, one may imagine that it may have beneficial consequences or side effects. To our knowledge, no immunological side effect of a probiotic has been reported in man, except one case of auto-immune hepatitis which might have been enhanced by ingestion of very large doses of yoghurt [31]. Prantera et al. observed in a randomised controlled clinical trial that *L. rhamnosus* GG increased the recurrence rate of endoscopic lesions in patients who had been operated for Crohn's disease (60% vs 35% in the placebo group); however this effect was not statistically significant and open trials have suggested that *L. rhamnosus* GG may even have beneficial effects in this disease so that no clear clinical conclusion can be drawn (32).

Gene transfer of antibiotic resistance

Some antibiotic resistance genes can be transferred from ingested lactobacilli to the endogenous flora in the gastrointestinal tract [33]. The probability of gene transfer depends on the nature of the genetic material to be transferred (plasmids, transposons...), on the nature of the donor and recipient micro-organisms, on their concentrations, and on selection pressure (especially the presence of antibiotics). Probiotics should therefore not carry transferable antibiotic resistance genes (34).

*To summarise, the tolerance of commercial probiotics is excellent. 1- the rare cases of infections observed with *S. boulardii* occurred only in hospitalised patients who had an indwelling catheter and who were treated with the yeast for severe medical conditions; 2- there is no evidence that ingested probiotic lactobacilli or bifidobacteria pose any greater risk of infection than commensal strains; 3- there*

is insufficient knowledge on the risks or benefits (or risk to benefit ratio) of probiotics in immunodeficiency.

Prebiotics

Prebiotics are not absorbed in the small bowel, exert an osmotic effect in the intestinal lumen, and are fermented in the colon into short chain fatty acids and gas. They influence the endogenous ecosystem: increase the population of bifidobacteria and often decrease the colonic pH (3,35). Relevant therapeutic effects have been obtained using lactulose, lactose and lactitol for the treatment of constipation and hepatic encephalopathy but many other applications such as colon cancer prevention, and effects on lipid metabolism or mineral are very promising as well as fructooligosaccharides (FOS), inulin and transgalactosides (4,5). Prebiotics and other non digestible oligosaccharides (NDOs) are usually well tolerated but may also have undesirable effects consisting of excessive flatus, borborygmi, abdominal pain, and diarrhoea (36).

Mechanisms of the adverse events

As long as they are not fermented, prebiotics exert an osmotic effect in the intestinal lumen which is negatively related to their molecular weight. This increases the water flow rate, and may induce borborygmi, abdominal pain, and eventually diarrhoea if the capacity of the colon to absorb water and electrolytes is exceeded. Fermentation produces gas which may also induce borborygmi, abdominal pain, and excessive flatus but it decreases or may even suppress diarrhoea by removing the osmolarity (37). Intolerance symptoms can be due to the osmotic effect and/or fermentation, and can originate from the small bowel and/or the colon. As a rule, the first symptoms occurring are borborygmi and excessive flatus; painful symptoms i.e. bloating and cramps occur for higher doses, and diarrhoea is always the last intolerance symptom occurring with high doses (Fig 1). The protective role of colonic fermentation in reducing the severity of NDO-induced diarrhoea has been demonstrated in studies comparing the output of faecal water in response to a nonfermentable osmotic load such as polyethylene glycol (PEG) or magnesium sulfate with a load of NDO. Hammer et al., (38) compared diarrhoea resulting from iso-osmolar loads of lactulose and PEG. Lactulose induced less diarrhoea than PEG, especially at low doses. Saunders & Wiggins (39) showed that while increasing doses of magnesium sulphate immediately increased faecal output, a "lag period" without diarrhoea was observed for low doses of NDOs (mannitol, raffinose and lactulose). This lag period is probably largely due to fermentation of the NDOs which suppresses a part of the osmotic force (as short chain fatty acids are absorbed by the colon). The maximal capacity of the colonic flora to ferment NDOs was estimated to be around 40-80 g/d.

Another mechanism for potential intolerance has recently been reported. (40). Colonic fermentation of carbohydrates is known to influence gastric and oesophageal motility in healthy subjects. Piche et al. investigated the effects of colonic fermentation induced by oral administration of FOS in patients with gastro-oesophageal reflux disease (GERD). Following a cross-over design, the patients were administered during two seven-day periods, either 6.6 g of FOS or a placebo three times daily after meals. Compared to placebo, FOS led to a significant increase in reflux as shown by an increase in reflux episodes, in the symptom score for GERD and in the number of transient lower oesophageal sphincter relaxation (40). Whether lower doses of prebiotics or NDOs would significantly increase the risk of GERD is purely speculative at the present time but this should be studied.

Variability and factors of tolerance

The tolerance of NDOs is clearly influenced by their nature, dose, and individual sensitivity factors.

The laxative threshold of prebiotics can vary between them because of their fermentation pattern, and osmotic effects (36). Clausen et al., (41) gave to 12 subjects increasing doses of FOS or lactulose in a crossover design. Both induced a dose-dependent diarrhoea but the slope for lactulose was twice as high as for FOS (41).

The risk of intolerance and severity of symptoms in a given individual and in populations are clearly dose-dependent (38, 41-43). A threshold is always found for diarrhoea, but not always for borborygms or gaseousness as these symptoms are frequent in the general population even in the absence of consumption of NDOs (Fig 1). Doses below 20 g/d are usually well tolerated.

Many studies have underlined a wide intersubject variability in the tolerance of NDOs which is probably due to differences in intestinal motility and sensitivity. In the study by Clausen et al., (41), the diarrhoea induced by 80 g of lactulose varied greatly between subjects. Indeed, 4/12 subjects had more than 1 L of diarrhoea, while 4 other had a faecal volume < 280 mL/d. It is likely that the difference in the dose-response between individuals was due to differences in the capacity for fermentation of the prebiotic. Indeed, when faecal samples from different subjects are incubated with the same NDO, the production of gas may vary in a 1/10 ratio (44). Teuri et al., (45) showed that the subjects with lactose maldigestion and those with self-reported milk intolerance were more often intolerant to low doses of prebiotics such as FOS and lactulose than lactose digesters. Irritable bowel syndrome may participate to the symptoms in subjects with NDO-intolerance although this has not been studied in depth with prebiotics (46).

Adaptation to regular consumption ?

Regular consumption of some NDOs such as lactulose results in changes in the metabolic activity of the colonic flora (bacterial adaptation) which increase its ability to ferment the sugar and includes a fall in breath hydrogen excretion (47,48). A lower risk of diarrhoea might thus be expected when prebiotics are consumed regularly (clinical adaptation). This was confirmed in some (but not all) human volunteers ingesting regularly low doses of lactulose (49). However, Briet et al., (42) reported that the symptoms and laxative threshold were similar during the occasional and regular consumption of FOS. In keeping, no decrease in breath hydrogen excretion has been observed by Stone-Dorshow et al. during chronic consumption of FOS (50).

To summarise, the tolerance of low doses of prebiotics is excellent. However, 1- prebiotics at high doses may induce gaseousness, bloating and eventually diarrhoea.; 2- these effects are dose-dependent but the threshold varies between individuals and is lower in subjects with irritable bowel syndrome, 3- another point which is not easy to solve is that prebiotics and other NDO may be consumed in several products during the same day so that the cumulative dose may be high in some subjects (for example ingestion of a glass of milk in the morning, 5 g of FOS in a sugar replacer, a glass of cider with meal and 5 g of another prebiotic in a fermented milk preparation leads to 20 g of NDO ingested).

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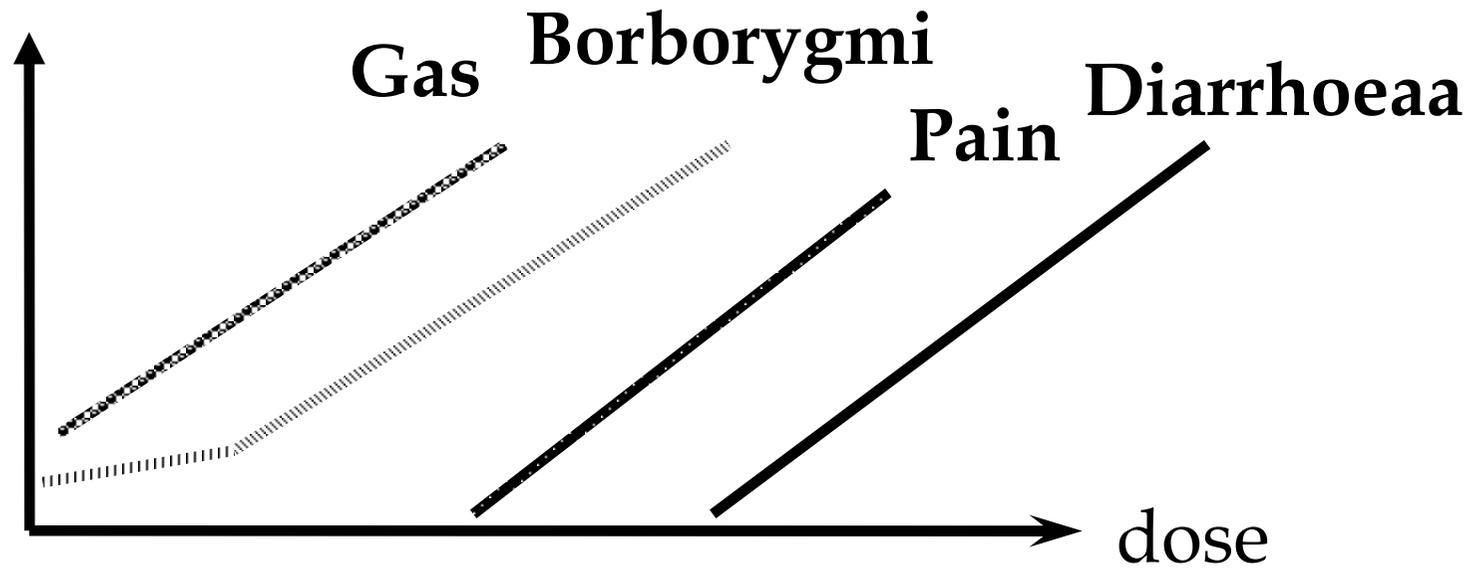
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Figure 1 :

% of subjects with side effects



Individual tolerance depends also on

- *the type of consumption (fasting state or with a meal)*
- *the subject (visceral sensitivity, IBS...)*
- *? adaptation of the flora*

Figure legend :

Figure 1 : Risk of intolerance to prebiotics

Prebiotics Promote Good Health: The Basis, the Potential and the Emerging Evidence.

Jan A. E. Van Loo

Summary (<200words)

Background: The concept of prebiotics was launched in 1995 and has attracted increasing interest since then. It concerns non-digested and selectively fermented carbohydrate food ingredients. It was thought that their effect in the colon could reduce risk for disease.

Aims : The prebiotic concept is revisited and, on the basis of the definition and the findings that have accumulated steadily, possible mechanisms are proposed. The physiological consequences of prebiotics consumption are evaluated in terms of potential to reduce risk for disease.

Patients : This paper is a compilation of several original research papers, each of which complied with the world medical association declaration of Helsinki (1989).

Methods : For the human dietary intervention trials, the aim was to perform double-blinded, placebo-controlled, cross-over studies. A parallel study design with adequate numbers of volunteers was used only in cases where long-term studies were carried out. By far the most nutritional research has been done with $\beta(2-1)$ fructans, so they are taken as an example of prebiotics here.

Results : The results are relevant to the fields of gut function, lipid metabolism, mineral absorption, bone formation, immunology and cancer.

Conclusions : It is observed that the modification of the intestinal flora by the inherently selectively fermented prebiotics is central in determining their nutritional properties. They interact positively through the large intestinal surface with various physiological processes, and are thought to improve health status by reducing risk for disease (markers).

Introduction

Prebiotics

Since the introduction of the term prebiotics [1], a vast amount of literature related to the topic has been generated. Prebiotics have been defined as non-digestible food ingredients that affect the host beneficially by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health [2]. As research has progressed, the definition has been fine-tuned.

There are three criteria that need to be fulfilled for a food ingredient to be designated a prebiotic.

(1) The food ingredient must be non-digestible by host enzymes. They need to be resistant to attack by digestive enzymes, such as carbohydrases from the brush border or those of pancreatic origin; as such, they remain available to the intestinal flora. The non-digestibility can be demonstrated by means of tests *in vitro*, using intestinal scrapings from various animals (pigs, rats, birds, fish). For human purposes, the best way to demonstrate non-digestibility is with ileostomised volunteers [3].

(2) The food ingredient must be fermented in the gastro-intestinal (GI) tract. This can best be shown by means of fermentation tests *in vitro*. These tests show whether an ingredient is fermented, but *in vitro* models alone cannot be considered adequate for study of the complex ecosystem of the colon [4].

(3) There must be selectivity in stimulation of intestinal flora and of metabolic activity. This criterion is of utmost importance. A prebiotic ingredient, by definition, is not available to all bacteria of the intestinal ecosystem. It must be particularly readily available to some groups of

bacteria (of which lactobacilli and bifidobacteria are considered indicator organisms) that are not related to a diseased state of the intestine. As a result, a prebiotic is less available to other groups of bacteria such as proteolytic bacteria, of which several are pathogenic. It is often the case that stimulating growth of certain groups of bacteria allows them to take a more dominant position in the GI ecosystem, which they substantiate by producing metabolites, including bacteriocin-type products, or compounds related to quorum-sensing cascades.

Demonstration of the fulfilment of this criterion is much more difficult, and can be achieved only by means of repeated human dietary intervention studies. Whereas data from studies in vitro can give some mechanistic indications (production of metabolites such as short-chain fatty acids), they cannot be fully representative of what happens in the human (animal) GI environment. Data from experimental models cannot be extrapolated to humans, as the GI architecture of different species of animals is dramatically different [5].

The prebiotic property has been adequately demonstrated for only a few food ingredients. According to a recent in-depth review, only the the inulin-type fructans [6-12], galacto-oligosaccharides [13-15] and lactulose [16] are proven prebiotics [5]. The latter however is considered rather a therapeutic ingredient than a food ingredient. For others (isomalto-oligosaccharides, lactosucrose, xylo-oligosaccharides, soybean oligosaccharides and gluc-oligosaccharides, polydextrose, there is some preliminary evidence.

The basis for prebiotic activity

The primary 'effect of prebiotics' (prebiosis) is an interaction with the GI ecosystem, which basically is an interaction with the fermentative capacity of the GI ecosystem. It is thought that all physiological effects that take place following prebiotic consumption originate in this altered fermentative functioning of the GI ecosystem. The initiation of physiological change originating in an altered fermentative capacity of the gut could happen through microbe/microbe interactions, host/microbe interactions and/or host/bacterial metabolite interactions. The latter may have the most important impact. The main metabolites are the short-chain fatty acids (SCFA) acetate, propionate and butyrate, which are all biologically active compounds [17]. Prebiotics can easily double the pool of SCFA in the GI tract, and the molar ratio between the different SCFA is altered [18,19].

This process takes place in the intestine, having a surface of about 300m² in humans, compared to 2m² for the skin or 100m² for the lungs. The lining of the GI tract is continuous with the external part of the body. As such, the surface of the intestine is the largest interface between our body and the environment. Via this large interface, we are in intimate contact with a very dense microbial ecosystem composed of up to 10¹² bacteria per ml, which is, volumetrically, about the highest possible microbial population one can be in contact with (Figure 1). Improvement of the GI ecosystem (less pathogens, more saccharolytic activity) thus represents an important improvement of the external environment in which we live. It is thought that improving the composition of the intestinal ecosystem has a positive impact on physiological functioning of an organism.

Nutritional properties of prebiotics

The nutritional properties of prebiotics depend directly on the physiological changes that they induce in the body of the host. Bacterial metabolites are thought to be the main effectors of most observed effects. In the strictly anaerobic environment of the gut, short-chain fatty acids (SCFA) are the common electron sinks of respiration, but other (unknown) metabolites that are produced in minor quantities could be signalling molecules that trigger cascades of biochemical processes in organs. In the case of immunological reactions, it was observed that bacterial cell-wall oligosaccharides can interact with the sensors of the gut-associated immune system (GALT) [20].

Bowel habit

A direct consequence of prebiotic consumption is improved bowel habit. As prebiotic carbohydrates become available to the intestinal bacteria, they grow on it, increase in numbers and eventually in volume; they induce a fecal bulking effect. Prebiotics that are fermented completely induce a fecal bulking effect of 1.5 to 2g of feces per gram of prebiotic consumed [8,21]. The SCFA products of fermentation stimulate intestinal peristalsis [22].

As a consequence of prebiotic consumption, there is a relief of constipation in chronically constipated people [23]. As prebiotic carbohydrates arrive in the colon, they modify the composition of the intestinal flora, and they influence its metabolic activity. These processes increase the 'colonisation resistance'[24]. This is the ecological resistance of an existing bacterial population against other bacteria to find a niche to colonise and, in the case of a

pathogen, to induce infection and/or inflammation. In a study with people travelling to ‘high risk’ countries, it was observed that prebiotic consumption reduced the incidence of diarrhoea by almost 50% [25]. In another study it was shown that the consumption of the prebiotics relieved diarrhoea [26].

This increased colonisation resistance also is becoming an important reason for the use of prebiotics in animal nutrition. In livestock production (poultry, pigs, calves, etc.), the use of antibiotics as growth promoters will be completely forbidden in European countries within the next few years. It was observed that adding 0.075 to 1% of prebiotic inulin or oligofructose to animal feed was able to reduce the numbers of pathogens and symptoms of intestinal infection, to a degree that was comparable with the effects of antibiotic supplementation [27-29]. Besides the impact on colonisation resistance, addition of prebiotics to post-weaning food reduced the incidence of diarrhoea [30].

It appears from a wide variety of studies that bowel function is improved by prebiotics. This includes the fecal bulking effect, intestinal transit (reduced constipation), and reduced risk for infection (reduced diarrhoea).

Increased mineral absorption

By means of experimental models it has been demonstrated repeatedly that dietary prebiotics improve the efficiency of mineral absorption from food [31-38]. Most studies showed effects on Ca and Mg absorption, others showed increased absorption of Fe and Zn [31,39] but not of heavy metals such as Cu or Hg [31,40].

Other studies have shown that the increase in mineral absorption results in increased bone density and bone trabecular structure [41-44]. Interestingly, it appeared that not all prebiotics

were able to promote mineral absorption to the same extent. The presence of prebiotic carbohydrates containing long chains (degree of polymerisation (DP) 12–65) always resulted in better effects. The best effects in rats were obtained with a $\beta(2-1)$ -type fructan designer prebiotic (Synergy1), where a ratio of 1/1 short chains (DP 2–8) and long chains (DP12–65) was used [32].

The absorption results have been evaluated in human dietary intervention studies. It was observed that in human volunteers, whose gastrointestinal architecture differs from that of the test animals, the short chains were able to increase the absorption of Mg, but the effect on Ca absorption was less pronounced [45,46]. The effect was more pronounced when long-chain fructans were present [47]. In a comparative study, under restrictive conditions of a high intake of calcium (>1500mg/day), it was shown that only the designer prebiotic (Synergy1) and not the short-chain fructan was able to further increase calcium absorption [48].

These and other observations have led to the hypothesis that short-chain $\beta(2-1)$ fructans (which are very soluble and are fermented quickly and selectively) are able to modify the composition of the intestinal flora in the proximal part of the colon, and that the long-chain fructans (which are less soluble, and are fermented selectively, but slowly) are a selective substrate. This implies that the improved intestinal flora is kept metabolically active for a prolonged period of time, and hence also in more distal parts of the intestine. This observation was the basis for the introduction of the concept of ‘designer prebiotics’.

Several mechanisms were proposed to explain the effect on mineral absorption. The intake of prebiotics acidifies the intestinal contents, which aids the solubilisation of minerals (mainly Ca or Mg salts) [49]. The increased presence of butyrate, which is a selective source of energy

for the intestinal epithelial cells, improves the absorptive capacity of the mucosa [50]. Others have observed an increased activity of Ca transporters (calbindin) in the colon [51].

Anticarcinogenic effects

A consistent anti-carcinogenic effect of dietary prebiotics has been observed in various experimental models.

The effect was shown in chemoprevention models in the short term, where colonic aberrant crypt foci (ACF) are the biomarkers. Most of these preneoplastic lesions heal, but the multifocal lesions are thought to deteriorate into tumors. Inulins reduced the incidence of %ACF as molecular mass increased. Again, the more pronounced reduction of ACF by the long chains (sustained activity of basically the saccharolytic fraction of the intestinal flora) especially in the more distal parts of the colon was demonstrated clearly. The effects of the designer prebiotic Synergy1 were as great as those of the positive control (Sulindac). These prebiotics have their effect in the initiation and post-initiation phases of carcinogenesis [52-54].

In long-term chemoprevention models, the incidence of tumours serves as the biomarker. The effects observed in the short term were confirmed [55,56]; again, long chains were more active.

In genetically predetermined Min-mice (apc-) models, there was a significant reduction of colonic tumours, and even of tumours in the small intestine (as shown by Dr Martin Lipkin, the father of the Min-mouse model), indicating that systemic effects are involved [57,58]. One study in a Min-mouse model failed to show anticarcinogenic effects [59], but the methodology and the conclusions of that study have been heavily criticised [60].

The systemic efficacy was confirmed in models where tumour cells were implanted in muscle tissue (with growth-rate of the tumour as a marker) or in peritoneum (with increase in life-span as a marker:[61,62].

The results from these experimental models provided a solid basis to perform a phase 2 human dietary intervention study.

At present, the anticancer effect of prebiotics is being investigated in a human volunteer study, in which a group of colon cancer patients and a group of polypectomised patients are given the designer prebiotic Synergy1 [63]. The results of this study are expected in early 2004.

The immune-potentiating effects

The research with prebiotics in this field of interest is very young. At present most data originate from experimental models. The general observations are that consumption of inulin-type fructans increases the phagocytic capacity of macrophages [64], and that more IgA is secreted in the intestine [65]. In the Peyer's patches of Synergy1-fed rats, a marked increase in interferon- γ and in IL-10 is observed [66]. In mice that were i.p. injected intraperitoneally with pathogens such as *Listeria* or *Salmonella*, a significant survival rate was observed as

compared to control diets. Here, the effect of slowly fermented fructans was markedly more pronounced than the effect of rapidly fermented prebiotics.

In human volunteers there are some indirect indications of improved immune status. In a multicentre study with children in kindergarten, it was shown that consumption of rapidly fermented inulin-type fructans resulted in less absenteeism and in reduced incidence of fever-associated diarrhoea [67].

Effects on lipid metabolism

It is thought that increased production of propionate is at the basis of altered lipid metabolism[68]. Propionate, which is produced from the fermentation of prebiotics, migrates via the portal vein to the liver, where it interacts with the regulation of the expression of genes that code for digestive hormones (incretins) such as GLP1 and GIP, and of insulin [69].

The human studies that have been performed have failed to show very uniform outcomes. In some studies, cholesterol levels are reduced [70]. In other studies, both cholesterol and serum triglycerides are reduced [71]. In other studies, serum triglycerides alone were reduced [72], and in still other studies, no effect on lipid metabolism was observed [73]. This is ascribed to the complexity of the biochemistry of lipid metabolism, which certainly complicates experimental protocols and outcomes [74-76].

Several studies with experimental models and in human dietary interventions, however, showed that the parameters of lipid metabolism are not unaffected. A closer look at the published data again reveals that especially the presence of long chains is associated with lipid-modulating effects.

Discussion

The purpose of this review is to show that carbohydrate prebiotics effectively interact with various physiological processes of the host (bowel habit, mineral balance, immunology, cancer, lipid metabolism, ...). This was observed by monitoring several biomarkers of these physiological processes. It is considered that the way in which prebiotics modify these various physiological processes results in a reduced risk for disease, or an improved health status.

To date, it can be considered shown that prebiotics are active food ingredients that have a good chance of showing 'improved health' effects in such hypothetical studies.

All prebiotics are fermented selectively, but those that are fermented slowly and those that are fermented rapidly have different effects; further, a combination of the two types can have even stronger physiological effects than their components [48,49,77]. As the physiological effect is dependent on the type of intestinal fermentation that is induced by a certain prebiotic, it can be concluded that the primary effect of prebiotics is on intestinal fermentation, and that all other physiological processes are a consequence (directly from the bacteria, or indirectly from the metabolites they produce) of these altered intestinal fermentation processes [78,79].

With respect to the prebiotic effect itself, the selectivity cannot be described in terms of the names of bacterial species that increase in numbers, and others that decrease in numbers, the more so because well over 80% of the bacterial species present in the intestine are unknown (i.e. cannot be grown in vitro, so cannot be classified taxonomically). The selectivity is

recognized in terms of groups of bacteria, i.e. saccharolytic versus proteolytic, each with representative marker organisms, e.g. Bifidobacteria and Clostridia.

Rapidly fermented prebiotics can change the composition of the intestinal flora dramatically, whereas slowly fermented prebiotics do so less markedly (compare [7] with [6]).

Nevertheless, the slowly (but always completely) fermented prebiotics have at least as much pronounced physiological effects [49]. This implies that the type and quantity (importance of the selectivity) of the bacterial metabolites may be central in the functioning of prebiotics.

The fact that not all the details of modification of intestinal flora are known to date does not contraindicate consuming prebiotics. Prebiotics have always been part of the human diet, and their consumption is likely to have been more important in pre-settlement eras of human history, when fruits and roots were the main food ingredients. Nowadays, 1 to 4g of prebiotic inulin is consumed as part of a western diet, and to date there are populations who eat at least 25 times that amount [80]. No complaint, besides in some cases some extra flatulence, has been reported in any of the carefully monitored volunteers in the various dietary intervention studies [81].

Prebiotics can be used in a wide variety of foodstuffs (dairy, bakery, meat products, drinks, spreads, confectionery, chocolate, ...) [82]. As such, they are considered a potent vector to import interesting nutritional properties into the western style diet, of which the high fat and low dietary fibre content are considered to be of major public health concern (cause of type 2 diabetes, syndrome X, cancer, cardiovascular disease, etc.). Of course, the best thing would be to make people eat a healthy varied food, low in fat, high in fibre and vitamins etc., combined with a lot of exercise, but the last half century has shown that attempting to change dietary habits (especially away from the very palatable and extreme tastes of a western style diet) of

populations simply by the agents of advice and education is unsuccessful. The addition of prebiotics could compensate for this in a convenient way, and this without negatively affecting the highly appreciated organoleptic properties of our food.

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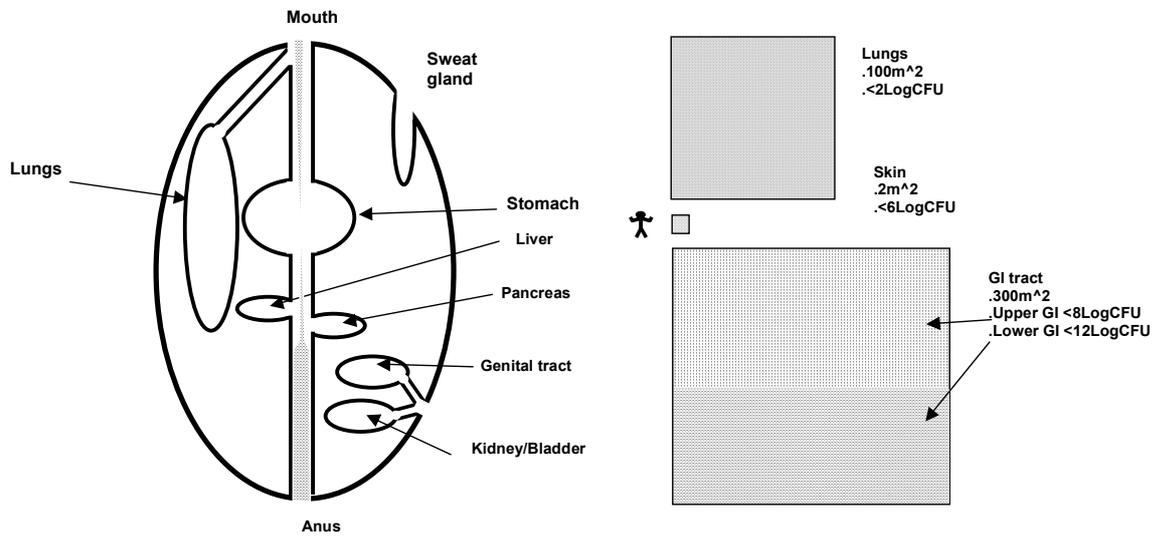


Figure 1

Figure legends

Figure 1. A diagram of the human body and its relation to the bacterial ecosystems (in log colony-forming units (CFU) per ml for GI content or per cm² for lungs and skin). In the left part of the Figure it is made clear that many organs are actually external to the body. There is an exogenic bacterial pressure. It is only in the GI tract that a bacterial population is allowed and maintained. The right part of the Figure presents the ratio of the surface of the skin, the lungs and the GI tract in proportion. It is clear that the intestine is by far the largest surface through which the mammalian body is in contact with the external environment, which represents about the densest possible bacterial population (not more than 10¹² bacteria can be contained within 1 cm³).

Probiotics and Immunity

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Until the end of last century, basic immunology has been imprinted by a static view in which all the cells belonging to the innate and to acquired immunity outside primary tissues (Thymus and Bone Marrow) had to be mature. The question " where do lymphocyte precursors differentiate once the Thymus has involved, in the adulthood" has not been yet answered. Under the conditioning of the clonal selection theory and of the Coombs and Gell classification, a lymphomononuclear cell infiltrate observed at the periphery has ever been considered as the result of a T-memory response towards specific antigens/allergens.

The obliged maturity and specificity of the immune response at periphery has been a myth lasting from 1955 as well as the power of placebo. Nowadays it is time to convey with JC Bailar III " Some myths really ought to be true. evidence against them is unwelcome and not be trusted. But some such myths are flawed and misleading" (1).

For decades evidence has been cumulated for maturational events occurring at periphery, outside primary organs, but only during the latest years the concept of peripheral leukocytopoiesis has emerged and is now achieving great acceptance (2-11) .

A different reading for the TH2/TH1 paradigm also develops under such influences.

Until cells at periphery have been regarded as terminally mature, only a divergent interpretation was possible with consequent discussions during the years (12-15). A consistent body of evidence raised a more recent version where TH2 represent the early and TH1 the late stages of a linear model of differentiation. TH2 can ultimate their differentiation into TH1 only under the maturational influence of IL12 and IFN γ (16-18). A defective expression of IL12 and IFN γ , as demonstrated in allergic diseases, restrains the differentiation progression, leaving TH2 cells partially immature. Such impaired

differentiation leads to the accumulation of immature cells (TH2↑) with respect to the mature forms (TH1↓) causing the TH2/TH1 imbalance.

As well as in lymphoid lineages, distinct helper populations (H2 and H1), with distinct secreting profiles, have been also identified in myeloid lineages of cells belonging to the native immunity compartment (Natural Killer and Dendritic Cells) (19-22). A defective expression of the maturational cytokines IL12-IFN, in fact, has been reported to impair the immature Dendritic Cells (DC2) linear differentiation into the mature type (DC1) (23,24).

If evidence for a compensatory peripheral leukopoiesis underlying TH2 allergic diseases is instinctively acceptable since, e. i., the association between eczema and immunodeficiencies has been extensively described, how can we interpret literature reports of TH2 skewed profiles in diseases other than allergy?

The linear differentiation model for H2/H1 populations allows impressive answers potential.

An impaired cells ontogeny induces a functional immunodeficiency; being the immune system redundant and compensatory it could activate a lymphoneogenesis occurring at extra-thymic sites.

An imbalance in helper TH2 and TH1 cells is discussed for Hepatitis C Virus infection (HCV). Standard treatment for HCV is Group I IFN that induces the maturation of the innate immunity cells (DC); mature cells (DC1) can secrete IL12 and IFN γ that allow TH2 develop into functionally mature TH1, capable of addressing a virus-specific response.

A main question regards the origin of such an immature TH2: are they the result of an external peripheral homing or they arise from intrahepatic resident Hemopoietic Precursor Cells (HPC). Emerging evidence suggests for the latter hypothesis (25, 26).

Lastly, what about inflammatory bowel diseases?

Compelling evidence supports the gut as the Thymus substitute-complementary organ for T-cells differentiation and education (27-33). T-cells are present, even when Thymus lacks, in gut. Athymic/euthymic mice experiments indicate that in euthymic animals the alternative lymphopoietic pathways are repressed except in conditions of severe lymphocyte depletion (34). Which are severe lymphocyte depletion conditions?

Adult inflammatory bowel diseases often develop as a consequence of severe stress.

This is a triggering condition shared with adulthood atopic dermatitis, a paradigmatic TH2 disease that has been extensively investigated to verify mentioned hypothesis (2, 3, 16, 17).

Chronic stress is immunosuppressive and induces a TH2/TH1 unbalance (35-37); the increased levels of glucocorticoids lead to a lymphocyte severe depletion that could activate a compensatory lymphopoiesis (concept of peripheral lymphopoiesis extra-demand).

How can probiotics impact the derangement underlying TH2 diseases?

Differently from immunosuppressant drugs that while control symptoms, paradoxically appear to worsen the causes (17, 38-50).

If we may hazard an immunological definition of probiotics, we could conclude: " Probiotics, as particular bacterial strain products, are biological response modifiers (BRMs) capable of inducing the indirect production of maturational cytokines (such as IL12 and IFNs)".

A consistent body of evidence supports such a view since it has been proved that Bacteria pay the toll to the immunity through the interaction with particular receptors of the innate compartment cells. Mentioned "Toll Like Receptors", belonging to the large IL1R family, activate the NF-kB factor transcription that controls a gene network including those codifying for the mentioned maturational cytokines (51-55).

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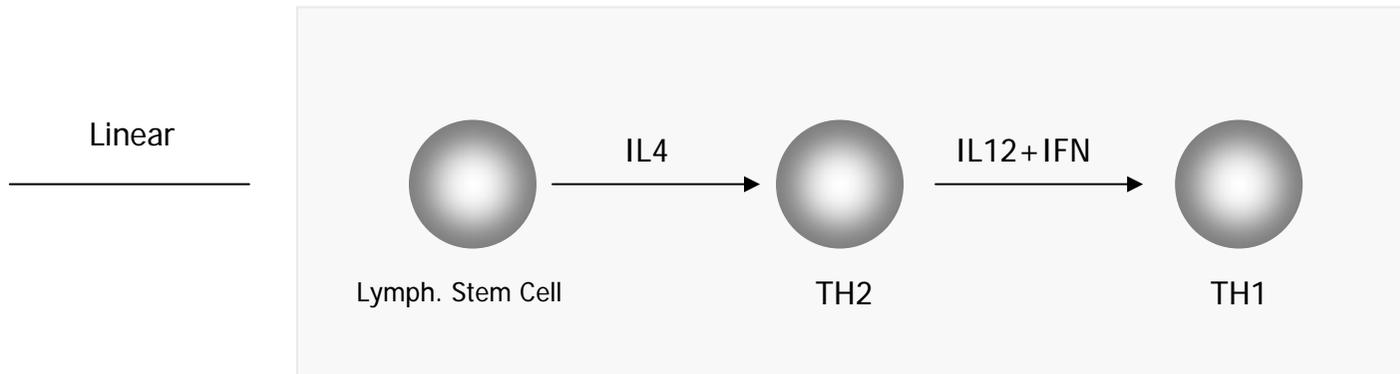
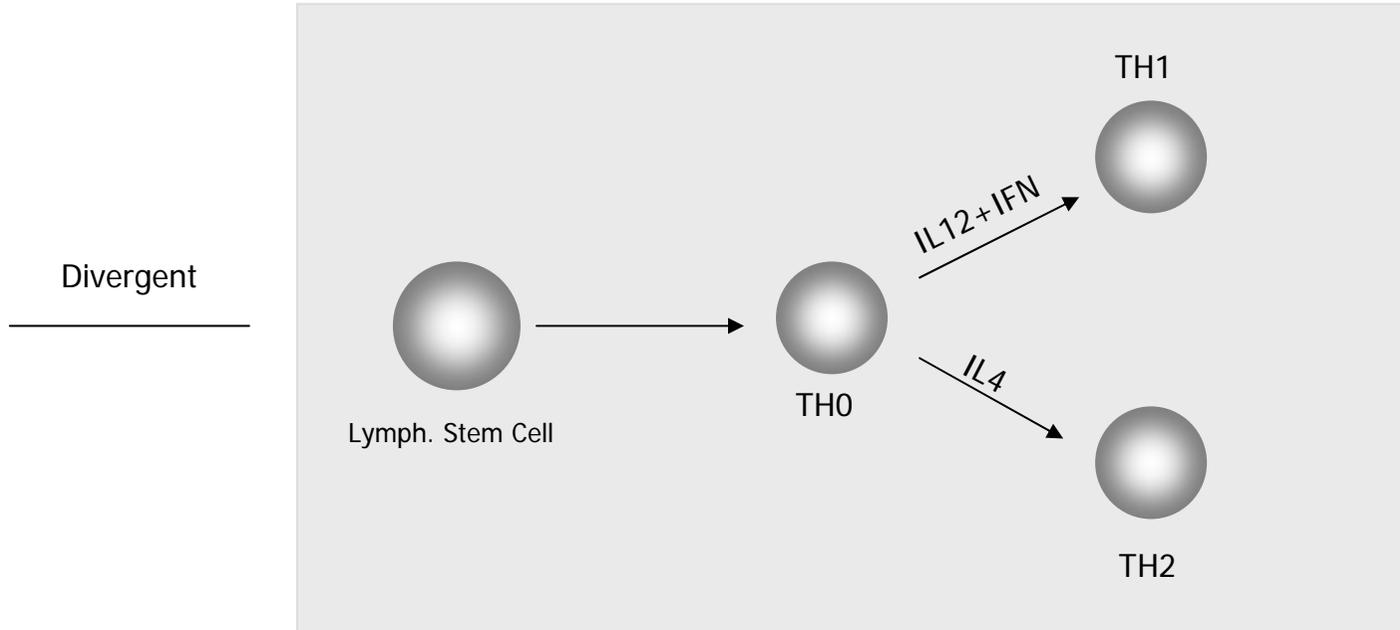
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Legend to

Fig. 1 Linear differentiation lecture of the TH2/TH1 paradigm.



Lactoferrin functions: current status and perspectives

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Lactoferrin (Lf) is a multifunctional iron binding glycoprotein which is known to exert a broad-spectrum of defence activity against bacteria, fungi, protozoa and viruses. Lf is found predominantly in secreted fluids and in granules of neutrophils. Lf functions are related and unrelated to iron binding capability. Its iron sequestering ability is at the basis of the bacteriostatic effect, which can be counteracted by microbial pathogens by the synthesis of siderophores which bind ferric ion with high affinity and transport it into cells, or of specific receptors capable of removing the iron directly from lactoferrin. Independently on iron chelating property, Lf can also show a bactericidal activity due to its binding to the lipid A part of bacterial lipopolisaccharide (LPS). This action is due to lactoferricin (Lfc), a peptide obtained from Lf by enzymatic cleavage, which is active against bacteria, fungi, protozoa and viruses. Additional antibacterial activities of Lf have also been described. They concern the inhibition of the bacterial adhesion, colonization and the intracellular invasion, including the increase of the apoptosis of infected cells by intracellular pathogens. Recently, it has been demonstrated the influence of Lf, iron modulated, on the bacterial aggregation and biofilm development. The antifungal activity of Lf and Lfc has been tested mainly towards *Candida* and *Trichophyton*, while the antiprotozoal action of Lf has been demonstrated against *Toxoplasma gondii*. As to the antiviral activity, it is exerted against several enveloped and naked viruses, with an inhibition which takes place in the early phases of viral infection, as a consequence of a binding to the viral particle or to the cell receptors for virus. The antiviral activity of Lf has also been demonstrated in in vivo model infections and proposed for a selective delivery of antiviral drugs. All these functions suggest that Lf may contribute to the protection of mucosal surfaces from the injury of bacteria and viruses. The new perspectives in the studies on the antimicrobial activity of Lf appear to be linked to its potential prophylactic and therapeutical use in a considerable spectrum of medical conditions, taking advantage of the availability of the bovine and recombinant human Lf.

PREBIOTICS IN INFANT FORMULAS

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At birth the infant's intestine is nearly sterile. The colonisation of the intestine starts with birth and depends mainly on the mode of delivery and on the nutrition during the first weeks of life. Breast fed infants develop an intestinal flora, which is characterised by a dominance of Bifidobacteria. In bottle fed infants the Bifidobacteria don't dominate and other bacteria more characteristic for the flora in later life became important.

There is a wide consensus that the intestinal flora, which develops during breast-feeding, is considered as beneficial for the infant. Although the intestinal flora is characterised by the dominance of Bifidobacteria we know that the entire flora of breast-fed infants is different from bottle fed infants. This for instance demonstrated by the differences in faecal short chain fatty acids between the both diets.

Due to the beneficial effects of the entire intestinal flora established during breast-feeding many attempts were made to mimic this intestinal flora also in formula fed infants.

In principle there are two ways to influence by dietary intervention the intestinal flora: The prebiotic and the probiotic approach. Probiotics are defined as living bacteria, which are administered to the infant to be colonised in the colon. First results of epidemiological trials with probiotics have been presented indicating that the intestinal flora play an important role in the development of the immune system.

More recently the prebiotic concept has been developed. Prebiotics are substances, which survive the passage through the small intestine, arrive the colon and can be utilised by the bacteria as substrate of their metabolism.

The composition of human milk represents the prebiotic concept. Since nearly a century many studies have been made to identify the ingredients in human milk which are responsible for this prebiotic effect. Among many factors, human milk oligosaccharides seem to be the most important dietary factor in human milk with respect to the development of the intestinal flora.

The composition of human milk oligosaccharides is very complex and many other functional effects than the prebiotic function have been identified. Therefore, it is a challenging questions which of the oligosaccharides in human milk are the most relevant oligosaccharides for the intestinal flora. There is evidence that the neutral fraction of the oligosaccharides in human milk reflects the most relevant prebiotic compound of human milk oligosaccharides. Furthermore, the molecule size distribution seems to be an important structural basis for the prebiotic effects.

The most relevant prebiotic oligosaccharides of non-milk origin are galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). GOS have some similarities with the structure of the core molecule of human milk oligosaccharides. There are no structures in human milk oligosaccharides comparable to FOS. The most promising results arrived from the mixture of GOS and FOS in which GOS represent 90% of the mixture. This mixture mimic the molecule size distribution of the neutral fraction of human milk oligosaccharides.

Several clinical trials have shown that this prebiotic mixture influence the entire flora of a formula fed infant in the direction to the flora of breast fed infants. This is relevant with respect to the number of faecal Bifidobacteria and the metabolic products of intestinal fermentation like short chain fatty acids and stool pH. This is accompanied by a reduction of the counts of faecal pathogens and with stool characteristics similar like in breast fed infants. The stimulation of the entire flora also indicates that the prebiotics can positively influence the postnatal development of the immune system.

In summary, the supplementation of infant formulas with ingredients able to establish an intestinal flora similar than during breast feeding is a new step to improve the quality of infant formulas.

MICROFLORA IN INFLAMMATORY BOWEL DISEASES

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The intestinal microflora and the microbiological imprinting

Microorganisms have traditionally been viewed as harmful to human health and efforts have been directed at increasing hygiene conditions also through the use of antibiotics. In recent years, the concept that microbes may be beneficial to human health is being increasingly recognized. Intestinal microflora is the result of a complex interaction between the external environment and the host. It is estimated that approximately 4 to 500 different bacterial species populate the digestive tract, with an increased density in the colon. Several bacteria are pathogenic but others exert beneficial effects. In addition, the load of specific bacteria may be more important than previously recognized. Investigations on intestinal flora have been traditionally based upon culture techniques, that are poorly sensitive for quantitative analysis. The development of molecular techniques has led to major changes in molecular taxonomy, allowing the evaluation of phylogenetic relationships among enteric bacteria and a more precise estimate of selected counts. By using direct sampling of 16S rDNA molecule, it has been recognized that intestinal microflora is a highly complex ecosystem in which anaerobes logarithmically exceed aerobic bacteria by a 1000/1 ratio (1). There

are 9 major genera that dominate the ecosystem and constitute a rather stable pattern, regardless modifications in feeding regimens (table 1). However, intestinal microflora include transient bacteria and resident bacteria. The former are detected as long as they are ingested, disappearing once their supply is withdrawn or shortly thereafter. Examples of these include probiotic strain of *Lactobacillus* and *Bifidobacterium*. Other strains, although ingested on a daily basis, are substantially incapable to colonize the intestine.

The pattern of intestinal microflora although quite stable in the adult host, undergoes an ecologic modification in the early stages of life. Intestinal colonization begins during the newborn period, when a true “microbiological imprinting” occurs. The imprinting lasts approximately 2 weeks in germ-free adult animals. It takes longer in human infants and proceeds along diet modifications until weaning after which the microbial pattern slowly shifts toward the adult pattern. It may be important the fact that infant microflora shows more variability than adult microflora. Infants born by cesarean delivery, are not directly exposed to maternal flora and either have some delay in acquisition of conventional flora or develop a peculiar microflora (2); intestinal flora in these infants is different from that of their mothers and reflects that found in the hospital environment.

Fecal flora of breast-fed infant is dominated by *Bifidobacteria*; in contrast infants fed with milk formula have a more complex microbiota with no bacterial genus predominance. The primary source of microorganisms to the newborn host is maternal vaginal and fecal flora (3). Several factors are able to modify the intestinal flora, including age, type of delivery, diet, use of antibiotics, gastric pH, intestinal peristalsis and immune system (table 2). It has been shown that administration of probiotics or prebiotics early after birth is effective in influencing the composition of intestinal microflora (Bhoem +LGG 2003)

The composition of the individual’s flora may change in selected circumstances, such as acute diarrheal disease, antibiotic treatment, dietary interventions. However, the individual’s flora composition, once established, usually remains constant. Thus, the manipulation of intestinal

microflora, particularly in the first weeks of life, lead to a specific microbiological imprinting which may reduce or increase the risk of developing disease.

Interaction between the enteric microflora and the immune system

Several data support a close interaction between microbes and the immune system, particularly in the early stages of life. Specialized cells, the M cells, act as immune sensor, and present sampled intestinal content to dendritic cells and lymphoid follicles. Plasma cells, in the lamina propria, produce large amounts of IgA that are reversed into the intestinal lumen. The intestine shows a physiologic degree of inflammation. Germ-free animals lack the inflammatory infiltrate normally present in the submucosal layer, their lymphoid structures are not well developed and serum immunoglobulins are low. In germ-free animals, suppressor cell activity is absent and animals are not tolerant to antigens given through parenteral route, but become so after oral administration of lipopolysaccharide. In addition, germ-free animals show a decreased mucosal tolerance involving the Th2 lymphocyte cells. Mucosal tolerance is more based on Th1 than on Th2 response. However, the picture is more complex and although the host response to intestinal bacteria involves mucosal Th2 cells, tolerance is more a systemic immune-related phenomenon, depending on bacterial colonization. The critical step of oral tolerance may be related to bacterial translocation to regional lymph nodes which up-regulates the Th2 response.

The hygiene hypothesis provides the background to the concept of “microbial education of the immune system”. It is based on the concept that overcrowding and poor hygienic conditions in the early life protects from atopic diseases, implying a protective effect by infections against atopy (4).

There are data showing a relationship between microbiological imprinting and the risk to develop atopic eczema. Neonates exposed to *Lactobacillus GG* had a decreased risk of eczema in the first 2 years of age. Administration of probiotics to children with cow's milk allergy resulted in a reduction of symptoms (5), which was associated with a decrease in eosinophilic cationic protein (6). In contrast, when probiotics were administered to adults with allergy, little effect was observed,

indicating an age related pattern of the relationship between intestinal microecology and immune response (7,8). Thus there is a close interaction between the enteric microflora and the immune system. The microbiological imprinting is related to the immunological imprinting and the immune system may be “educated” by enteric bacteria. This process however, occurs in an age frame, corresponding to the early stages of life, peaking around, and immediately after, delivery (9).

Role of infectious agents in the pathogenesis of inflammatory bowel disease (IBD): epidemiologic and clinical links

Ulcerative colitis and Crohn’s disease are chronic and relapsing intestinal disorders. In the last years the incidence of IBD is increased, in adults as in children. It is progressively becoming clear that several chronic inflammatory states are the result of three distinct interactive conditions: genetic-based host susceptibility, enteric microflora and immune dysregulation.

Several studies point to genetic factors predisposing to these diseases. Intestinal microflora plays a major role in the development, recurrence and complications of the IBD. The chronic and recurrent inflammation of IBD may be caused by a defective clearance of enteric pathogens, by the infection with a specific unusual enteric pathogen or by the induction of an abnormal immune response.

The role of microbes is strongly supported by the observation that IBD occurs in the distal intestine, the topographic location where is found the highest luminal bacterial concentrations. In addition, improvement of symptoms is observed when luminal bacterial concentrations are decreased upon antibiotic treatment. It is well known that several enteric infections strongly resemble the inflammatory bowel diseases not only in their clinical features but also in histological findings, raising the problem of differential diagnosis (table 3). In parallel, selected intestinal infections show local and extraintestinal manifestations similar to that observed in IBD (table 4). However there are histological hallmarks that may help differentiating IBD from intestinal infections (table 5)

Because of the similarities of Ulcerative Colitis with *Campylobacter*, *Shigella* and amebic colitis and of Crohn’s disease with ileocecal tuberculosis, *Yersinia enterocolitica* and *Chlamydia* infection, it has

been hypothesized that a specific pathogen could be involved in pathogenesis of IBD. A large number of organisms have been proposed to have a triggering role in IBD (table 6). *M. paratuberculosis* has been cultured from several patients with Crohn's disease and an increased antibody titer against mycobacterial antigen in patients with Crohn's disease has been reported (10). However, clinical and immunohistochemical studies have failed to provide evidence of a causative role for *Mycobacterium* in Crohn's disease. In recent years, the use of PCR showed that mycobacterial sequences are detected in intestinal specimens of adults and children with Crohn's disease by molecular techniques (11,12).

Other studies have suggested that persistent measles infection, particularly if exposure occurs in utero or early in life, may lead to Crohn's disease (13,14). Paramyxovirus-like particles have been detected in vascular endothelium of patients with Crohn's disease by electron microscopy and measles antigen has been detected in areas of granulomatous inflammations. However, no epidemiological link between measles infection and Crohn's disease has been observed (15,16).

Similarly, conflicting data is reported for adherent *E. coli*. There is evidence of an increased rates of adherent or toxigenic *E. coli* from patients with Crohn's disease or ulcerative colitis (17,18), but other studies did not confirm this association (19,20). Overall, available data are not strong enough to conclude that IBD are induced by specific enteric agents. However, epidemiologic and clinical data suggest a close link between IBD and selected enteric.

Role of infectious agents in the pathogenesis of IBD: experimental models and modifications of intestinal microflora

Experimental data support the role of intestinal microflora in IBD. The most convincing evidence has been obtained using a transgenic rodent model. IL-2 and IL-10 knock-out mice and HLA-B27 positive aptotype spontaneously developed intestinal inflammation, whose features were similar to human IBD; inflammation did not occur when animals were grown under germ-free conditions (21,22).

Madsen and coworkers demonstrated that in IL-10 gene-deficient mice, colitis is associated with altered colonic microflora. At 2 weeks of age, and prior to developing colitis, microflora was characterized by an increase of adherent Clostridia and a reduction of Lactobacilli and Bifidobacteria. Treatment with metronidazole, an active agent against anaerobic bacteria, was able to prevent the disease (23).

Enteric bacteria differ in their capacity to induce inflammation and act in a synergical manner. HLA-B27 transgenic rats develop colitis after colonization with *Bacteroides vulgatus* alone or in combination with five other bacterial species but not when they are colonized with the five bacterial species without *Bacteroides* or when colonised with *Bacteroides* and *E. coli* (24). Selected animal models show a specific response to bacterial stimuli. For example IL-10-deficient mice developed only a mild colitis when colonised with the same bacteria that caused severe inflammation in HLA-B27 transgenic rats (25).

Evidence that bacteria do play a role in the pathogenesis of Crohn's disease exists also in man. Relapse of inflammation, in neoterminal ileum of patients undergoing ileocolonic resection was observed in 40-60% of cases within 3 months and in almost 80% within 1 year. This incidence was drastically reduced by performing a diverting ileostomy, but increased again following reanastomosis (26).

A recent study performed in 61 patients with Crohn's disease and colon cancer patients after ileocelectomy demonstrated that colonization of neoterminal ileum is increased in both, but in IBD relapsing patients, there is a prominent increase of gram-negative bacteria mainly including *Bacteroides*, *E. coli*, *Fusobacteria* and nitrate-reducing bacteria, whereas gram-positive anaerobes were increased in cancer patients (27). This suggests that a specific pattern of intestinal microflora exists in IBD.

Kleessen et al. analysed the composition of intestinal flora in patients with Crohn's or with ulcerative colitis and compared it to that of non-IBD controls. The results showed an increased number of colonic bacteria in IBD patients and a difference between Crohn's disease and ulcerative

colitis with respect to counts and frequency of selected bacterial groups. In ulcerative colitis there was a predominance of Proteobacteria, Enterobacteriaceae, Bacteroides/Prevotella, Clostridium histolyticum, Clostridium coccoides, together with high G+C Gram-positive bacteria or sulphate-reducing bacteria. In Crohn's disease, fecal samples were rich in bacteria belonging to the former three groups (28). However, it is not yet clear whether these associations are to be interpreted as a cause or a consequence of a specific IBD.

Abnormal immune response to commensal bacteria in the pathogenesis of IBD

In normal condition the intestinal mucosa is relatively free from adherent bacteria. Recent data suggests that increased numbers of bacterial antigens and nucleic acid sequences are found associated to the mucosa in IBD patients (29,30). Patients with IBD may have abnormalities in the mucus-epithelial layer composition which allow a closer interaction between colonic bacteria and intestinal mucosa. This interaction could trigger inflammation thereby increasing intestinal permeability. Changes in intestinal permeability would, in turn, induce a mucosal secretion of IgG antibodies against a broad spectrum of commensal bacteria (31) consistent with an hyperactive immune response, characterised by T lymphocyte against bacterial antigen in Crohn's patients (32). A pathogenic role for bacterial-responsive T lymphocytes has been demonstrated in an animal model of colitis (33). Interestingly the cell line developed from this Th1 lymphocytes is able to induce colitis in SPF T-cell-deficient recipients (SCID) (34).

Recent studies performed in adults with IBD, showed a high concentration of mucosal bacteria in patients but not in controls. The concentrations of mucosal bacteria correlated with the severity of the disease, being highest in severe Crohn's disease. Isolated species were of fecal origin in all groups; the main anaerobic microorganism isolated from the mucosa was Bacteroides whereas the main aerobe included Enterobacteriaceae (primarily E. coli). In the light of the location of mucosal bacteria also in non-inflamed mucosa, the lack of apoptosis in the nucleus of invaded enterocytes,

the normal electronic appearance of the adjacent cells, it was concluded that the alterations of mucosal flora in IBD patients were not secondary to inflammation but rather a result of a specific host response (35), which is probably genetically determined. Some patients with Crohn's disease (17-25%) have mutations in the NOD2/CARD15 gene, which regulates host responses to bacteria (36). This may suggest a specific host-agent relationship based on genetic susceptibility leading to an abnormal immune response, at least in a subset of Crohn's patients.

Probiotics in IBD

Data on probiotics administration support the role of enteric agents in IBD. In an experimental model of colitis, provided by IL-10 knock out mice, the administration of Lactobacillus GG (37) or of a mixture of different probiotics (VSL3) (38) induced a significant improvement in the severity of colitis. Clinical studies were undertaken to investigate the role of administration of probiotics to patients with IBD. Administration of E. coli Nissle strain 1917 was equally effective than maintenance doses of mesalazine in preventing relapse of ulcerative colitis (39). Similarly a cocktail of eight probiotics (Lactobacillus, Bifidobacterium and Streptococcus species), named VSL3, significantly prevented relapse in adults with pouchitis (40). Preliminary data showed a possible use of probiotics in pediatric children with Crohn's disease; the administration of Lactobacillus GG was effective in reducing the severity of the disease as judged by a reduction of PCDAI score, probably reducing the intestinal permeability (41). However data in adults with Crohn's disease showed no efficacy by Lactobacillus GG, either in preventing recurrence or in reducing the severity of recurrent lesions (42).

We have recently shown that fecal concentration of Nitric Oxide (NO) is increased in children with IBD, due to the activation of the inducible NO Synthase isoform of NOS (43). NO concentration was highly increased in children with active ulcerative colitis. It was also increased, though to a lesser extent, in those with inactive UC and also in children with active Crohn's disease and rectal involvement, but not in those with rectal sparing (figure). We also found that concentrations of NO

were increased in children with Cystic fibrosis, a chronic inflammatory condition, with frequent intestinal involvement (44). In these patients, the administration of Lactobacillus GG for 6 months was effective in reducing abdominal pain. An evident decrease of NO concentration, as determined by the dialysis bag was also observed (Bruzzese E, unpublished data). These data provide indirect evidence of a role of enteric microflora in intestinal inflammation.

Conclusions

Several lines of evidence indicate that enteric microflora plays a key role in IBD. Clinical evidence includes the similarities in symptoms and histological pictures of IBD and intestinal infections caused by selected pathogenic bacteria. Epidemiologic links have been found for either Crohn's disease and ulcerative colitis with enteric infections caused by selected microorganisms, but conclusive evidence has not been reached.

Some evidence suggests that selected enteric agents trigger inflammatory changes that are then perpetuated by an abnormal immune response in genetically susceptible host. On the other side, immunologic abnormalities may be an underlying condition, on which selected bacteria may then operate to perpetuate or re-exacerbate intestinal inflammation.

Finally there is indirect but compelling evidence that differences exist between adults and children in terms of clinical presentation, enteric microflora, immune abnormalities and response to treatment. Several data show that microbiological imprinting and immunological imprinting are closely related, particularly in the earlier stages of life, and may affect the development of immune related diseases.

This may be a key for better understanding the pathophysiology of IBD and the role of enteric microflora. It may also provide an option for active interventions aiming at modifying intestinal microflora to "better educate" the immune system in its developmental stages.

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Table I

Bacterial genera dominating the intestinal ecosystem

- Bacteroides
- Bifidobacterium
- Clostridium
- Coprococcus
- Eubacterium
- Fusobacterium
- Lactobacillus
- Peptostreptococcus
- Ruminococcus

Table II

Major determinants of intestinal microflora in children

- Age
- delivery mode and setting
- feeding type
- gastric PH
- intestinal motility
- immune system
- drugs (antibiotics)
- x-ray
- probiotics, prebiotics

Table III

Enteric pathogens causing infections resembling inflammatory bowel disease

Crohn's disease like	Ulcerative colitis like
Mycobacterium tuberculosis	Campylobacter jejuni
Mycobacterium avium complex	Salmonella species
Yersinia enterocolitica	Shigella species
Entamoeba histolytica	Clostridium difficile
Chlamydia trachomatis	E. coli O157: H7
E. coli O157: H7	Aeromonas species
Cytomegalovirus	Plesiomonas species
Histoplasma capsulatum	Treponema pallidum
Cryptococcus neoformans	Legionella species

Table IV

Intestinal complications and extraintestinal manifestations of intestinal infection resembling IBD

Intestinal complication	Extraintestinal manifestations
Toxic megacolon	Reactive arthritis
Perforation	Spondyloarthropathy
Abscesses	Epatobiliary inflammation
Stricture	Erythma nodosum
Fistula	Uveite

Table V

Hallmarks histological findings in IBD and in intestinal infections.

Inflammatory bowel disease	Specific enteric infection
Distorted crypt architecture	Pseudomembrane (Clostridium difficile, E.coli O157:H7, Staphylococcus aureus)
Crypt atrophy	Viral inclusion (CMV, HSV2)
Basally located giant cells	Caseating necrosis (Mycobacterium tuberculosis)
Villous colonic epithelium	Diagnostic organisms (Entamoeba histolytica, Cryptosporidium)
Mixed inflammatory cell population in lamina propria	Specific staining (Mycobacterium, Histoplasma capsulatum)
Non caseating granulomas	
Lymphoid aggregates	

Table VI

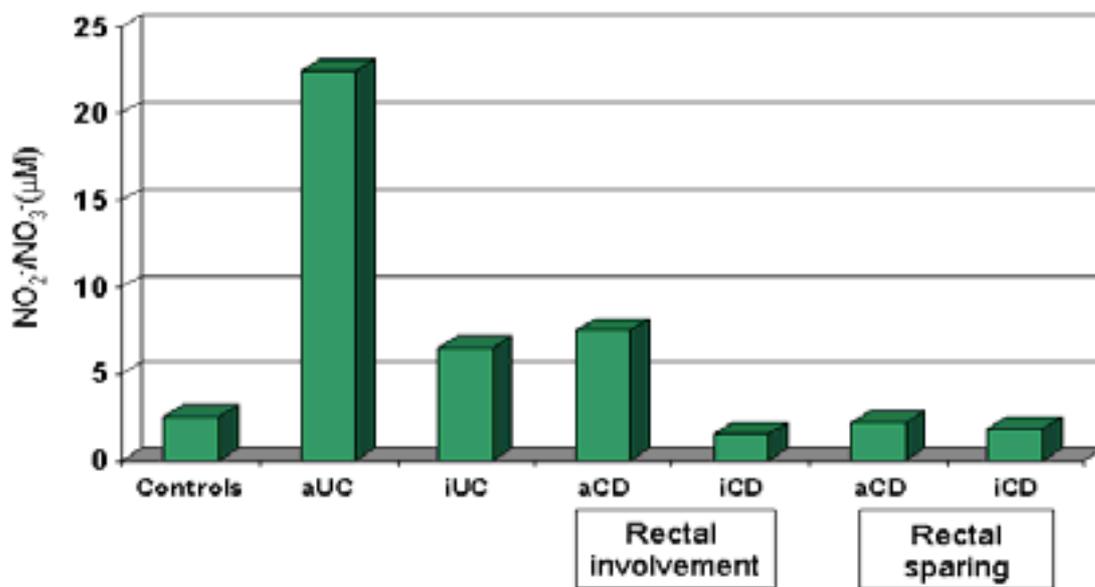
Pathogenic microorganisms implicated in the pathogenesis of IBD

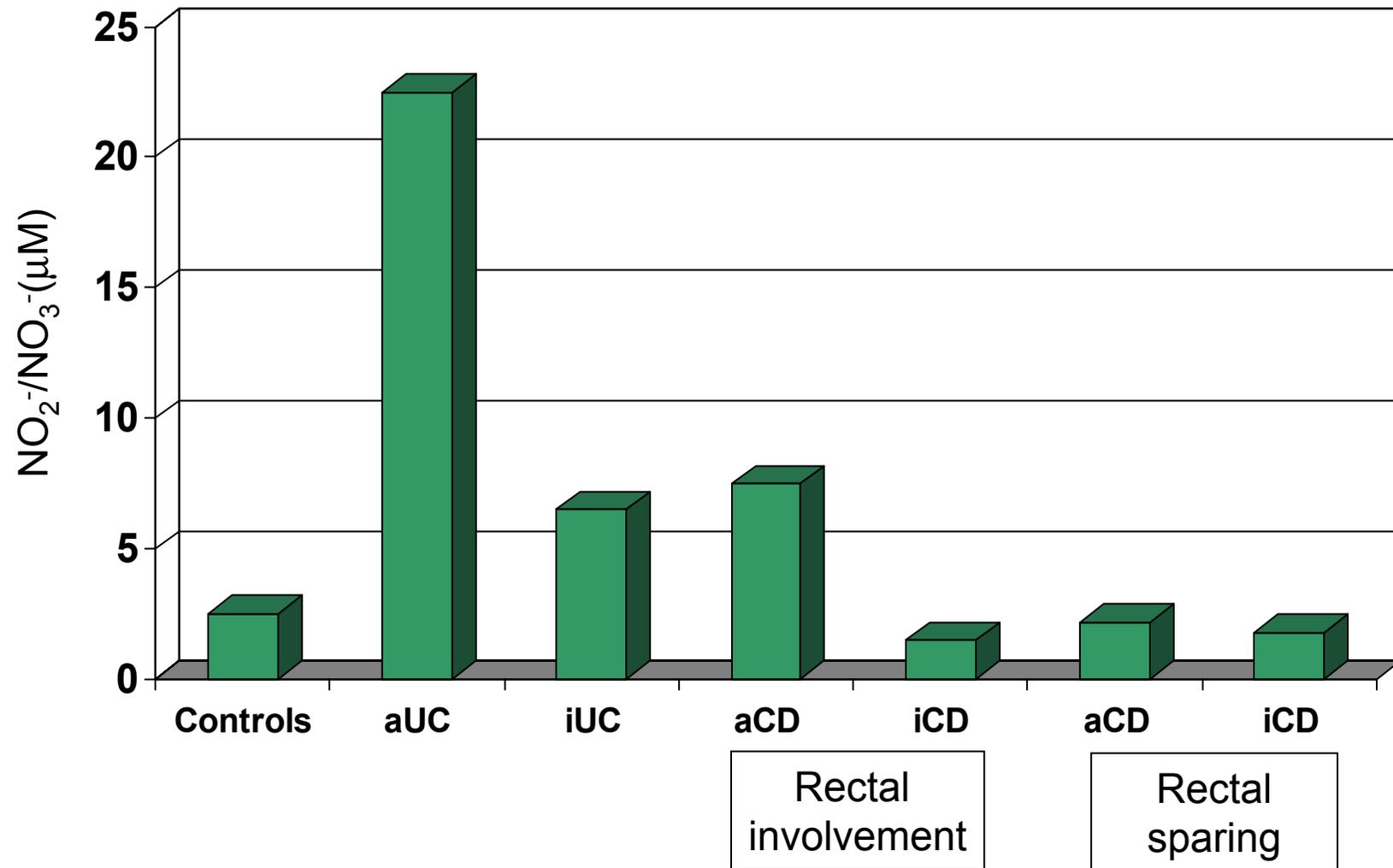
Ulcerative colitis	Crohn's disease
Diplostreptococcus	Chlamydia
Bacteroides necrophorum	Mycobacterium Kansaii
Shigella	Mycobacterium paratuberculosis
RNA virus	Paramyxovirus (measles)
Toxigenic/adherent E. coli	Listeria monocytogenes
	Adherent/invasive E. coli
	L forms of Pseudomonas maltophilia
	Pseudomonas fluorescens

Figure legend

NO end stable metabolites ($\text{NO}_2^-/\text{NO}_3^-$) determined by rectal dialysate in children with active UC (aUC) or inactive UC (iUC), children with active CD (aCD) or inactive CD (iCD) with or without rectal involvement, and controls.

Modified from ref. 43





PROBIOTICS, *E. COLI* FROM CHILDREN WITH CROHN'S DISEASE AND ENTEROCYTE FUNCTION

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Background: Probiotics are living microorganisms origin, which are associated with therapeutic or preventive health benefits. Impaired paracellular permeability is a known feature of CD patients. It has been suggested that probiotics may ameliorate the intestinal permeability defect and disease activity in CD patients. The exact mechanism by which probiotics influences mucosal barrier function is not well defined. We have identified mucosa adherent *E. coli* strains from CD patients that affect the paracellular permeability of human intestinal cell monolayers. The paracellular defect was associated with degradation of the TJ proteins ZO-1 and Occludin as well as disruption of the actin cytoskeletal architecture. In this study we examined the role of LGG in preventing the CD *E. coli*-induced loss of TJ proteins and alteration in actin cytoskeletal architecture.

Methods: The human intestinal villous epithelial cell line Caco-2/bbe subclone C2 exhibits decreased transepithelial electrical resistance (TEER) when exposed to CD *E. coli* isolate CD1033 for 10 hr. The laboratory *E. coli* strain DH5 α does not affect TEER. Cells were preincubated with LGG for overnight before adding the *E. coli*. Cells not exposed to bacteria or exposed only to LGG or VSL#3 were included as controls. TEER was measured at 0, 2, 4, 6, 8 and 10 hr. Levels of the TJ-associated proteins ZO-1 and Occludin were assessed in C2 cells using quantitative Western Blots. Actin cytoskeletal structure of the bacteria-exposed C2 cells was examined at 10 hr using staining with FITC-Phalloidin.

Results: Baseline TEER increased following overnight incubation with LGG. Decrease in TEER of CD1033- exposed cells was prevented by pre-incubation with LGG. LGG blocked CD1033-induced degradation of the TJ protein ZO-1 at 10 hr. Staining of CD1033-exposed cells revealed a widening in the intercellular bands, with noticeable beading. LGG pre-treatment prevented these effects of CD1033 on the actin cytoskeleton.

Conclusion: LGG increased the baseline TEER of human intestinal epithelial cell monolayers and prevented CD *E. coli*-mediated changes in paracellular permeability only following preincubation but not seen with co-incubation. LGG appeared to mediate these effects at least in part by preventing the degradation of the TJ protein ZO-1 and by preventing the disruption of the actin cytoskeletal architecture. We speculate that LGG is useful in maintaining the remission of crohn's disease.

PROBIOTICS: REQUIREMENTS AND NEW PERSPECTIVES IN ATOPY

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At the beginning of last century Ilja Metchnikoff hypothesized that health in Bulgarian peasants was in part a consequence of consumption of fermented milks (1). Metchnikoff attributed such health effects to shifts of the intestinal microbial balance; as such, the ukrainian Nobel Prize winner began to modify the colonic microflora through ingestion of soured milks containing lactic acid bacteria (LAB) such as a Gram-positive rod which he called *Bacillus bulgaricus*. Since these early observations, attempts have been made, especially during the last two decades, to improve the health status by modulating the intestinal microflora composition.

The definition of probiotics has evolved concomitant with a resurgence of research interest in host-microbe crosstalk. Parker was the first to use the term "Probioticum", meaning for life ("*organisms and substances which contribute to intestinal microbial balance*") (2). In 1989 Fuller (3) emphasized the requirement of viability for probiotics and introduced the aspect of a beneficial effect on the host, which was, according to his definition, an animal ("*a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance*"). The definition was later changed to include microbial food supplements for human use; according to Guarner "*oral probiotics are living microorganisms which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition*" (4). More recently Schrezenmeir and de Vrese propose the following definition: "*A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host*" (5). It is up to the experts in microbiology, nutritional sciences, and food technology to formulate unequivocal criteria for probiotic bacterial strains and products that contain them. Such criteria should be the basis for establishing requirements for the use of the term probiotic, which could be established by national or international authoritative bodies. The selection of suitable bacterial strains (identity, counts, stability, growth conditions, survival gastrointestinal passage, non pathogenicity, nontoxinogenicity) can be regarded as the primary requirement for the use as a probiotic. It is important to ensure that the specific properties of a probiotic used originally as selection criteria are also targets for quality

Furthermore, probiotic bacteria selected for commercial use in foods and in therapeutics must retain the characteristics for which they were originally selected (6): human origin, characteristics for growth and survival during manufacture and storage, safety in human use, bile and acid resistance, adhesion to the mucosa, at least temporary colonization of the human gut, survival in the intestine, ability to inhibit known gut pathogens and antimicrobial substances production. These abilities are common among gram-positive lactic acid bacteria represented by the genera *Lactobacillus* and *Bifidobacterium*.

Strains used for new probiotics should be chosen from the commensal flora of humans. The significance of human origin has been debated, but most current successful strains are indicated to be of human origin: it can be argued that a probiotic strain can function better in a similar environment to where it was originally isolated from.

Safety remains a major concern, especially when the use of living bacteria is promoted as prophylactic in a consistent proportion of infants from the the general population. Although lactobacilli and bifidobacteria have a generally favourable profile there have been sporadic cases of adverse effects in immunodeficient patients (7). The ability to adhere to the intestinal mucosa is one of the more important selection criteria for probiotics because adhesion to the intestinal mucosa is considered to be a prerequisite for colonization (8). As substrata, enterocyte-like Caco-2 tissue culture cells (human colon carcinoma cell line), intestinal mucus, mucus-secreting HT29-MTX tissue culture and human ileostomy glycoproteins are currently used (9). Possible changes in adhesion stability should be examined by using more than one model. Only *Lactobacillus* GG (ATCC 53103) and *Lactobacillus johnsonii* La-1 were adhesive, by using Caco-2 and human ileostomy glycoproteins as in vitro models for intestinal epithelium and mucus, respectively assurance (10). *Lactobacillus* GG isolated from the fecal samples of subjects consuming a fermented whey drink containing *Lactobacillus* GG had adherence properties equal to those of the original strain (11).

The long term exploitation of probiotics would depend on the desire by consumers to use natural methods for health maintenance and effective marketing strategies. Much of this growth will also depend on the reliability of claims that these products will bare. Probiotics are presently marketed as conventional foods and, therefore, the general rules for labeling and claims, nutrition labeling, and nutrition claims apply. There is an ongoing lively discussion on the terms *prevention of disease* and *health claims*. The Codex General Guideline on Claims (12) prohibited claims that cannot be substantiated and claims on the suitability of a food for use in the prevention, alleviation, treatment or cure of a disease, disorder, or particular physiologic condition. The new guidelines proposed by FAO/WHO (13) will require that probiotic strains and products be identified properly, be proven to actually confer specific health benefits, and be manufactured and labelled in a way that delivers the optimum benefit to the patient or consumer. The crucial point is to show a distinct health benefit achieved by consumption of a specific strain. The effect of a “friendly bacterium” is strain specific and cannot be extrapolated even to other strains of the same species. For demonstration of probiotic activity, well-designed clinical trials are needed, which should be controlled, randomised and double-blinded. Such standards are badly needed to move this area to one of even greater respectability.

In recent years it has become accepted that healthy human intestinal microbiota may have a crucial role in the maturation of the immune system to nonallergic mode (14). Cohort and cross-sectional studies have indicated different microflora-associated characteristics in faeces from allergic and non allergic infants: a lower incidence of atopic skin and respiratory tract hypersensitivity complaints was found among children with stable gut populations of lactic acid bacteria than among those with a paucity of these bacteria and high counts of *Clostridia* and *Stafilococcus aureus* (15-17).

The intestinal microflora beneficially affect gut permeability by providing potent stimuli to build and strengthen the mucosal barrier. The evidence that the gut associated lymphatic tissue (GALT) evolves through bacterial colonisation (18,19) supports the concept that the intestinal microbiota are an essential component in the mucosal immune defence. Recent evidence suggests that lactobacilli and bifidobacteria are far from inert commensals and that they may provide important regulatory signals to the immune system.

Increasing evidence, including human studies, is supporting the immunomodulatory role attributed to given lactic acid bacterial strains. GALT underlying the epithelia must constantly distinguish innocuous antigens present in food and gut commensals from invasive pathogenic microbes. The gut microflora is known to contribute to gut mucosal barrier functions (20) and to regulate local immune responses against ingested antigens (21,22). An obligatory input from the normal microbiota to the innate immune system is essential for the establishment and maintenance of mucosal immune tolerance. Appropriate immunoregulatory responses can invoke the down-regulation of T helper 2 (Th2)-type responses; intestinal microorganisms could down-regulate the

allergic inflammation by counter-balancing T-helper cell type 2 responses and by enhancing antigen exclusion through induction of an IgA response (23). Although this effects alone might suggest a direct link with decreased Th2-mediated allergy, it has become clear that the Th1/Th2 paradigm is inadequate to explain mucosal immune responses (24). Specific immunological unresponsiveness to dietary components (oral tolerance) and to commensal bacteria is critically dependent to inhibition of potential lymphocyte reactivity “*bystander suppression*”. Two recently recognized suppressor-cell population are central in this process: Th3 and T regulator (Tr) 1 cells, which produce *transforming growth factor-β* (TGF-β) and interleukin-10 (IL-10), respectively. These cytokines possess an important regulative role in the development of allergic type immune response by promoting potentially anti-allergenic processes and reducing the immunogenicity of food antigens. Both the commensal flora and pathogens are recognized by *toll-like receptors* (TLRs), a group of evolutionarily conserved *pattern-recognition receptors* (PRR), expressed on both enterocytes and antigen-presenting cells (25-28). Connection of TLRs by specific microbial components leads ultimately to the production of Th1 proinflammatory cytokines through a process dependent on the transcription nuclear factor-κB (NF-κB). Antigen-presenting cells orchestrate specific mucosal immune responses which might induce suppressive Th3 and Tr1 responses in the gut (29). There are, however, a few potential reasons why commensal bacteria normally do not cause constant inflammation in the gut by the activation of the immune system via TLRs (30,31). For a variable period after birth, the GALT is functionally immature and the intestinal permeability is transiently increased; the consequent enhanced antigen load may cause aberrant antigen transfer leading to sensitisation (32). In early infancy some non pathogenic bacteria do gain access to the GALT because of the immature gut barrier function. *Lactobacillus rhamnosus* GG (ATCC 53103), a probiotic strain, has been shown to induce the production of Th1, Th3 and Tr1 cytokines, particularly activating the NF-κB pathway (33).

Dietary modulation of the gut microbiota is a topical area of nutritional sciences and the main focus of probiotics. Experimental studies have led to the hypothesis that stimulation of the immune system by probiotics may prevent allergic diseases. Current approach in the management of food allergy aims at complete avoidance of food proven to cause symptoms. In infants with cow’s milk allergy (CMA), extensively hydrolyzed formulas are used to eliminate cow’s milk antigens from the diet. The preliminary heat treatment of cow’s milk mainly affects the conformation of proteins and facilitates their hydrolysis. Subsequent enzymatic hydrolysis with pepsin and trypsin causes progressive destruction of sequential epitopes and refines the formulas into the least antigenic and allergenic form. However, even the extensive degree of hydrolysis does not render the formula non-antigenic: adverse clinical reactions after the ingestion of hydrolyzed formulas have been reported in a subgroup of infants with CMA (34).

Recent studies draw attention to the potential use of probiotic supplementation of infant nutrition to control atopic eczema and local and systemic inflammatory responses, and in early prevention of onset of atopic disease. Isolauri reported that atopic infants (exhibiting eczema and/or food hypersensitivity) who were fed an extensively hydrolyzed milk whey formula, supplemented with *Lactobacillus rhamnosus* GG or *Bifidobacterium lactis* Bb12 exhibited reductions in ex vivo markers of intestinal inflammation and in vivo dermal hypersensitivity (35,36). Kalliomaki showed that consumption of *Lactobacillus* GG by at-risk babies born to atopic parents, or by their mothers pre-partum and post-partum, halved the subsequent occurrence of atopic dermatitis (37,38). In patients with food allergy increased intestinal permeability is a result of a local hypersensitivity reaction (39). Oral introduction of probiotics can help in the treatment of such food allergies by alleviating intestinal flogosis (40) and reversing increased intestinal permeability induced by cow milk antigens (22). Specific probiotic strains have been shown to increase the production of cytokine-suppressive factors, IL-10 (41) and TGF β (42), raising the suggestion that production of these anti-inflammatory cytokines may be just as important in limiting atopic disease as pro-Th1 immune signalling (37). It has also been suggested that probiotics modify the structure of

potentially harmful antigens and thereby alter the mode of their immunogenicity (43,44). Pro-Th1 activation is not the only possible means by which probiotics may effect anti-allergy immunoregulation: active down-regulation of atopic responses could be due to induction of generic cytokine-suppressive factors (TGF- β and IL-10) as much as anti-Th2 immunoregulatory factors. *Lactobacillus* GG and *Lactobacillus paracasei* (strain NCC2461) can induce these lymphosuppressive factors (41,45). Finally, it has been speculated that the early preventive effect of *Lactobacillus* GG on atopic eczema might at least partly be mediated by the activation of the NF- κ B pathway via TLRs.

“A more complete understanding of the relationship of the intestinal flora with the human host may reveal new understanding of human diseases and suggest novel approaches towards reating them” (27).

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GUT-IMMUNE SYSTEM MATURATION: ROLE OF PRE-PROBIOTICS

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Summary

The neonatal period is crucial in term of mucosal defence, mainly with regard to infections and priming for allergic disease. This is believed to be due to the immaturity of gut associated lymphoid tissue (GALT) functions. In our study we evaluated the capacity of three groups of children, breast-fed, bottle-fed and fed with a formula-milk supplemented with probiotics, to produce IgA *in vitro* in response to activation by anti-CD40 and IL10. Our data suggest that the capacity to produce IgA is present at birth, but it need to be stimulated by antigens to develop.

Introduction

In healthy conditions the human child's intestine is sterile at birth, but it can be colonised by 10^8 - 10^9 bacteria in less than 48 hours. Shortly after birth, only the facultative anaerobic bacteria *Escherichia Coli (E.Coli)* and *Streptococcus* can colonise the intestine of a baby, coming from maternal faecal flora. Two to four days later, anaerobic bacteria as *Bifidobacterium*, *Bacteroides* and *Clostridium* can be found. The factors responsible for the growth of this selected flora can be the degree of maturation of the intestinal mucosa, growth promoters or inhibitors present in the meconium or exogenous factors as the type of diet: breast-feeding versus bottle-feeding. In exclusively breast-fed babies, *Bifidobaterium*, *E.Coli* and *Streptococcus* are the predominant faecal microflora, while in bottle-fed babies *Bifidobacterium*, *Bacteroides*, *Clostridia*, and other *Enterobacteria* are present. In the weaning time the intestine can be colonised by opportunistic pathogens and can rich the adult microflora at about two years of age.

IgA, and mainly secretory IgA, represent the first line of defence in the mucosa and the development of this Ig isotype is important to prevent infections and priming for allergic disease.

The aim of the present study was to evaluate the functional maturity of B lymphocytes in infants at birth and after feeding at breast, with a conventional formula or with a milk supplemented with probiotics.

Patients and methods

Twenty exclusively breast-fed babies, 20 formula-fed babies and 20 babies fed with milk added with probiotics were enrolled in the study. All were healthy babies, born at term with a natural delivery.

At birth and after 1, 3 and 6 months the capacity of infants' B lymphocytes to produce IgA *in vitro* was evaluated as previously described (1). Lymphocytes from peripheral blood were cultured in presence of antiCD40 and IL10 (T-cells independent activation system) for 10 days at 37°C in 5% CO₂ (1). IgA concentration in the supernatants was determined by an ELISA technique previously described (2).

Results

The results are reported in Table.1.

	Breast- fed		Bottle- fed		Probiotic- fed	
	IgA r	IgA s	IgA r	IgA s	IgA r	IgA s
T0	0.66±0.74	98.89±96.95	0.66±0.74	98.89±96.95	0.66±0.74	98.89±96.95
T1	1,81± 2,00	60,18 ± 59,70	1,51 ± 1,86	91,27 ± 90,10	1,96 ±2 ,99	73,25 ± 81,31
T2	0,95 ± 0,78	15,04 ± 13,74	0,92 ± 0,99	39,22 ± 36,73	0,4 ± 1,36	20,4 ± 23,08
T3	1,14 ± 1,24	60,35 ± 56,77	1,93 ± 2,40	94,53 ± 93,96	2,37 ± 1,43	70,4 ± 68,87

Tab.1: T0 at birth before feeding, T1 at 1 month, T2 at 3 months, T3 at 6 months of age. The IgA production is expressed in ng/ml. IgAr =IgA resting (without stimulation); IgAs = IgA production after antiCD40 and IL10 stimulation

Discussion

The GALT structures generally develop in fetal life. At birth, the new-born's Peyer's patches (PP) contain primary lymphoid follicles, T-cell dependent area, a dome region and follicle-associated epithelium, but secondary follicle with germinal centers are absent. They appear only shortly after birth, when antigenic stimulus occurs (3). Antigen constituents of food have a stimulatory effect on the intestinal B lymphocytes as well as the indigenous microflora as shown by the fact that the intestinal IgA system of germ-free mice is poor, but it can be developed and normalised after 4 weeks of conventionalization (4). *Bacteroides* and *E.Coli* seem to be particularly important for the development of intestinal IgA immunocytes (5). It is assumed that PP largely give rise to the small intestine B-cell population (6), while the appendix primes B-cells for the large intestine (7). The IgA, in human, are present in two subclasses, IgA1 and IgA2, which seem to develop at different rates and respond to different antigenic stimuli. IgA1 is predominant in human serum and IgA- cells are in large number in duodenum and jejunum (77%) and produce antibodies directed towards protein antigens, while IgA2 predominate in the colon (64%) and IgA2 antibodies are more resistant to microbial proteases and direct to lipopolisaccharides (LPS) from gram-negative bacteria (6).

In our study we found that new-borns are able to produce IgA *in vitro* shortly after birth. The system utilised was a T-independent stimulus, given by anti CD40 and IL10. In this way we could show that breast-fed infants produce less IgA than infants fed with formula milk. The infants fed with formula milk enriched with probiotic produce an amount of IgA more similar to the breast-fed children. These results can be explained by the fact that large amount of maternal IgA present in human milk can be lightly inhibitory on IgA production by infants' B-cells. The probiotic present in the formulated milk can represent a tolerogenic more than immunogenic stimulus, whereas the conventional formulated milk gives a stronger antigenic stimulus that lead to a greater IgA production. Interesting is the fact that at 3 months of age the three groups of children have a less response to the anti CD40-IL10 stimuli, in term of IgA production. This fact can reflect the starting production of an IgG response, mainly after conventional vaccination.

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PROTECTIVE EFFECTS AND IMMUNOMODULATION ON INTESTINAL CELLS BY PROBIOTICS ¹

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Introduction

Probiotics were recently defined as: “viable microorganisms used as functional food in sufficient number, which alter intestinal microflora of the host by adhesion and colonization, and exert health beneficial effects in this host” (Schrezenmeir et al., 2001). Special interest has been focused recently on the use of bifidobacteria and lactic acid bacteria as probiotics. The several benefits ascribed to the ingestion of these microorganisms include the following: 1) maintenance of proper balance in the human intestinal microbiota; 2) protection against intestinal pathogens; 3) modulation of immune response; 4) improvement in food allergies, autoimmune diseases and oral tolerance (Ballongue 1993; Fuller 1989; Salminen et al., 1996; Sanders 1993; Walker and Duffy 1998). Probiotics must be able to survive the gastric transit and colonize intestinal mucosa, and in such way maintain or induce a healthy intestinal microflora. The interaction between epithelial cells and microflora is fundamental to establish gut mucosal barrier and thus to promote intestinal integrity and prevent cell dysfunction (Donnet-Hughes et al., 1992; Bouhnik et al., 1992; Goldin et al., 1992; Ouwehand et al., 2002)

Protection against pathogen adhesion, invasivity and membrane disruption

Intestinal microflora represent a barrier against the adhesion of pathogens to intestinal mucosa, that is the first step in the infective process. Pathogen adhesion leads to brush border lesions in intestinal cells, that allows the entry of bacteria and the consequent death of epithelial cells. Several studies showed protective effects of probiotics in intestinal inflammations and ulcerations experimentally induced in animals to mimic the human ulcerative colitis, Crohn's disease and pouchitis. In fact, *Lactobacillus reuteri* diminished the severity of

colitis induced by acetic acid in rats (Fabia et al. 1993), *Lactobacillus plantarum* induced a low intestinal permeability and bacterial translocation in rats with enterocolitis (Mao et al. 1996) and *Lactobacillus brevis* was able to prevent or improve disorders of intestinal permeability (García-Lafuente et al., 2001).

One of the mechanisms of protection against pathogens by probiotics is the adhesion competition which may likely lead to inhibition of invasivity. Indeed, some probiotics such as *L. rhamnosus*, *L. acidophilus* and *Bifidobacterium lactis* have been shown to compete with pathogens for adhesion to differentiated human intestinal cells-lines and to reduce the invasiveness of *Escherichia coli* (Gopal et al., 2001). Inhibition of cell invasion associated to inhibition of adhesion of enteropathogenic *E. coli* (EPEC), *Yersinia pseudotuberculosis* and *Salmonella typhimurium* has been reported also in intestinal cells treated with *L. acidophilus* LA1 (Bernet et al., 1994). Recently, a protection against adhesion and disruption of brush border integrity induced by enteroinvasive *E. coli* (EIEC) was observed on intestinal cells after treatment with *Streptococcus thermophilus* and *L. acidophilus* (Resta-Lenert and Barret, 2003). Some authors, using a human intestinal cell-line secreting mucus (HT-29), found an increase in mucine gene expression (MUC 2 and MUC 3) induced by *L. plantarum* and *L. rhamnosus* GG and suggested that this led to a decrease in EPEC adhesion (Mack et al., 1999).

Despite the increasing number of studies on the inhibitory effect of mucosal injury by probiotics, there is a need to extend the investigations to other probiotic strains, as well to study their activities against a wide range of pathogenic bacteria.

We have recently investigated whether *Bifidobacterium animalis* and *Lactobacillus casei* may protect human intestinal epithelial cell-line (Caco-2) against the membrane damage induced by enterotoxigenic *E. coli* (ETEC), strain K88. This bacteria is a principal cause of diarrhea and mortality in piglets. ETEC k88 has been shown to adhere to Caco-2 cells. *B. animalis* can survive and colonize intestinal tract (Mengheri et al., 1999). *L. casei* has been used as probiotic in previous studies (Kato 1998; Sanders 1993). In our experiments, Caco-2 cells were grown on Transwell filters which allow the separation of apical from basolateral compartments. These cells spontaneously differentiate, after 15 days of confluency, to mature enterocytes, which are characterized by structural polarization, tight junctions and microvilli. When differentiated, these cells were

treated with ETEC and *B. animalis* or *L. casei*, added to the apical compartment. The membrane permeability was assayed measuring the transepithelial electrical resistance (TEER). The results showed that these probiotics did not affect the TEER but they were not able to prevent the ETEC induced permeability increase when added together with ETEC.

To assay whether probiotics could reduce ETEC invasivity, Caco-2 cells were infected with ETEC and treated with *B. animalis* or *L. casei*. Cells were also treated with gentamicin to kill extracellular bacteria. The number of viable internalized bacteria were quantified by agar plating. Probiotics were able to reduce ETEC internalization. These results indicate that, despite the lack of protection on membrane permeability, probiotics were able to protect the cells against ETEC infection.

Immune modulation

Immune activation has been suggested to be responsible for prevention of intestinal disease by probiotics. The immune effects include a modulation of cytokine expression. Indeed, several studies have reported that probiotics were able to regulate anti- and pro-inflammatory cytokine production. In a study on mice treated with *L. paracasei*, an increase of interleukin (IL)-10 and IL-12 in splenic lymphocytes was observed (Von der weid et al. 2001). In other studies, the level of interferon (IFN)- γ and IL-12 in human peripheral blood mononuclear cells (PBMC) were found increased after treatment with *L. sakei* whereas the level of transforming growth factor (TGF)- β increased after treatment with *L. johnsonii* (Haller et al., 2000). Moreover, plasmatic values of IFN- α and IFN- β increased in mice injected with lactic acid bacteria (Solis-Pereyra et al. 1997).

Epithelial cells can participate to regulation of the mucosal immune response to bacteria by modulating the secretion of cytokines/chemiokines. It has been reported that intestinal cells remained hyporesponsive to a challenge of non pathogenic *E. coli* and *L. sakei*, whereas when these cells were co-cultured with leucocytes an increased expression of IL-8, IL-1- β , TNF- β and monocyte chemoattracting protein 1 was observed. In our study on the effect of *B. animalis* or *L. casei* on immune homeostasis and ETEC associated immune perturbations on Caco-2 cells, we have found that these probiotics actually induce a modulation

on cytokine gene expression of Caco-2 cells. Indeed, we observed no changes on expression of TGF- β and IFN- γ , a small increase of tumor necrosis factor(TNF)- α and an increase of chemoattractant cytokines IL-8, growth related oncogene(GRO)- α and epithelial neutrophil-activating protein(ENA)-78. In addition, when the probiotics were added together with ETEC, they were able to reduce expression of IFN- γ , TNF- α IL-8, GRO- α and ENA-78.

We have also studied whether probiotics could regulate the migration of neutrophils induced by ETEC. Caco-2 cells were grown on inverted Transwell filters and neutrophils were added to the basolateral compartment, whereas ETEC and/or probiotics were added to the apical compartment. ETEC induced a strong neutrophil transmigration after 2 hours of infection, but this migration was markedly reduced when *B. animalis* and/or *L. casei* were added together with ETEC. Probiotics without ETEC did not induce neutrophil transmigration.

Effects on food allergy, autoimmune disease and oral tolerance

The gut immune system has to be able to protect the mucosa against pathogens but also must avoid hypersensitivity reactions to food proteins, normal bacterial flora and other environmental macromolecules. Oral tolerance is a specific suppression of cellular and humoral cell-mediated immune responses to orally administered antigen upon subsequent immunization with the same antigen to prevent immune reactions to dietary antigens (Mowat, 1994; Brandtzaeg, 1997; Mayer, 2000; Husby, 2000). Breakdown of oral tolerance may lead to the development of adverse reactions to food, mucosal immunopathologies directed against environmental antigens or autoantigens and thus to autoimmune disease (Weiner, 1997; Strobel et al., 1998).

Food allergy is currently treated by antigen elimination, but such approach is not always satisfactory. Very recently, the use of probiotics to favorably alter the indigenous flora and modulate intestinal immune response has been suggested as a new strategy to control allergic inflammation. Indeed, inflammatory response against food antigens in allergic subjects was lower after treatment with probiotics (Pessi et al., 1999; Pelto et al., 1998). Moreover, symptoms of atopic dermatitis were diminished in infants receiving *L. rahnosus* GG and atopic disease development was reduced in newborns from mothers which received the

same probiotic during pregnancy and breastfeeding (Majamaa and Isolauri 1997; Kalliomaki et al., 2001, Kalliomaki et al., 2003). There are also studies on the effect of probiotics during the development of autoimmune diseases. Mice treated with *L. casei* strain Shirota during the development of arthritis induced by type II collagen, which is similar to human rheumatoid arthritis, were less susceptible to the induction of the disease (Kato et al., 1998).

However, more evidence is necessary to confirm a role of probiotics in the management of allergic disease.

We have recently investigated whether feeding with *B. animalis* or *L. casei* influenced oral tolerance induction and maintenance in rats immunized with a food antigen normally not present in their diet, that is ovalbumin (OVA). We assayed splenic and mesenteric lymphocytes proliferation after *in vitro* stimulation with OVA or with a polyclonal mitogen Concanavalin A (ConA). Proliferation of mesenteric lymphocytes of immunized rats after *in vitro* stimulation with OVA was lower in probiotics treated than untreated rats, whereas it was similar in splenic lymphocytes of the two groups of rats. We did not observe a reduction in proliferative response of splenic and mesenteric lymphocytes after stimulation with ConA indicating that bacteria feeding did not cause an immunosuppressive status. The analysis of cytokine gene expressions showed that probiotic treatment induced an increase of IL-10 only in mesenteric lymphocytes as compared to untreated rats. ELISA assay confirmed the increase of IL-10 level in both OVA unstimulated and stimulated cells, indicating an increase in the basal level of IL-10 in immunized rats treated with probiotics. These results indicate that the immune response to probiotics may be different at intestinal or peripheral site, and that the achievement of oral tolerance may be influenced by the commensal bacteria.

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Clinical Experience with *Lactobacillus reuteri*

Running title: Clinical Experience with *Lactobacillus reuteri*

Eamonn Connolly, PhD

Summary

Lactobacillus reuteri (*L. reuteri*) is considered to be one of the few true autochthonous (indigenous) *Lactobacillus* species in the gastrointestinal tract of man and is widely used for its probiotic properties as a food additive to improve gastrointestinal health. The safety of *L. reuteri* (ATCC 55730) on administration to man has been repeatedly demonstrated in healthy and immunocompromised adults, children and newborn term and pre-term infants, making *L. reuteri* a suitable and safe supplement for all age groups. Recent clinical data show that *L. reuteri* (ATCC 55730), delivered via a tablet formulation, colonises the gastric and intestinal epithelium in healthy man and modulates the immune response of these epithelia particularly through the CD4 positive T-helper cells in the ileum. These data confirm preclinical findings in animal models and such immunomodulatory effects may be a key mechanism of action explaining the health promoting properties of *L. reuteri*, including prevention of diarrhoea, inhibition of

pathogen infection and consequent host responses to these pathogens, as well as the potential modulation of the Th1/Th2 immune response balance in the human intestinal epithelium, a response that has been strongly linked to the prevention and treatment of allergy. This review article presents accumulated clinical data on *L. reuteri* and links earlier preclinical observations with the latest findings in human clinical trials.

***Lactobacillus reuteri* - background**

Lactobacillus reuteri (*L. reuteri*) was originally described by Gerhard Reuter as *Lactobacillus fermentum* type II¹ but is now recognized as a distinctive species². *L. reuteri* is a heterofermentative species that resides in the gastrointestinal (GI) tract of humans and all animals tested³⁻⁶ and is considered to be one of the few true autochthonous (indigenous) *Lactobacillus* species in man⁶.

The probiotic mode of action is generally attributed to the ability of *L. reuteri* to exert an inhibitory effect of pathogenic microorganisms by a combination of different mechanisms including excretion of lactic acid, hydrogen peroxide, antimicrobial substances and bacteriocins⁴. *L. reuteri*, like other lactic acid bacteria, is able to convert milk sugar (lactose) into lactic acid and has been shown to produce hydrogen peroxide. In addition to lactic acid, *L. reuteri* ferments carbohydrates into short chain fatty acids, e.g. acetic acid and these fatty acids have an antibacterial effect.

Axelsson⁷ reported that *L. reuteri* converted glycerol into a potent, broad-spectrum antimicrobial that was termed “reuterin.” Reuterin is a low molecular weight, neutral,

water-soluble compound (3-hydroxy propionaldehyde) that is capable of inhibiting growth of species representing several bacterial genera including *Escherichia*, *Salmonella*, *Shigella*, *Proteus*, *Pseudomonas*, *Clostridium* and *Staphylococcus* as well as yeasts, fungi, protozoa and viruses⁷, many of which are pathogenic in humans. Reuterin is excreted by *L. reuteri* during anaerobic growth in the presence of glycerol⁸ and both of these conditions are satisfied by the milieu of the human bowel. Chung⁹ showed that reuterin was synthesized under environmental conditions similar to those that exist in the GI tract. Reuterin synthesis was stimulated by contact with other bacteria, that to a varying degree are found in the human gut, such as *E. coli*, *Salmonella typhimurium*, *Shigella*, *Proteus*, *Pseudomonas fluorescens*, *Staphylococcus epidermidis*, *Bacillus megaterium*, *Clostridium sporogenes*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides* and *Streptococcus cremoris*. The β -hydroxy moiety of reuterin renders its aldehyde function reactive, capable of spontaneous reaction with available amino and sulfhydryl functional groups in the gut lumen. Thus, reuterin is not systemically absorbed.

The structure of another unique compound with antimicrobial effect produced by *L. reuteri*, reutericyclin (a tetramic acid), has recently been elucidated and reported^{10,11}. Reutericyclin has been shown to exert an inhibitory effect on several bacteria that are pathogenic to humans or considered as food contaminants, such as *Bacillus cereus*, *Staphylococcus aureus*, *Listeria*, *Enterococcus fecium*. Further studies have demonstrated that *L. reuteri* exerts a strong antibiotic action on GI tract pathogens but that it is unique in that it does not negatively affect the normal, beneficial microflora^{4, 12-14}.

Survival and colonization of the GI tract demand the ability of the probiotic bacteria to tolerate the low pH in the stomach and the bile salts. *L. reuteri* tolerates both bile and acid^{15, 16}. Adherence to intestinal mucosa and growth at the site of action are mandatory properties as well and this has been demonstrated for *L. reuteri*^{4,12,17}. Thus, oral administration of *L. reuteri* delivers live bacteria to the entire GI tract and leads to faecal shedding of live bacteria (see below).

Lactobacillus reuteri is widely used as a probiotic in dairy foods. Milk supplemented with *L. reuteri* (ATCC55730) was first introduced in Sweden already in 1991 under the BRA brand name and SymBalance, a fermented milk product with *L. reuteri* (ATCC55730) was sold on the Swiss market during the 1990's. Currently, dairy products containing *L. reuteri* (ATCC55730) are sold in the US, Finland, Japan, Korea, Spain, Portugal and the UK. In 2000, chewable tablets containing *L. reuteri* (ATCC55730) were introduced in the US and these tablets have since been introduced into Europe, S. Africa and Asia. To date the equivalent of more than 200 million doses of 10^8 CFU *L. reuteri* have been sold through the various products described above (BioGaia internal market information). No clinical infection or untoward side-effect involving *L. reuteri* has yet been reported.

Other *Lactobacillus* strains have been linked to anecdotal case reports of infection, in which the patients were either immunocompromised or otherwise suffering from other severe clinical conditions¹⁸⁻²¹. There are no reports of isolation of *L. reuteri* in connection

with any disease or pathological process in either animals or humans. Indeed, an excellent study recently reported on bacteremia in the Finnish population²² did not detect any cases of *L. reuteri* in the circulation, although several other well-known *Lactobacillus* probiotic species were detected in the blood. These observations were made during a period when *L. reuteri* was widely consumed in Finland in dairy and juice products.

Many of the studies below refer to work with *L. reuteri* ATCC 55730. This strain is that used by BioGaia in all human product applications and is sometimes referred to as *L. reuteri* SD2112 (an earlier ATCC designation).

Overview of safety of *L. reuteri*

Adults:

Wolf et al.²³ studied the safety and tolerance of *L. reuteri* ingestion in healthy males in a randomized, double-blinded placebo controlled trial. Thirty healthy males (n=15/group) were randomly assigned to receive either *L. reuteri* (ATCC55730) (1×10^{11}) or placebo capsules for 21 days. The incidence of subjective tolerance factors such as flatulence, diarrhea and cramping were infrequent and similar between the groups. *L. reuteri* levels in the feces and the ratio of *L. reuteri* to *Lactobacillus* spp. was significantly higher in the *L. reuteri* supplemented group on days 7, 21, 14 and 28 as compared to the control group. The results indicate that *L. reuteri* can be ingested at a level of 1×10^{11} CFU/day without any clinically significant safety or tolerance problems.

Further, Wolf²⁴ also examined the safety and tolerance to *L. reuteri* (ATCC55730) in individuals with HIV infection in a randomized, double-blinded placebo controlled trial. The subjects were supplemented either with *L. reuteri* (10^{10} CFU/day, n=15) or placebo (n=20) capsules for 21 days. *L. reuteri* supplementation caused an increase in fecal levels of *L. reuteri* on days 7, 14 and 21 compared to baseline. These results indicate that HIV positive (immunocompromised) individuals can ingest *L. reuteri* at 1×10^{10} CFU/day without any clinical safety and tolerance problems.

The ability of *Lactobacillus* spp. to survive *in vivo* was examined in 12 healthy men in a double-blind cross-over trial¹². Three groups were enrolled in a three period crossover trial. One feeding period consisted of *L. reuteri* (DSM 12246; 2×10^{10} CFU/day) combined with *L. rhamnosus* 19070-2. A second period consisted of *L. rhamnosus* LGG, *L. delbrueckii* subsp. *lactis* CHCC 2329 and *L. casei* subsp. *alactus* CHCC3137. The third period was a placebo. Each treatment lasted for 18 days with a 17-day washout period after each treatment. The investigators did not report any adverse health effects. *L. reuteri* was found to colonise the GI tract in 8/12 subjects during the administration period and was washed out when administration of ceased. Notably, this study also showed that *L. reuteri* was the only *Lactobacillus* that did not negatively affect the growth of the normal bacterial residents of the gastro-intestinal tract, whilst having the most widespread and strong inhibitory effect on pathogenic bacteria.

Children:

Ruiz-Palacios et al.²⁵ established the tolerance and dose response of a probiotic mixture containing *L. reuteri*, *L. acidophilus* and *B. infantis* in children (n=72), ages 12 to 36 months. The children were randomly assigned to one of three different amounts of daily supplementation with *L. reuteri* together with unchanged levels *L. acidophilus* and *B. infantis* or non-probiotic placebo. No significant differences in the incidence of vomiting, abdominal discomfort, gas, and stool characteristics were observed among the groups. The *L. reuteri* supplementation caused an increase in fecal *L. reuteri* levels in a dose dependent manner. The results show that *L. reuteri* supplementation was well tolerated by the children up to a dose of 1×10^{10} CFU/day. In further studies^{26,27} in 248 children (aged 12 to 35 months) children receiving a probiotic blend including *L. reuteri* (ATCC 55730) for 14-16 weeks, no adverse health effects were reported.

In a recent study in India, 340 children in a rural area in India were given either *L. reuteri* (ATCC 55730) tablets at a dose of 10^8 cfu/day or placebo for 35 days (BioGaia data on file). Extensive safety data on blood parameters revealed no influence of the *L. reuteri* on any blood parameter tested

Infants and neonates:

Shornikova et al.^{28,29} studied children (ages 6 to 36 months) with infectious diarrhoea were treated with *Lactobacillus reuteri* (ATCC 55730). In one of the studies, children received 10^{10} to 10^{11} CFU of *L. reuteri* once per day, and in the other 10^7 or 10^{10} CFU/day

for 5 days. No adverse effects of *L. reuteri* supplementation on weight gain, consumption of oral rehydration solution or electrolyte, or on acid-base balance could be detected.

Karvonen et al.³⁰ studied the safety of doses of *L. reuteri* between 10^5 to 10^9 CFU/day given to 90 healthy neonates (as powder additive to breast milk) for 28 days. Adverse effect symptoms like abdominal discomfort, abdominal pains or cramps were recorded. No difference compared to placebo could be detected for these parameters in any group.

Two recent studies have examined the safety and colonising efficacy of an oil suspension of *L. reuteri* (ATCC 55730) in newborn and premature infants (Vesikari & Karovonen, in preparation, 2003). In these placebo-controlled studies infants were given the *L. reuteri* oil suspension from birth up to 28 days of life at doses between 10^7 to 10^9 CFU of *L. reuteri* per day. In both studies no safety issues were reported and the formulation was well-tolerated by the infants. Significant fecal colonisation was observed in all infants given the active *L. reuteri* oil formulation.

Most recently, a clinical safety study has been presented with *L. reuteri* supplemented infant formulas given to very young infants³¹. Healthy infants between 3-65 days of age were given *L. reuteri* (ATCCC 55730)-supplemented (approx 10^8 CFU/day), *B. bifidum*-supplemented or placebo infant formula for 4 weeks. All groups were similar with regard to growth, gastrointestinal function and well-being.

The infant studies above, as well as a pre-clinical study in infant rhesus monkeys³², are of particular interest with reference to the existing CODEX guidelines for use of live bacteria as fermentation aids for the acidification of infant formulas. These guidelines refer to clinical data from the late 1950's and perpetuate the idea that lactobacilli that produce both D-lactic acid are unsuitable for use in infant formulas and even recent publications misleadingly refer to this³³. However, there is a major difference between acidifying agents for fermenting milks in production and the addition of freeze-dried probiotics to the final infant formula product. Further, a review of the existing literature (Connolly, manuscript for publication) and data from our own clinical trials with *L. reuteri*, clearly show that there is no evidence describing D(-)-lactic acidosis in healthy infants (or humans of any age), despite extensive use of probiotic *Lactobacillus* supplements around the world. This is, of course, not a surprise since these bacteria are a natural component of mothers milk and cannot be expected to adversely affect the newborn. Fortunately, the EU Scientific Committee on Foods has recognized this in their Opinion (http://europa.eu.int/comm/food/fs/sc/scf/outcome_en.html, see out199IF SCF May2003_en.pdf, section XI) which will come into force early 2004 and potentially very efficacious lactobacilli normally found in mothers milk will be acceptable for use as additives in infant formulas, irrespective of the lactic acid metabolic profile. The CODEX guidelines need to be adjusted to also recognize this issue.

Overview of efficacy of *L. reuteri*

Ruiz-Palacios et al.²⁶ investigated the use of probiotic bacteria to prevent the outbreak of community-acquired diarrhoea in healthy children. Children (ages 12 to 35 months) were

randomly assigned to a probiotic blend treatment group (n=119) or to a control group (n=120) for 14 weeks. The probiotic blend contained *L. acidophilus*, *B. infantis* and *L. reuteri* (ATCC 55730). The number of children with diarrhoea was lower in the probiotic group compared to the control group, 29/119 vs 43/120 and the incidence of diarrhoea per child was also lower in the treatment group; 0.27 vs 0.42.

A second blinded RCT study by the same research group²⁷ compared the diarrhoea preventing effect of two probiotic blends given to healthy children (ages 12 to 32 months). One feeding group was given a blend of *L. acidophilis* and *B. infantis*. The other group received the same blend plus 1.5×10^8 CFU *L. reuteri* (ATCC 55730) per day. A third group received placebo. The number of children in each treatment group was 129 and in the placebo group 130. The relative risk of contracting diarrhoea compared to placebo was significantly reduced to 0.67 for the *L. reuteri* fed group and to 0.75 (not significant) for the group given the probiotic blend without *L. reuteri*.

Two double-blind, placebo controlled studies examined the effects of *L. reuteri* (ATCC 55730) on acute infectious diarrhoea in children^{28,29}. In the first study, 41 children were included to receive 10^{11} to 10^{10} CFU per day or placebo. Duration of diarrhoea was reduced to 1.7 days compared to 2.9 for the placebo group. Vomiting on the second day was absent in the *L. reuteri* treated group whilst 19% of the placebo group had vomiting episodes, and some even lasted for 6 days. In the second study (66 patients), the duration of diarrhoea was 2.5 days in the placebo group and was reduced to 1.9 days in children

receiving 10^7 CFU and to 1.5 days in children receiving 10^{10} CFU of *L. reuteri* per day. Notably, the low dose gave almost the same efficacy as the higher dose.

Karvonen³⁰ studied the effect of different doses of *L. reuteri* on stool consistency in a study of 90 healthy neonates. *L. reuteri* was administered for 28 days, in breast milk or infant formula base, in daily doses of 10^5 CFU, 10^7 CFU, 10^9 CFU or placebo. The number of watery stools per day was significantly reduced to 0.1, 0.3 and 0.4 in the respective treatments groups, compared to 1.0 for placebo indicating again the efficacy of the lower doses. Colonization of the infants was dose dependent.

The ability of *Lactobacillus* strains to colonize the human intestinal mucosa was studied in thirteen healthy volunteers³⁴. Nineteen different strains of multiple species of lactobacilli (two of which were *L. reuteri* 108 and *L. reuteri* 47 (=R2LC)) were administered as freeze-dried fermented oatmeal soup for 10 days. Biopsy samples of the upper jejunum and rectum mucosa were taken for microbial identification. The *Lactobacillus* numbers increased in the gut, and both phenotypic and genotypic identification showed colonization by *L. reuteri* 108 and four other strains of *Lactobacillus*. At the eleventh day after terminating the administration, three persons still were colonized with *Lactobacillus reuteri* in jejunum or rectum.

Recent clinical findings

Asli et al.³⁵ have just presented data from a double-blind, placebo controlled trial in infant formulas supplemented with either *L. reuteri* ATCC 55730 (approx 10^8 CFU/day) or B.

bifidum (Bb12) for 12 weeks. Infants given the probiotics showed less febrile episodes and fewer GI illnesses and infections than the placebo group and the authors noted that *L. reuteri* was found to be superior to both placebo and supplementation with *Bifidobacterium bifidum* (Bb12) in maintaining the gastro-intestinal health of the infants.

Interesting new clinical data is being generated by Rosenfeldt et al.³⁶⁻³⁸ with a mixture of *L. reuteri* (DSM 12246) and *L. rhamnosus* (19070-2) indicating amelioration of rotovirus induced acute diarrhoea as well as potential effects on acute dermatitis. Unfortunately, the mixture of strains does not allow the determination of which of the bacterial strains has effect. BioGaia is currently sponsoring a major multicentre trial on the effect of supplementation of newborn infants with *L. reuteri* (ATCC 55730) on the incidence of atopic allergy in the first two years of life, so new data should soon be available.

Further new clinical data (Valeur et al. submitted for publication 2003) demonstrates in situ colonization of the human gastro-intestinal mucosa by dietary supplementation with *L. reuteri* (ATCC 55730) tablets and subsequent immune responses at these sites. In this open clinical study, 10 healthy volunteers and 9 subjects with ileostomy underwent gastroscopy or ileoscopy and biopsies were taken from the stomach, duodenum or ileum before and after supplementation with 4×10^8 CFU live *L. reuteri* per day for 28 days. Biopsies were analysed for colonization (using FISH with a molecular beacon probe) and for immune cell populations. Endogenous *L. reuteri* was detected in the stomach of 1 subject and the duodenum of 3 subjects (of 10). After *L. reuteri* tablet supplementation, the stomachs of 8 and the duodenums of all subjects were colonized. Three ileostomy

subjects (of 6 tested) had endogenous *L. reuteri* at baseline whilst all 6 displayed colonization after *L. reuteri* supplementation. Our data provide the first clear and direct evidence of colonization of the healthy human stomach, duodenum and ileum by any exogenously delivered probiotic. Colonisation of the stomach and upper GI tract (the sites of *H. pylori* infection) by *L. reuteri* from the tablet supplement as well as the known ability of *L. reuteri* ATCC 55730 to kill *H. pylori*, encourages our continued investigations in this area.

Most notably, *L. reuteri* administration in our study induced a significantly higher amount of CD4-positive T-lymphocytes in the ileal epithelium of the subjects. New emerging evidence suggests that the probiotic effects of *L. reuteri* may be related to its ability to modulate the immune system of the gastrointestinal tract^{4, 39-44}. Our findings are in good agreement with earlier observations in poultry⁴⁵, where an increased CD4:CD8 ratio in the ileum mucosa was found after intake of *L. reuteri* in a model where colonization by *Salmonella typhimurium* is markedly reduced by *L. reuteri* supplementation with a consequent dramatically improved survival of the chicks^{4,45}. Further studies performed by our group have shown that ileal growth is stimulated by *L. reuteri* supplementation in the mouse with consequent reductions in *Salmonella*-induced inflammation and mortality (C&D 2000; BioGaia unpublished data). Further Mao et al.⁴⁶ studied methatrexate-induced enterocolitis in rats and found that *L. reuteri* could increase both ileal and colonic secretory IgA levels as well as CD4+ and CD8+ cell populations in the gut lamina propria and that these changes were associated with decreased intestinal permeability, increased mucosal mass and recovery from enterocolitis⁴⁷. It is also worth

noting that Ferreira et al.⁴⁸ have shown that activated T-lymphocytes in the human small intestinal lamina propria are involved in enhancing proliferation of intestinal epithelial cells and that probiotics⁴⁹ including *L. reuteri*⁴ have been shown to stimulate mucosal growth in animals. *L. reuteri* is known to be a predominant indigenous species in the ileum⁴ and thus, *L. reuteri* stimulation of T-helper cells cells in the ileum may be a central mechanism of symbiosis for improving the health of the host gut and a key mechanism of action for this probiotic bacteria.

In conclusion, this study shows the attachment, growth and colonization of the human gastrointestinal tract by *L. reuteri* ATCC 55730/SD2112 delivered in a tablet formulation and that such a colonization activates a local immunological response in the human gut mucosa. Based on confirmatory pre-clinical data, these responses to supplementation with the probiotic *L. reuteri* will improve the gastro-intestinal immune response and defence against pathogens in an already healthy recipient.

Conclusions

Extensive clinical trials have shown that *L. reuteri* ATCC 55730 effectively colonizes the human GI tract, is safe for use in humans of all ages and further that *L. reuteri*, administration significantly reduces the incidence and the severity of diarrhoea of different origins, reduces gastrointestinal illness and infections and may be able to activate basic immune responses in the human gastrointestinal tract that ultimately lead to a improved gut function and further enhanced health of the host through more global immunological effects.

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THE ROLE OF PROBIOTICS IN THE CLINICAL FIELDS

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The probiotics restore the normal intestinal permeability and gut microecology, improve the intestine's immunological barrier function, reduce the intestinal inflammatory response and generation of proinflammatory cytokines characteristics of local and systemic allergic inflammation (1)

In clinical trials probiotics appear to be useful for the treatment of various clinical conditions as food allergy and atopic dermatitis, but even in primary prevention of atopy (2).

Majamaa et al. evaluated the effects of probiotics in food allergy in 27 infants with atopic eczema and cow's milk allergy (3). All the children received an extensively hydrolyzed whey formula without (n. 14) and with (n. 13) the addition of *Lactobacillus* GG (5×10^8 colony-forming units/gm formula). In a second group of 10 breast-fed infants who had atopic eczema and cow's milk allergy, *Lactobacillus* GG (2×10^{10} cfu) was given to nursing mothers. The results show a significant improvement of atopic dermatitis (as seen by SCORAD score) after 1 month's intervention only in those receiving *Lactobacillus* GG (WhGG: $p=0,008$) but not in the other group (Wh: $p=0,89$). In the breast-fed infants SCORAD score was 26 (20 to 36) before treatment and 11 (0 to 25) 1 month later ($p = 0,007$). To evaluate the intestinal inflammation the concentrations of fecal α_1 -antitrypsin, tumor necrosis factor- α and eosinophil cationic protein were determined before and at the end of the study. The concentration of fecal α_1 -antitrypsin and tumor necrosis factor- α decreased significantly only in the group receiving *Lactobacillus* GG.

Interleukin-10 has anti-inflammatory properties by downregulatory effects on IL-2, IL-6, IL-12, TNF α and INF γ and IgE synthesis. Pessi et al. studied the IL-10 generation in atopic children: they added *Lactobacillus rhamnosus* GG (2×10^{10} cfu) for 4 weeks to the diet of 9 children (mean age 21 months) with AD and cow milk allergy (4). The rise in IL-10 concentration in the sera of atopic children demonstrates the anti-inflammatory properties of specific probiotic bacteria strains. Also in another study, *Lactobacillus rhamnosus* administration had a positive effect in 20 allergic children.

The effects of probiotics in atopic eczema have been studied by Isolauri et al. (5). In a randomized double-blind study 27 infants (mean age 4,6 months) who manifested atopic eczema during exclusive breast-feeding were divided into three groups: probiotic-supplemented, *Bifidobacterium lactis* Bb-12 or *Lactobacillus* strain GG, extensively hydrolyzed whey formulas or to the same formula without probiotics. A significant reduction in the SCORAD score was seen after 2 months in the probiotic-supplemented groups as compared to the unsupplemented group ($p=0,002$), in parallel with a reduction in the concentration of soluble CD4 in serum and eosinophil protein X in urine.

Probiotics have been also evaluated in primary prevention of atopic disease. In a double-blind, randomized placebo-controlled trial Kalliomäki et al. (6) gave *Lactobacillus* GG (1×10^{10} cfu) prenatally to mothers with a family history of atopic disease and postnatally for 6 months to their infants. Children were valued at 3, 6, 12, 18, 24 months and the primary study endpoint was atopic disease at 2 years. The results show a significant increase in the frequency of atopic eczema in the placebo group when compared to the probiotic group. On the other hand Prist, Rast and skin prick tests were very similar between the groups.

In a double-blinded, placebo-controlled study of 62 mother-infants pairs, Rautava et al. (7) has demonstrated that administering *Lactobacillus rhamnosus* GG (2×10^{10}) during the 4 weeks before giving birth and during breast feeding (3 months), increased the immunoprotective potential of breast milk, as assessed by the enhancement of anti-inflammatory transforming growth factor 2 in the milk of mothers receiving probiotics vs placebo. The best results of maternal probiotic supplementation were found in children with an elevated cordon blood IgE concentration.

High numbers of bacteroides and E.coli were associated with the extent of atopic sensitization. To characterize the relationship between gut microflora and the extent of allergic sensitization Kirjavainen et al. (8) analysed the faecal microflora in 21 children with atopic eczema, 8 intollerant (HSG) and 13 tolerant (SG) to extensively hydrolysed whey formula (EHF). In the tolerant group, 6 children were weaned to EHF+placebo and 7 to EHF+*Bifidobacterium lactis* Bb-12. In the intolerant group lactobacilli/enterococci counts were higher than tolerant group ($p=0,002$). The bifidobacterial supplementation determined a decrease in the numbers of E.coli and protected against an increase in bacteroides numbers during weaning.

On the basis of these experimental data probiotic therapy appears to alleviate allergic inflammation demonstrated as control of clinical symptoms and reduction of local and systemic inflammatory markers and enhancement of anti-inflammatory markers.

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PROBIOTICS AND IRRITABLE BOWEL SYNDROME

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It is generally accepted that symptoms of Functional Bowel Disorders, and of IBS in particular, are generated by abnormalities of gut function, including altered sensory perception, abnormal motility and, in some patients, abnormalities of epithelial function [1,2], but what is lacking is an understanding of the pathogenic mechanisms that alter gut function. It is well known the “classic” hypothesis that central and peripheral factors acting on the neuro-hormonal control of gut motility and perception have been implicated in the pathogenesis of IBS [3], but evidences supporting infection and inflammation and/or changes in intestinal microflora, as basis for altered gut function in IBS, are strongly emerging [4,5].

Probiotics are viable microorganisms that have a beneficial effect in infective diarrhea and Inflammatory Bowel Diseases [6-8], while contrasting results are reported in IBS clinical studies. Most of these trials demonstrated a statistically significant improvement in at least one symptom [9-15], whereas in two studies

treatment with probiotics was not more effective than placebo at all [16,17], and in others, essential symptoms for IBS, such as pain, decreased significantly also in placebo group [15]. Administration of a single strain or a mixture of bacteria, the wide range in the duration of treatment but, overall, important differences in the dosage or in the use of live/dead preparations, are probably the main reason because the results of these trials are different and the efficacy of probiotics in IBS is not fully accepted yet. It has still to be determined if efficacy is related with a specific type of probiotics or rather with high dosage or use of mixture rather than a single strain. Finally, it needs to be known whether one of the three IBS patients subgroups (constipation, diarrhea or alternation between the two) and/or FD patients may benefit more than others.

We addressed a study to evaluate the effect on IBS patients of a probiotic preparation containing at least ten folds more lactic acid bacteria than the previous employed products, and with a combination of several bacterial species and not a single strain. In fact, it contains 4 strains of lactobacilli, 3 strains of bifidobacteria and one strain of *Streptococcus thermophilus*, lyophilized in a concentration of 3×10^{11} viable cells per gram. Bacteriotherapy with VSL#3[®] have already shown to be effective in prevention of Ulcerative Colitis relapse [18] and in the treatment of pouchitis [19].

Specific aims of the study were to assess if colonization by VSL#3[®] exogenous bacterial strains occurred in a well defined group of patients with D-IBS and FD and if this was associated to changes in clinical picture. Secondly, in a restricted number of patients, we investigated if changes in mechanical distention-induced colonic motor pattern and in concentration of microbial groups and enzymatic fecal activities were shown during probiotic treatment.

We studied 84 patients with well documented functional origin of chronic diarrhea. Diagnosis was confirmed by exclusion and positive clinical criteria: 60 patients fulfilled the Rome II criteria for Diarrhea-predominant IBS (D-IBS) and 24 for Functional Diarrhea (FD), without a history of malabsorption, and with normal blood tests (Hb, C-reactive protein, thyroid-stimulating hormone, T₄ and electrolytes) and dosages for tissue-transglutaminase auto antibodies and for Vanillylmandelic acid. The patients should also have a normal colonoscopy with biopsy within 8 months before study entry and at least 3 months later the diarrhea onset.

The probiotic preparation VSL#3[®] (VSL Pharmaceutical, Ft. Lauderdale, FL - USA) was used in this study. It consists of bags, each containing 450 billion of viable lyophilized bacteria taxonomically referred to lactic acid bacteria and bifidobacteria: 4 strains of the genus *Lactobacillus* (*L. acidophilus*, *L. plantarum*, *L. casei* and *L. delbrueckii* subsp. *bulgaricus*), 3 strains of *Bifidobacterium* (*B. longum*, *B. breve* and *B. infantis*), and 1 strain of *Streptococcus salivarius* subsp. *thermophilus*, designated as *S. thermophilus* throughout this paper. Preparation has to be kept in refrigerator and it can be administered alone or in any cold food.

This was a 21-days trial. Two VSL#3[®] bags were administered to each patient in the morning on empty stomach after one week of wash-out period from any previous drug administration and one week run-in observation period. During the run-in week (T0) and the last 7 days of treatment period (T1), symptom assessment, motility studies and fecal recovery for microbiological evaluations were performed according to method already published [20,21].

This study showed presence of the ingested bacteria in the feces during assumption of the probiotic preparation VSL#3, at a dosage of 2 packets/day (900

billion bacteria). According to the definition of “probiotic”, exogenous bacterial strains were no more detected two weeks later administration suspension. This evidence of transient colonization were associated with symptom relief, changes in the motor activity and sensitivity threshold patterns stimulated by mechanical distention of the colonic wall and with variations in the fecal biochemistry.

Several scenarios prompt consideration of a role for a rupture in intestinal ecosystem as a cause of the gut dysfunction, which characterizes IBS pathophysiology. Apart the direct evidences for an association of intestinal flora changes [4,22], we have to consider that IBS are part of the Western diseases and their incidence is higher in countries where dramatic changes in food habit occurred during the past fifty years. It is well known as of all the organisms harbored by the human body only ten per cent are eucaryotic and the remaining ninety per cent are microbes constituting what has been called “the microbe organ”, an organ of 1-1.5 kilogram, similar in size and in metabolic activity to the liver [23-25]. Many factors affect the composition of the intestinal microbiota in humans, but certainly the amount and type of growth substrate, in other words, what we use to eat, has the most influential role: it is not possible to think that changes in food ingredients, presence of probiotics produced by natural fermentation and so on did not induce changes in the composition and physiology of the intestinal flora. Physiologically, nearly 80% of the fecal dry weight consists of bacteria, viable in the 50%: this “stool production” is influenced by substrates arriving with the ileal affluent [26,27]. In this sense, a stimulation of the colonic bacterial growth may be the rationale because dietary fibers are undoubtedly effective in many patients with constipation- predominant IBS, but they are effective also in diarrhea [28,29].

Considering this strong rationale for changes in intestinal microflora as basis for altered gut function in FBD, almost all the previous clinical trials failed to show a fully convincing efficacy of probiotics on IBS patients, such as a recent review concluded probiotics may offer some relief to suffers of IBS as an overall picture of reporting [30]. In our study satisfactory improvements were reported after few days of treatment by significant percentages of patients. Three main differences from previous experiences characterize our study: an extreme homogeneity of patient population, a severe degree in terms of bowel frequency and the properties of the employed preparation.

As regards the first two points, it is remarkable that we distinguished between FD and IBS and in this last one only patients with diarrhea were selected, excluding alternate- and constipation predominant IBS ones. Actually, we enrolled a subgroup of symptomatic patients, having ≥ 3 bowel motions/day, feature which is not mandatory for D-IBS and FD diagnosis, but selects certainly the most severe clinical conditions. In fact, a large percentage of patients enrolled in recent studies had less than 2 stools per day and/or hard stools [12,15]: we think it may compromise a complete evaluation of the treatment efficacy on number of defecation and fecal consistency. Finally, we excluded patients with markers for probable important psychological disturbances. In our opinion this is a key point, since bowel motion may be a psychovisceral response in case related to panic disorder [31,32]; furthermore, during stress, g.i. symptoms are increased without orocecal transit alterations [33]. Lactose intolerance was not detected in our patients since its relationship with FBD is still debated. Furthermore, it has to be considered that milk was reintroduced in many patients during treatment and that probiotics seem to be effective also in case of this intolerance [34].

More remarkable is perhaps the second point: VSL#3[®] preparation provided several hundred folds of viable bacteria than every previous preparation employed. Since there is a general agreement that success of probiotics may be due to recolonization of the intestine with a more suitable flora, the arrival of sufficient amounts of bacteria into the large bowel is a crucial point for this effect.

Probiotics preparations, as it is for substances having “antibiotic” activity, are not equal in all case: we can speculate that composition (i.e. mixture versus single strain), very high dosage and viability are the most discriminate elements for probiotic efficacy. This study supports that the newer higher potency probiotic preparations, both in terms of the bacterial concentration and the number of bacterial strains, have a greater potential for clinical effectiveness than traditional preparations [35]. This conclusion is sustained by preliminary results of a study from our group showing that 4 bags of VSL#3[®] (i.e. 3600 billions of bacteria) per day have beneficial effects to prevent pouchitis in patients with ileal pouch anal anastomosis after proctocolectomy for Ulcerative Colitis [36].

According to previous observations, this study shows that HAPCs are present also during fasting in patients with diarrhea [37]. In our patients, balloon-elicited HAPCs are not qualitatively different from spontaneously occurring ones (data not shown), and onset thresholds are remarkably lower in comparison to normal subjects [38]. However, the dramatic reduction in HAPC after VSL#3[®] administration confirms that distention *per se* is unlikely to be an important physiological stimulus for spontaneously occurring propagating colonic motor activity. In other words, it seems that whatever mechanisms underlie functional diarrhea make themselves colonic wall sensitive to mechanical distention. Previous observations in subjects with normal

bowel habit, demonstrated that to elicit HAPCs by colonic wall distention with inflating volume similar to those employed in the present study, it was necessary “to premedicate” the large bowel with exogenous stimulants as bisacodyl [39]. We can speculate that intraluminal factors related to colonic bacteria alterations, characterizing D-IBS and FD patients, sensitized the colon so much that minimal mechanical stimuli, and likely even other ones, are able to rouse the explosive motor activity occurring in these patients. Further work has to be done for understanding relationship between colonic microflora and motility phenomena in health and disease, but the present work shows that changes in the bacterial colonic concentrations due to probiotic assumption are associated with differences in motor patterns.

The employment of molecular tools for specific detection and quantification of *S.thermophilus* allowed to demonstrate the colonization of administered VSL#3[®] strains, since this bacterial species is not a component of the normal intestinal microflora; in fact, we failed to detect it before treatment, at T0.

The more complete microbiological evaluation showed that no decrease in well-established intestinal microbial groups occurred during the probiotic ingestion, whereas an expected increase in lactic acid bacteria and bifidobacteria was detected. PCR analysis, allowing to distinguish endogenous and exogenous ingested bifidobacteria, demonstrated that the bifidobacteria contained in VSL#3[®] can be recovered in the feces of all the patients treated with the probiotic preparation, even if these strains do not colonize permanently the gastrointestinal tract. These results are in accordance with the property of probiotics, which should survive in the intestinal lumen but not persist in the long term [40]. Whether the beneficial effects observed in our patients were related to the

exogenous health promoting bacterial groups present in VSL#3[®] preparation, we can speculate that similar findings could not be obtained by prebiotic administration, since it favors only the resident bifidobacteria flora.

Changes in β -galactosidase and especially urease activities observed during the treatment with VSL#3[®] can be considered one of the most interesting finding of the present study. The increase of β -galactosidase is presumably a direct consequence of the elevated numbers of bifidobacteria, lactobacilli and *S.thermophilus*, which possess high levels of this enzyme. As regards the decrease of urease activity, although the number of some important ureolytic bacterial groups such as bacteroides, clostridia and enterobacteria did not change, it may be supposed that the increased concentration of bifidobacteria and lactic acid bacteria and their metabolic activities can indeed significantly alter biochemistry of the colonic flora, including its urease activity. The consequent decrease in ammonia production can be considered of particular importance for IBS patients, in terms of reduction of toxic compounds and intestinal gas, as well as the β -galactosidase increase can play a positive role since its action prevents the metabolism of unabsorbed carbohydrates by gas-forming colonic bacterial groups.

In conclusion, these data indicate an important impact of VSL#3[®] on intestinal microbiota, suggesting relevant consequences for its host health. The present study is certainly an open, non-controlled trial, but to our knowledge, this is the first observation showing a clinical improvement related to changes in the composition of the fecal bacterial flora and in fecal biochemistry and, remarkably, in colonic motility pattern, all effects being induced by probiotic administration in patients with functional diarrhea.

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PROBIOTICI E SINDROME DEL COLON IRRITABILE NEL BAMBINO

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I probiotici sono micro-organismi vivi con effetti benefici sulla prevenzione della pouchite ricorrente e nella diarrea da *Clostridium difficile*. Alcuni dati di letteratura ed alcune esperienze cliniche supportano l'uso dei probiotici per prevenire o trattare i disordini funzionali intestinali. Non esiste ancora, comunque, una solida base scientifica per l'uso di questi micro-organismi; è necessario, pertanto, lo sviluppo di nuovi studi. I micro-organismi usati come probiotici comprendono il *Lactobacillus* sp., il *Bifidobacterium* sp., l'*Escherichia coli*., lo *Streptococcus* sp., il *Propionibacterium* sp. e diversi funghi. Alcune preparazioni di probiotici contengono più specie di batteri. Essi sembrano essere vantaggiosi nella prevenzione o nel trattamento di diversi disordini intestinali, quali la diarrea infettiva, quella da antibiotici e quella del viaggiatore. Vi è evidenza clinica, basata su trials aperti, che i sintomi prevalentemente diarroici dei pazienti con sindrome dell'intestino irritabile migliorino con terapia probiotica; nessun trial formale randomizzato, comunque, ha dimostrato una totale efficacia in questa sindrome o identificato il meccanismo d'azione del probiotico. In un trial controllato randomizzato di 4 settimane, 5×10^7 unità formanti colonie/ml di *Lactobacillus plantarum* e 3.6 g di farina di avena furono comparati con placebo in 60 pazienti con sindrome dell'intestino irritabile; la flatulenza diminuì significativamente nel gruppo trattato con *Lactobacillus* rispetto a quello trattato con placebo. Il dolore addominale era meno intenso rispetto all'inizio dello studio in entrambi i gruppi ma non si osservò nessuna differenza tra i due trattamenti. Dopo un anno, la funzione gastro-intestinale globale era significativamente migliore nel gruppo trattato con i probiotici. L'efficacia clinica nella sindrome dell'intestino irritabile con diarrea predominante e nella diarrea acuta (infettiva e del viaggiatore) suggerisce che questi agenti possono ritardare il transito colonico e facilitare il riassorbimento dei fluidi e degli elettroliti, migliorando in tal modo l'alvo. La flatulenza, la diarrea, il dolore addominale ed il gonfiore sono sintomi comunemente osservati nella sindrome dell'intestino irritabile, ma gli ultimi due sono difficili da trattare in maniera tale da avere, così, un impatto significativo sulla vita dei pazienti. Hahn *et al.* hanno esaminato la frequenza, la durata e la severità dei sintomi in 122 pazienti adulti con sindrome dell'intestino irritabile; medialmente, i pazienti presentavano alvo irregolare, gonfiore severo e fastidio/dolore da moderato a severo rispettivamente nel 25%, 28 % e nel 33 % dei giorni. La durata di ciascun episodio di gonfiore e di dolore addominale era in media di 5 giorni, con 1-4 episodi al mese. Questi risultati confermano la rilevanza di questi sintomi. Attualmente sono in corso studi placebo controllati sull'efficacia di una nuova preparazione probiotica, VSL#3, per valutare i suoi effetti sui sintomi e sul transito intestinale.

PROBIOTICS AND CHILDHOOD DIARRHOEA

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Childhood diarrhoea accounts for substantial morbidity and mortality worldwide. Recently, probiotics have been proposed as adjunctive therapy in the treatment of acute diarrhoea in children. Probiotics are a group of live micro-organisms thought to have beneficial effects on human health when they colonize the bowel. The delivery of non-pathogenic bacteria to the intestine is thought to provide protection via a number of mechanisms. First, antibacterial substances produced and secreted by such organisms, which include lactobacilli and bifidobacteria, may inhibit enteric pathogens. In addition, competition for mucosal receptor sites may prevent the adhesion and overgrowth of enterotoxigenic gram-negative aerobic bacteria and enteropathogenic viruses and allows more beneficial organisms to adhere to the intestinal surface. Increased short chain fatty acid production and reduction of faecal pH may also play crucial roles in the inhibition of enterotoxigenic organisms. Furthermore, orally ingested *Lactobacillus GG* may also have an immunomodulatory effect. Probiotics have been studied for over 30 years. The best-studied probiotic is *Lactobacillus GG*, strain of *L. rhamnosus*, developed in 1985 by Drs. Gorbach and Goldin. This particular strain of *Lactobacillus* was isolated because of its specific abilities to resist degradation by human acid and bile, to bind to human intestinal epithelial cells, and to colonize the human intestinal tract. Effective colonization of the gut has been demonstrated in healthy human volunteers, as documented by faecal cultures and colonic biopsy samples. In addition, all available data indicate that no harmful effects occur in association with *Lactobacillus GG* in healthy individuals. Prior animal studies have shown the effectiveness of bifidobacteria in treating and preventing *Rotavirus* and *E.coli* –

induced diarrhoea. In addition, a number of randomized, placebo – controlled trials have evaluated several variations of bacterial probiotics in the treatment of acute diarrhoea in children in various areas of the world. Recent meta-analyses, evaluating the use of probiotics in acute infectious diarrhoea, provided confirmatory evidence of the efficacy of probiotic supplementation in reducing duration of symptoms among children with acute, non-bacterial diarrhoea. Further studies are recommended to identify properly probiotic strains and products, to prove that they are able to confer specific health benefits through an elucidated mechanism, and are manufactured and labelled in a way delivering the optimum benefit to the patient. Such standards are needed to move this area to one of even greater respectability.

PROBIOTICS: A POTENTIAL TARGET FOR THE PREVENTION
AND TREATMENT OF STEATOHEPATITIS

Short title: probiotics and steatohepatitis

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Non Alcoholic fatty liver disease (NAFLD) represents a spectrum of disorders ranging from fat accumulation in hepatocytes without concomitant inflammation or fibrosis (steatosis) to hepatic steatosis with a necroinflammatory component (steatohepatitis, NASH) that may or may not have associated fibrosis.¹ The NASH may progress to cirrhosis in up to 20% of patients, is now recognized to be a leading cause of cryptogenic cirrhosis, but the pathogenesis has not been fully elucidated.²

Most cases of NASH appear to have a multifactorial aetiopathogenesis. Obesity and diabetes are undoubtedly associated with the development of NAFLD and the most widely supported theory implicates insulin resistance as the key mechanism.^{3,4} However, only a minority of obese and/or diabetic patients develop progressive disease while many patients with NASH are neither obese nor diabetic.⁵ Thus, it would seem likely that other factors are involved in determining the development and progression of NAFLD. Among these factors intestinal bacterial overgrowth may play a role. Indeed, a) NASH was encountered as a common complication of jejunoileal bypass surgery for morbid obesity,⁶ b) NASH has been reported in individuals with jejunal diverticulosis and intestinal bacterial overgrowth,⁷ and c) various rat models of intestinal bacterial overgrowth have been associated with liver lesions similar to NASH.⁸

These considerations have led to the development of theory that intestinal bacterial overgrowth may be implicated as a potential source of hepatotoxic oxidative injury. To support the pathogenetic role of intestinal bacteria is the observation that the administration of antibiotics, such as polymyxin B and metronidazole, improved steatosis grades in both rats and humans on total parenteral nutrition, following intestinal bypass surgery and in alcohol exposed rats.^{6, 9-11.}

Recently, intestinal bacterial overgrowth has been observed significantly more often in patients with NASH compared to controls.¹²

Intestinal bacteria are involved in the mechanism of intestinal barrier function and their metabolic competition counterbalance opportunistic and pathogenic microbes.¹³ Old age, treatment with

antibiotics, and immunocompromised states can all negatively affect intestinal flora that can lead to emerging new and serious events.¹⁴

Intestinal bacteria overgrowth could increase intestinal permeability and absorption of endotoxin. Endotoxin can induce steatohepatitis, mediated chiefly via the cytokine tumor necrosis factor alpha (TNF- α).^{12,15} Yang et al have suggested that systemic endotoxaemia contributes to TNF- α production and steatohepatitis in genetically obese rats.¹⁶ Therapy with anti-TNF- α antibodies can improve steatohepatitis.¹⁷ Therefore, it seems plausible that gut derived endotoxins perhaps generated from intestinal bacteria are important in the pathogenesis of NASH via Kupffer cell stimulation and TNF- α production.

Probiotics are defined as viable microorganisms that have a beneficial effect in the prevention and treatment of specific pathologic conditions when they are ingested.^{18,19} There are many mechanisms by which probiotics enhance intestinal health, including inhibition of intestinal bacterial enzymes, stimulation of immunity, competition for limited nutrients, inhibition of epithelial and mucosa adherence, inhibition of epithelial invasion and production of antimicrobial substances.²⁰ The probiotic therapy offers an intriguing approach to controlling negative metabolic or pathogenic activities of microbes and to increasing the numbers and activities of those microorganisms suggested to possess health-promoting properties. The administration of probiotics per four weeks to mice with NAFLD led to improvements in steatosis, hepatomegaly, and nuclear factor kappa B (NF- κ B) activity. The NF- κ B, a transcription factor that regulates cellular viability, promotes the synthesis of TNF- α and other inflammatory cytokines.

However, any postulated benefit should be accepted as fact only after extensive, randomized, multicenter testing in human clinical trial.²² There are still many unsolved issues such as preparation (single or multiple species) concentration of ingested bacteria and characteristics of patients that have to be answered

In conclusion, probiotics represent an exciting and potentially significant therapeutic advance, particularly if the application of genetic engineering⁽²³⁾ to enhance the ability to survive in

the intestinal tract and to produce metabolites that are responsible for the probiotic effect will be available in a short time.

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**Inulin and fructooligosaccharides:
physiology and kinetics of bifidobacteria in pure and faecal cultures**

Inulin FOS bifidobacteria

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STRUCTURED SUMMARY

Background: Despite the large use of FOS and inulins as prebiotics, relatively little is known about the physiological behaviour of bifidobacteria in response to differently lengthened fructofuranosides and to the availability of carbohydrates mixtures.

Aims: To compare *in vitro* fermentation properties of FOS and inulin in pure *Bifidobacterium* cultures; to observe the kinetics of *Bifidobacterium* fed with mixtures of five sugars; to study the effects of FOS and inulin on faecal cultures (microbial population and short-chain fatty acids).

Methods: The comparative studies of *Bifidobacterium* pure cultures were carried out in minimal medium containing FOS and inulin. Mixed faecal cultures on FOS and inulin were characterized by pH, SCFA (capillary chromatography) and bacterial composition (FISH) analysis. Carbohydrate concentrations were determined by HPAEC-PAD and HPTLC-AMD.

Results: Most bifidobacteria pure cultures fermented FOS but not inulin, whereas, in faecal cultures, they grew well also on inulin acting as scavengers of oligosaccharides generated by primary degraders. When grown on carbohydrate mixtures, they fermented di- and oligosaccharides prior than monosaccharides. The effect of FOS and inulin in mixed faecal cultures is quite different concerning SCFA production.

Conclusions: FOS and inulin are differently utilized by pure cultures of bifidobacteria and exert different physiological effects on faecal cultures.

INTRODUCTION

Fructo-oligosaccharides (FOS) and inulin consist of mixtures of fructose units linked by β -(1 \rightarrow 2)-glycosidic bonds, with a terminal glucose unit. FOS have a degree of polymerization (DP) of 2-10, whereas inulins are more heterogeneous with respect to the polymer chain length, with a DP ranging from 3 to 60. They are considered prebiotics because they promote the proliferation of bifidobacteria, which constitute a significant portion of the intestinal microflora and are claimed to have several beneficial effects on the host.

In vivo human studies have shown that dietary addition of FOS or inulins led to an increase of bifidobacteria (1, 2, 3). Despite the large commercial use of FOS and inulins as prebiotics and several studies describing the *in vitro* fermentation of FOS with pure cultures of *Bifidobacterium* (4, 5, 6), relatively little is known about the physiological and fermentative behaviour of bifidobacteria in response to differently lengthened fructofuranosides. *In vitro* studies on three selected strains of bifidobacteria suggested that short chain FOS were fermented with higher rate and biomass yield than longer FOS (7).

Only a limited amount of experimental data are available to understand the structure-function relationships in FOS and inulin and the metabolism of these prebiotics by probiotics. In particular, it was not definitively understood which mechanisms are involved in hydrolysis and uptake of oligo and polysaccharides in bifidobacteria. There is evidence of cell-associated exo-glycosidases degrading oligo and large polysaccharides to monosaccharides successively transported into the cell (8). However, it remains to be investigated if this is the only mechanism or if bifidobacteria possess specific transport systems for FOS.

The purpose of this paper was to develop a greater understanding of the physiology of bifidobacteria in pure cultures and of the degree of cross-feeding with non probiotic bacteria into the faecal microbial ecosystem. We compared the bifidogenic properties of FOS and inulin via their *in vitro* fermentation by human gut bacteria and their effect on short chain fatty acid production.

Furthermore, two important factors distinguish the experimental growth conditions from the conditions found in the colon: the composition and the concentrations of the substrates present. In fact, intestinal bacteria usually grow under carbon-limited conditions in the presence of complex mixtures of potential substrates. A further development of this work was the study of the behaviour of a *Bifidobacterium* strain in a batch culture containing a mixture of five carbon substrates at high initial concentrations, in order to evaluate if it exhibited diauxic behaviour and what was the sequence of carbohydrate utilization.

METHODS

Strains and culture conditions

55 strains of *Bifidobacterium*, belonging to 11 different species of human and animal origin, were obtained from ATCC and DSMZ culture collections, from the collection of the Institute of Agricultural Microbiology of the University of Bologna, from commercial probiotic products or were isolated from faeces of healthy volunteers that followed a prebiotic- and probiotic-free diet for 1 month and were not treated with antibiotics for at least 3 months.

Fermentation of carbohydrates was tested in a minimal synthetic medium, with composition (g L⁻¹): glucose, Raftilose Synergy or Raftiline HP, 10; Casaminoacids (Difco), 5; Yeast Nitrogen Base (Difco), 6.7; ascorbic acid, 10; sodium acetate, 10; ammonium sulfate, 5; urea, 2; MgSO₄ · 7H₂O, 0.2; FeSO₄ · 7H₂O, 0.01; MnSO₄ · 7H₂O, 0.007; NaCl, 0.01; Tween 80, 1; cysteine, 0.5; pH adjusted to 7.0.

Chemicals

Raftilose Synergy, Raftiline and Raftilose P95 were supplied by Orafiti, Tienen, Belgium. They are mixtures of chicory fructo-oligosaccharides and inulin containing selected DP distribution. In Raftilose Synergy, fructo-oligosaccharides represent the largest portion, but a fraction of long-chain inulin is also present. Raftiline HP consists mostly of long chain inulin (average DP=25, absent fructo-oligosaccharides with DP<5). Raftilose P95 is composed by 95% fructo-oligosaccharides (DP 3-10).

Actilight[®] (Beghin Meiji, France) is composed by 1-kestose (GF₂) 37%, nystose (GF₃) 53% and fructosyl-nystose (GF₄) 10%.

Carbohydrate analysis

The amount of residual soluble polysaccharides or oligosaccharides from Raftilose Synergy, Raftiline HP and Raftilose P95 was measured with HPAEC-PAD. The degree of fermentation of the polymers was estimated from the shift in molecular weight and from the lowered amount of polymer left in the supernatant after fermentation. The fermentation of the oligosaccharides was judged by comparing the HPAEC elution patterns of the oligosaccharides before and after fermentation.

Carbohydrate analyses were performed with a Dionex (Sunnyvale, CA, USA) Model 4000i gradient pump module equipped with a pulsed electrochemical detector (PED) working in pulsed amperometric mode and consisting of an amperometric flow through cell with a gold working electrode and a silver-silver chloride reference electrode. Separations were performed at room temperature using a Dionex CarboPac PA100 column, connected to the associated guard column. Chromatographic data were collected and plotted using the Dionex AI-450 chromatography workstation. Fructooligosaccharides were separated using a sodium hydroxide and a sodium acetate gradient, as previously reported (rif1). The assignment for the chromatographic peaks with DP higher than 3 was based on the generally accepted assumptions that the retention time of a homologous series of carbohydrates increased as the DP increased, and that each successive peak represented a glucofructan which had a fructose more than that of the previous peak. Under the optimised conditions we achieved the selective

separation of both low molecular weight and high molecular weight fructan mixtures in the whole molecular weight range.

The amount of residual soluble polysaccharides or oligosaccharides from Raftilose Synergy, Raftiline HP and Raftilose P95 was measured with HPAEC-PAD. The degree of fermentation of the polymers was estimated from the shift in molecular weight and from the lowered amount of polymer left in the supernatant after fermentation. The fermentation of the oligosaccharides was judged by comparing the HPAEC elution patterns of the oligosaccharides before and after fermentation.

The analysis of mixtures of sugars (glucose, fructose, galactose, lactose, sucrose, raffinose, 1-kestose (GF₂), nystose (GF₃), fructosyl-nystose (GF₄) was carried out using planar chromatography (HPTLC - High Performance Thin Layer Chromatography) coupled with AMD (Automated Multiple Development). Using "Diol" layers, a proper program of gradient elution using mixtures of acetonitrile/acetone/water has been set up with the aim of separating and determining quantitatively the concentrations of the various sugars in the broth. For the separation of glucose and galactose it has been necessary to properly modify the elution gradient. The content of sucrose in the broth is due to its presence as impurity in the Actilight utilized as source of fructo-oligosaccharides and to the hydrolysis of GF_n.

Short-chain fatty acids analysis

Qualitative profiles of short chain fatty acids in faecal cultures were obtained by capillary zone electrophoresis (CZE) and indirect UV detection. Peaks were identified by comparing migration times and spiking samples with known quantities of standard solutions of SCFA.

Growth experiments and batch cultures fermentations

Cells from MRS cultures were inoculated into 10 ml of minimal synthetic medium containing 10 g L⁻¹ of carbohydrate. The cultures were propagated three times in the same medium. After 48 h of anaerobic incubation at 37 °C growth was determined by measuring the increase in absorbance at 600 nm (A_{600}) and the final pH.

For induction experiments, growth on Raftiline HP 10 g L⁻¹ was compared to growth on Raftiline HP 10 g L⁻¹ supplemented of a suboptimal quantity of Raftilose P95 (0.3 g L⁻¹).

Batch cultures were carried out into a 3 L bioreactor whose pH was adjusted to 6.5 by automatic addition of NaOH 4 M. Anaerobic conditions were maintained by sparging filter-sterilized N₂ into the culture. The fermenter was inoculated (10 % v/v) with exponential phase pre-cultures grown on the same medium.

Comparative growth of bifidobacteria in faecal slurries

Faecal samples obtained from 7 healthy volunteers were transferred into an anaerobic chamber, homogenized and diluted 100 fold in prerduced half-strength Wilkins-Chalgren Anaerobe Broth (Oxoid). 400 µl of this suspension were used to inoculate serum bottles containing 40 ml of the following medium (g L⁻¹): yeast extract, 5; ascorbic acid, 10; sodium acetate, 10; ammonium sulfate, 5; urea, 2; MgSO₄ · 7H₂O, 0.2; FeSO₄ · 7H₂O, 0.01; MnSO₄ · 7H₂O, 0.007; NaCl, 0.01; Tween 80, 1; hemin 0.05; cysteine, 0.5; Raftilose P95 or Raftiline HP as test carbohydrates, 10; pH 7.0. The bottles were insufflated with N₂ gas for 5 min and autoclaved.

Bifidobacteria were directly accounted by FISH technique in faecal cultures at the beginning of the experiment (T_0) and after 24 h incubation at 37°C (T_{24}). Commercial kit Bifidobacterium 10-ME-H001 (Microscreen B.V., Microbial Diagnostics, Groningen, The Netherlands) was used.

Further investigation of the samples involved pH measurement, SCFA analysis by capillary chromatography and determination of residual oligo and polysaccharides by HPAEC-PAD.

Extracellular hydrolitic activity in pure and faecal cultures

Bifidobacterial and faecal cultures, grown on Raftilose P95 or Raftiline HP, were centrifuged to precipitate cells. The supernatant was filtered (0.22 µm) and splitted into 2 portions, supplemented of 0.5 g L⁻¹ Raftilose P95 or Raftiline HP, respectively. Degradation of fructo-oligosaccharides and inulin by extracellular enzymes was monitored using HPAEC-PAD at the beginning and after 96 h of anaerobic incubation at 37 °C.

RESULTS

Growth of *Bifidobacterium* pure cultures on FOS and inulin

55 strains of *Bifidobacterium* were examined for their ability to growth on 10 g L⁻¹ glucose, Raftilose Synergy (mainly FOS) or Raftiline HP (inulin) as carbon source. After 48 h of anaerobic incubation, growth was determined by measuring the A₆₀₀ and the pH. The higher was the absorbance, the lower was the final pH. All strains grew well on glucose and FOS, whereas only 8 strains were able to grow on inulin. There was no evidence of a relationship among the human or animal origin of the strains and the capability to ferment inulin.

The induction experiments aimed to define whether short chain fructo-oligosaccharides could induce the expression of enzymes involved in hydrolysis of long-chain inulin. They revealed that, whenever Raftiline HP did not support the growth, the presence of a suboptimal amount of FOS did not result in any growth on inulins.

The degradation of FOS and inulin was determined by HPAEC-PAD in triplicate for 15 strains after 48 h incubation in minimal medium supplemented of 1% Raftilose Synergy or Raftiline HP. The results showed that almost every strain presented a different degradation pattern. For all strains a clear relationship between degradation and growth was observed, i.e. the strains showing the lowest final pH and the highest A₆₀₀ presented the highest degree of degradation.

The comparison of HPAEC patterns of the eight strains growing on inulin highlighted the wideness of the metabolic behaviour of bifidobacteria against inulin. Among them, only one strain, *Bifidobacterium* ALB 1, degraded completely also the longest chains of inulin.

Batch fermentations of the strains *B. adolescentis* MB 239 and *Bifidobacterium* ALB 3 were carried out under control of pH on Raftilose Synergy or Raftiline HP as carbon source, respectively. Optimization of the medium composition ensured that the culture shifted to the idiophase when the carbon source was depleted or the left-overs could not be degraded and fermented.

B. adolescentis MB 239 grew on Raftilose Synergy with a constant specific growth rate $\mu = 0.60 \text{ h}^{-1}$ and shifted to the idiophase when FOS were depleted. Interestingly, its specific growth rate on glucose as carbon source was much lower ($\mu = 0.15 \text{ h}^{-1}$).

Bifidobacterium ALB 3 grew on Raftiline HP with a constant specific growth rate $\mu = 0.21 \text{ h}^{-1}$. The comparison of elution patterns showed that growth stopped suddenly when all the picks with retention time lower than 69 min disappeared. The simultaneous degradation of differently lengthened chains demonstrated the absence of a polyauxic behaviour respect to the DP. The constant specific growth rate confirmed that the kinetic of degradation was constant and independent of the chain length. The inulin chains eluted after 69 min were not degraded at all.

Furthermore, enzyme location experiments were performed to define whether bifidobacteria produced extracellular enzymes degrading FOS or inulin. Experimental data demonstrated that: some strains growing only on FOS but not on inulin didn't present any extracellular fructofuranosidase activity; some of those growing on inulin showed a strong extracellular hydrolytic activity against both FOS and inulin; some others presented

extracellular activity only when grown on inulin, whereas if the same culture grew on FOS the extracellular activity was absent. These data confirmed the various response of bifidobacteria to FOS or inulin, described not only in terms of fermentation capability, but also by induction of differently located enzymes.

Effects of FOS and inulin on faecal cultures: bifidobacteria, pH and SCFA

In order to evaluate the bifidobacterial growth in mixed faecal cultures, strictly anaerobic fermentation vessels of minimal medium were inoculated with faecal slurries. They contained all the nutrients that could support growth of intestinal bacteria and Raftilose P95 (FOS) or Raftiline HP (inulin) as sole carbon source. During 24 h of incubation at 37°C, the concentrations of bifidobacteria were monitored by FISH.

Into the faecal samples bifidobacteria fermented directly FOS of Raftilose Synergy or the oligosaccharides derived from microbial hydrolysis of inulin (Raftiline HP), with a net increase of 4.0 and 3.4 log₁₀ units, respectively, suggesting that in the mixed faecal culture they grew similarly on FOS and inulin. Although pure bifidobacterial cultures didn't grow on inulins, in the mixed culture they grew abundantly on this polysaccharide by cross-feeding with other faecal bacteria, since HPAEC-PAD profiles demonstrated that supernatants of faecal cultures were able to quickly hydrolyze both FOS and inulin.

Concerning SCFA production in mixed faecal cultures grown on FOS or inulin, a marked difference was observed, both in quantitative and qualitative terms. Inulin (Raftiline HP) led to a remarkable accumulation of butyric acid and to lower amounts of acetic and propionic acids. No lactic acid was present at the end of the experiment. On the contrary, as a

consequence of FOS (Raftilose P95) fermentation, lactic and acetic acids were the major products. Butyric acid also appeared, even if in low amounts. Propionic acid was not present.

The different SCFA final composition of faecal cultures grown on FOS or inulin was confirmed by the different pH reached on the two sugar mixtures. Respect to inulin, the availability of FOS as carbon source led to a more acidic final pH. In presence of Raftilose Synergy the concentration of bifidobacteria was higher and the outcome of their metabolic products, lactic and acetic acids, was higher too.

Growth kinetics of *Bifidobacterium adolescentis* MB 239 on carbohydrate mixtures

As a rule bacteria exhibit diauxic growth in batch cultures containing a mixture of carbon sources at high initial concentrations, utilizing first the substrate that supports the highest growth rate and repressing the consume of the others. In order to evaluate whether bifidobacteria exhibit diauxic behaviour, a pH controlled batch fermentation of *B. adolescentis* MB 239 was performed. The minimal medium contained a defined mixture of glucose, fructose, lactose, raffinose and FOS (Actilight[®]), each 7 g L⁻¹, as carbon source.

Growth was monitored (dry weight) and single carbohydrate concentrations were determined by automated multiple development planar chromatography (HPTLC-AMD).

First, only lactose was fermented; after 6 h of exponential growth on this sole carbon source, when its concentration reached 4.5 g L⁻¹, the simultaneous utilization of raffinose began. After 2 more hours, when lactose and raffinose concentrations were 3 and 4 g L⁻¹ respectively, FOS and glucose utilization started. The balance of fructose remained constant during the fermentation, because of the continuous generation from hydrolysis of FOS and raffinose. When lactose, raffinose and galactose ran out, fructose started a net decrease.

These results confirmed the strong preference of bifidobacteria for oligo and disaccharides respect to monosaccharides, in agreement with kinetics data from single sugar fermentations.

DISCUSSION

The utilization of FOS (Raftilose Synergy) or inulin (Raftiline HP) as carbon sources by 55 pure cultures of bifidobacteria was investigated. Growth and pH measurements suggested that, despite fermentation of FOS was common among all the 55 strains screened, only 8 were able to grow in a minimum medium fermenting long-chain inulin. There was no evidence of correlation between the animal or human origin of the strains and their ability to use inulin as carbon source. These results contradicted previous findings which showed that inulin was degraded preferably by strains of animal origin (9). Induction experiments showed that suboptimal amounts of FOS didn't induce any enzymatic activity against inulin.

HPAEC-PAD chromatograms of spent broths suggested a strict correspondence between the amount of carbohydrates fermented by bifidobacteria and the growth and pointed out the different capability of each strain to attach fructan chains of variable DP.

Batch fermentations of *B. adolescentis* MB 239 supported the evidence of a simultaneous consumption of all the fructo-oligosaccharides constituting the Raftilose Synergy mixture. Switch from exponential growth to idiophase occurred as soon as FOS ran out, when the longest chains of inulin, not fermentable, were still present. Also the fermentation of *Bifidobacterium* ALB 3 on Raftiline HP demonstrated the absence of DP priority in the disappearance of the carbohydrates. As a consequence, growth occurred through a single uninterrupted exponential phase which arrested when the strain exhausted fructans eluted before 69 min.

The physiology of bifidobacteria on FOS and inulin was incomplete unless these studies were supported by data describing the response of bifidobacteria to fructans of

different DP on mixed cultures. In fact the fermentation of oligo and polysaccharides in the colon is the result of a sequence of different metabolic pathways carried out by several microorganisms. Therefore, batch culture inoculated with mixed faecal bacteria were performed in order to evaluate the different bifidogenic effect of FOS (Raftilose P95) and inulin (Rafiline HP).

A strong and rapid fructofuranosidase hydrolytic activity was observed in the supernatant of all the faecal cultures. Although this study supported the evidence that most of pure bifidobacterial cultures cannot hydrolyze and ferment long-chain inulins, these results suggest that bifidobacteria are able to compete as scavengers of partially degraded oligosaccharides released by primary degraders of inulin.

Studies with mixed faecal cultures demonstrated that FOS and inulin strongly affect the outcome of fermentation, with different types and amounts of SCFA produced, likely as a consequence of different effects on microflora composition. Butyrate was the major fermentation product formed during growth on inulin, whereas acetate and lactate were produced when FOS were present. Therefore, the nutritional relationship between members of the colonic microflora are not well known and still unpredictable, as demonstrated by the strong differences on physiological responses exerted by structurally related, differently lengthened, carbohydrates.

Although the general metabolic steps involved in fermentation of sugars by bifidobacteria were well understood, an unresolved area was their behaviour in presence of a mixture of carbon substrates at high initial concentration. The experiment described above, performed with mixtures of glucose, fructose, lactose, raffinose and FOS (Actilight®), demonstrated a strong preference for oligo and disaccharides respect to monosaccharides.

Even if diauxy generally happens at starving concentrations of carbon sources, our result showed that the shift from the consumption of a carbon source to the next didn't occur when the first ran out, but happened at a very high concentration (g L^{-1}), causing several carbon sources to be consumed simultaneously.

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PROBIOTICS IN BACTERIAL VAGINOSIS

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Bacterial vaginosis (BV) is the most common vaginal disorder among reproductive age women. BV is not caused by one specific pathogenic microorganism but rather by an imbalance of vaginal microbial flora. Lactobacilli, that represent the prevalent microorganisms of the healthy human vagina, are reduced or absent in BV and are replaced by *Gardnerella vaginalis* and other anaerobic microbiota such as *Bacteroides*, *Prevotella*, *Mobiluncus*, *Porphyromonas* and *Peptostreptococcus* species, and *Mycoplasma hominis*. Lactobacilli, particularly the H₂O₂ producing ones, play a pivotal role in controlling the microenvironment of the vagina and inhibiting the overgrowth of potentially pathogenic organisms. Metronidazole is currently the treatment of choice for BV, even though vaginal pathogens, particularly *G. vaginalis*, are showing increasing drug resistance and the cure rate one month after therapy is 61-71% and 78% for local and oral treatment, respectively. Recent attention has focused on new approaches involving alternative "natural" treatments that could be effective on the microbiological and clinical resolution of the condition without side effects. Several attempts have been performed to treat BV with such alternative substances as acid gel or lactobacilli-containing products. The possibility of utilizing lactobacilli in the maintenance of a healthy state in the human female urogenital tract is based on the capacity of these probiotic microorganisms to produce a barrier population. Lactobacilli are able to interfere with genitourinary pathogens by different mechanisms, including competitive exclusion of pathogens from the cell surface and production of antimicrobial compounds. We analyzed different *Lactobacillus* strains for properties relevant to mucosal colonization or antagonism (adhesion to epithelial cells, hydrogen peroxide production, antimicrobial activity towards *Gardnerella vaginalis* and coaggregation with the pathogen). The purpose of the work was to characterize and select *Lactobacillus* strains for the preparation of tablets to be used to treat vaginal infections as an alternative to antibiotics. The capacity of the strains to maintain their biological characteristics during manufacturing of the vaginal tablets was evaluated. Lyophilization reduced the adhesion capacity to epithelial cells and, therefore, the colonization ability of some isolates whereas pharmaceutical formulation and tablet production did not further influence the attachment capacity of the microorganisms. The loss of adhesion was not associated with a reduction in lactobacilli viability suggesting that the lyophilization process could modify the conformation of surface bacterial adhesins, thereby limiting their function. The development of a tablet formulation containing a high number of viable lactobacilli is therefore of major importance in order to obtain a high rate of multiplication, thus quickly restoring a high adhesion capacity. Three strains (*Lact. brevis* CD2, *Lact. salivarius* subsp. *salicinius* FV2 and *Lact. gasseri* MB335) were selected for their optimal properties to design a product for local application to the vaginal tract. *Lact. salivarius* FV2 and *Lact. gasseri* MB 335 strains are strong H₂O₂ producers, and *Lact. gasseri* MB 335 is also strongly adherent to epithelial cells. Both strains were able to coaggregate very efficiently with *G. vaginalis*. The coaggregation could be an important factor in establishing and maintaining a healthy urogenital flora because of the production of a microenvironment around the pathogen where the concentration of inhibiting substances produced by lactobacilli is exacerbated. *Lact. brevis* CD2, although not producing H₂O₂, was chosen for its strong adherence capacity and for the production of high levels of the enzyme arginine deiminase, which is able to down regulate polyamine synthesis in human cells. Polyamines are commonly found in elevated concentrations in vaginal discharges of women with bacterial vaginosis and contribute to the elevated pH of vaginal microenvironment and also to the clinical symptoms of bacterial vaginosis, in particular the "fishy" odour that is characteristic of vaginal discharges from affected women. Vaginal tablets containing a mixture of the three strains of lactobacilli were produced and tested for the capacity to interfere with

G. vaginalis adhesion to the cell surface, for the production of antimicrobial compounds and for the maintenance of lactobacilli viability. The selected strains adhered to epithelial cells displacing more than 60% of the pathogen; they produced high levels of H₂O₂, coaggregated with and inhibited the growth of *G. vaginalis*, and maintained a high viability for at least 18 months.

A preliminary randomized, placebo-controlled trial was designed to test the efficacy of the preparation containing the selected strains of lactobacilli for the therapy of bacterial vaginosis, and the ability of the product to restore physiological conditions in the vaginal environment by the re-implantation of a normal, healthy flora. All the patients treated with the probiotic preparation were BV-free after a one week therapy and 89% showed a very good colonization with the administered *Lactobacillus* strains. Three weeks after the therapy with vaginal tablets 78% of treated patients still harboured lactobacilli and the cure rate was 50%. The persistence of re-implanted lactobacilli and the absence of vaginosis were independent from the menstrual cycle (occurred in 89% of the recovered patients) before the control end point. At this time malodorous vaginal discharge and vulvar discomfort disappeared in most of the actively treated patients (61%) including women not BV-free and, if persisted, was of a lower intensity. This effect could be ascribed to the production by *Lact. brevis* CD2 strain of high levels of the enzyme arginine deiminase. In conclusion, we designed a pharmaceutical formulation of a probiotic preparation containing a mixture of three strains of lactobacilli with antimicrobial and adhesive properties as well as biochemical characteristics effective to treat the clinical signs and symptoms of vaginal infections.

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HYDRATION AND SPORTING ACTIVITY

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Introduction

Water is life. It constitutes 90% of cell weight and 65% of total body mass. Water is a fundamental nutrient which has no energy value but exerts a plastic, hydrating function which is protective and bioregulating. Besides being a solvent and an efficient physical means of transport for nutritional substances, it is an excellent medium for the chemical reactions which are necessary for the production of energy destined for the vital functions, and obviously for any muscle work. Sporting activity is a physiological condition in which it is necessary to lose the body heat accumulated through exercise, in order to maintain the inner body temperature constant (1).

The most efficient thermoregulation mechanism during physical activity is sweating. Water and mineral loss increases with sweat production and may reach very high values in unfavourable climactic conditions and during intense muscular work (2-3). Among the dangers of dehydration are reduction of cardiovascular efficiency, decrease in cutaneous blood flow with increase in inner temperature, reduction in plasma volume and consequent alteration of kidney functioning (4).

Normohydration is thus a fundamental condition for the practice of sport and particularly for muscular performance. A loss of water equivalent to a mere 1% of body weight may affect physical performance (5), while a loss of 2.5% of body weight may decrease sporting performance by 35% (4). Since we neither have to or are able to prevent fluid and mineral loss through sweating, adequate rehydration

must be obtained after or during exercise, and this can only be achieved if water and saline losses are correctly reintegrated. The speed of this reintegration process becomes especially important in situations of prolonged, continuous or intermittent muscle work, and it depends on the mineral, particularly sodium and potassium, concentrations of the beverage ingested.

Hydro-saline homeostasis

Most of the water which enters the organism daily is that which is ingested orally, both as simple water and in the form of other beverages, as well as the water contained in foodstuffs. A small amount is obtained by synthesis in the organism itself as a consequence of the oxidation of the hydrogen in the metabolised substances. This volume varies (from 150 to 250 ml/die) depending on the metabolism. Normally the amount of water entering the organism is about 2300 ml/die (6).

Water intake is physiologically compensated for by output; thus it is possible to assert, of course only schematically, that at a temperature of 20°C 1400 ml are lost with urine, about 100 ml with sweat, another 100 ml in the stools and the remaining 700 ml by evaporation from the respiratory tract or by diffusion through the skin (*perspiratio insensibilis*). When the environmental temperature is high, the loss of water through sweating may increase up to 3.5 l/hour. The loss of fluids also increases in muscular exercise in relation to the increase in pulmonary ventilation and in sweating as a compensation for the increase in body heat which occurs with effort. Water losses which occur with sweating involve all the various body compartments, since there is free communication among them. This distribution is influenced by the amount of sweat lost and by its composition, by the total amount of water and salt loss. At low levels of dehydration (< 3%), most water losses derive prevalently from

the extracellular compartment. With the increase in water loss, a greater percentage of water is of intracellular origin.

The osmolarity of extracellular fluids, as of intracellular ones (since the latter are in equilibrium with the former), is determined almost wholly by the extracellular fluid sodium concentration. This depends on the fact that sodium is the most abundant ion at extracellular level.

Two distinct control systems operate in close cooperation for the regulation of sodium concentration and osmolarity:

- 1) The osmo-sodium receptors and antidiuretic hormone system: this is a feedback system which works through consequential stages. An increase in extracellular fluid osmolarity excites the hypothalamus supraoptic nucleus receptors, which release the antidiuretic hormone. The latter brings about an increase in the permeability of the renal collecting ducts, provoking greater water absorption. Renal retention of water, but not of sodium and other osmotically active substances, provokes a decrease in their concentration in the extracellular fluid, thus correcting its high initial osmolarity.
- 2) The thirst mechanism: the decrease in water reserves causes onset of the sense of thirst and the consequent behaviour of drinking, which restores the homeostatic equilibrium between the inside and outside of the cell (homeostatic thirst). Sometimes water restocking may also occur in the absence of water deficit in order to provide the organism with water for a predicted future need (anticipatory thirst).

Peripheral mechanisms of thirst regulation (theory of dryness of the mouth, 7), and central mechanisms (theory of lateral hypothalamus, 8) have both been identified. The osmoceptors positioned in the lateral hypothalamus, which are sensitive to variations in intracellular fluid, signal when the amounts decrease to below threshold level, triggering the sense of thirst. These brain regions also receive afferences from systemic receptors which are able both to record the variations in blood osmolality

and particularly in circulating sodium, and also to modulate neuronal reactivity on the basis of arterial pressure and blood volume variations.

A 2-3% increase in blood osmolality is enough to evoke a deep sense of thirst, associated with an increase in circulating vasopressin concentration.

The mechanisms which respond to volume and intravascular pressure modifications seem to be less sensitive to plasma osmolality. In fact hypovolaemic thirst is evident only after a 10% decrease in blood volume.

In response to water deficit, the absorption of beverages drunk takes place rapidly for more than 50% of the total, this being followed by an intermittent use of relatively small amounts of fluids for a prolonged period (9). Initial thirst relief occurs before the absorption of a considerable amount and hence before the increase in circulating mass. Thus although the decrease in osmolality and increase in extracellular volume cause a reduced sense of thirst, other factors existing before the absorption of fluids intervene to modulate the ingestion of fluids. Signals from mouth, oesophagus and stomach receptors have the function of estimating the volume of fluids ingested, while gastric distension tends to diminish the sense of thirst.

The main processes affecting the post-exercise rehydration process depend on the volume and compositions of the ingested fluids. The volume ingested depends on numerous factors, including palatability of the beverage and its effects on the thirst mechanisms.

It is important to distinguish between the beverages which are necessary for hydro-saline reintegration after effort and those which should be drunk during the sporting activity itself. During effort it is mainly necessary to reintegrate with water the hydro-saline losses due to sweating, avoiding hypertonic solutions which, through osmosis, would draw a further amount of water into the intestinal lumen from already-dehydrated tissues. The maximum absorbable amount of water is thought to be about 1000 ml per hour, to be drunk in small sips so as to avoid vagal stimulations from gastric distension. After effort it is useful to reintegrate with even greater amounts of saline solutions. The oral reintegrating solution recommended by the World Health

Organisation for the acute treatment of diarrhoea establishes a sodium concentration of between 60 and 90 mmol/L, reflecting the high sodium losses which occur in some forms of diarrhoea. Many beverages used by sportsmen as reintegrators have a sodium concentration of 10-25 mmol/L and sometimes lower. The problem related to the poor palatability of the solution with a high saline concentration limits the use of these beverages. However sodium-free beverages undoubtedly have poor rehydrating potential, as well as decreasing the sense of thirst through osmolar decrease. The addition of an energy source is not necessary for rehydration, although a small amount of carbohydrates may increase intestinal sodium and water absorption, at the same time improving palatability (10).

Final remarks

The need for correct hydration in the athlete is now a fundamental one in the practice of daily sport, both in order to achieve the best performance and also to avoid situations which might damage the athlete's health. The means of maintaining and recovering this state are still the object of discussion, although the usefulness has now been established of reintegrating water and salt losses through sweating with a substance that is hypotonic for the plasma, but rich in salts, particularly sodium, potassium, calcium and bicarbonate (11).

Sodium regulates total volaemia through the renin/angiotensin/aldosterone axis, and regulates the sense of thirst (1). Intestinal water absorption is a purely passive process determined by a local osmotic gradient, which the sodium ion enhances thanks to the stimulation exerted on glucose absorption in the small intestine. The benefits of saline reintegration with sodium also include avoidance of excessive decrease in plasma osmolality, which would reduce the sense of thirst and further stimulate urine production (12-14).

Potassium on the other hand plays a fundamental role in controlling cell homeostasis (15), since it is the main intracellular ion. Personal research (14) is in agreement with Yawata's studies on rats (16), confirming the usefulness of this ion in reintegrating

intracellular water. In fact, the main damage from any dehydration is done to the intracellular compartment rather than to the overall amount of water.

An equally important role is played by **calcium** which, by helping gastric emptying, favours a more rapid hydrosaline absorption by the intestinal compartment, and **bicarbonate-ion**, which is able to buffer the tissue acidity following increased cell metabolism during exercise (17).

While a higher saline concentration than the one normally present in pure water is required, rehydration must be obtained with solutions which remain hypotonic towards the plasma, in order to avoid an immediate negative osmotic effect with a flow of water towards the intestinal lumen.

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Effects of bicarbonate-alkaline water on gastric functions: preclinical and clinical studies

Short title: Bicarbonate-alkaline water and gastric functions

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Background. Crenotherapy may play a positive role in the medical management of functional digestive disorders.

Aims. To evaluate the influence of bicarbonate-alkaline water (Uliveto[®]) on preclinical models of gastric functions, and digestive symptoms in patients with functional dyspepsia

Patients. Selected patients complained of dyspeptic symptoms in the absence of endoscopic digestive lesions or *Helicobacter pylori* infection within the previous 3 months.

Methods. Preclinical experiments were performed on rats, allowed to drink bicarbonate-alkaline or oligomineral water for 30 days. Animals then underwent pylorus ligation to evaluate gastric secretion of acid, pepsinogen, and mucus. In separate experiments gastric emptying was assessed. Patients were treated with bicarbonate-alkaline water (1.5 l/day) for 30 days. Frequency and severity of dyspeptic symptoms were assessed at baseline and day 30 by a score system.

Results. In preclinical studies, bicarbonate-alkaline water increased acid/peptic secretions and emptying, without changes in bound mucus. The enhancing actions on gastric secretions and emptying were prevented by L-365,260, a gastrin/CCK-2 receptor antagonist. At clinical level, crenotherapy was associated with relief of epigastric pain, retrosternal pyrosis, postprandial fullness and gastric distension.

Conclusions. Intake of bicarbonate-alkaline water promotes an improvement of dyspeptic symptoms. The preclinical study suggests that this clinical actions depend on an enhancement of gastric motor and secretory functions.

Key words: bicarbonate-alkaline water; gastric secretion; gastric motility; crenotherapy

Introduction

Functional dyspepsia is a heterogeneous syndrome characterized mainly by epigastric pain or discomfort in patients with normal endoscopic findings. The occurrence of dyspeptic symptoms represents a frequent reason for medical consultation, and it is recognized that organic lesions of digestive tissues are found only in a minority of patients with dyspepsia ¹. The current rationale for drug treatment of functional dyspepsia is based on altering the pathophysiological mechanisms believed to underlie the development of symptoms. However, the pharmacological treatment of functional dyspepsia remains unsatisfactory, as clinical studies indicates that drug therapy achieves only a partial relief of symptoms ². Clinical observations suggest that changes in the lifestyle and the use of natural products, including mineral water, may play an adjuvant role in the management of functional dyspepsia. In particular, it has been proposed that crenotherapy may be of benefit in patients complaining for functional dyspepsia ³.

Uliveto[®] is a bicarbonate-alkaline water, characterized by a high content of bicarbonate, calcium, and magnesium ions, which was reported to enhance the effects of high-fiber diet on stool frequency in patients with functional disturbances of distal intestine ⁴. Previous studies showed also that bicarbonate-alkaline water can enhance the rate of gastric emptying, an action that may contribute to the relief of dyspeptic symptoms ^{3,5}. In the present study, *in vivo* experiments were performed on preclinical models to gain insight into the influence exerted by Uliveto[®] water on gastric secretory and motor functions. An attempt was also made to assess whether Uliveto[®] water may affect the occurrence and/or severity of dyspeptic symptoms.

Methods

Preclinical investigation

Experiments were carried out on male Wistar rats, weighing 200 g. The animals were allowed to drink either Uliveto[®] water or a commercial oligomineral water (control) for 30 days. During this period, both changes in body weight and water intake were carefully monitored. On day 30, animals were subjected to evaluations of gastric acid and pepsinogen secretions, mucus bound to epithelial surface, and gastric emptying. In experiments aiming to examine the role of gastrin in the gastric effects of Uliveto[®], animals were treated with L-365,260 (5 $\mu\text{mol/kg}$ i.p.), an antagonist of gastrin/CCK-2 receptors, 10 min before starting subsequent procedures ⁶.

Gastric acid, pepsinogen and mucus secretions were determined in pylorus-ligated rats ⁷. Briefly, during anesthesia with diethyl ether, the duodenum was exteriorized and the pylorus was ligated. Two hours later, the stomach was excised and the volume of luminal fluid was measured (ml/2 h). The acidity in gastric juice was measured by potentiometric titration to pH 7.0 with 0.01 N NaOH and expressed as H⁺ output ($\mu\text{EqH}^+/\text{2 h}$). To determine pepsinogen, 2 ml of 2.5% bovine haemoglobin were added to 0.5 ml of 0.3 N HCl and 0.5 ml of gastric juice. Samples were then incubated for 10 min at 37°C and the reaction was stopped by 5 ml of 0.3 N trichloroacetic acid. The optical density was measured at 280 nm, and the results were compared to a standard curve generated by porcine pepsin. Data were expressed as μg of pepsin/2 h. For the estimation of mucus levels, stomachs were immersed for 2 h in 0.1% Alcian blue in 0.16 M sucrose buffered with 0.05 M sodium acetate (pH 5.8). The unbound dye was removed by washings in 0.25 M sucrose, and the mucus-bound dye was eluted 0.5 M MgCl₂ for 2 h. The optical density of aqueous phase was read at 605 nm, and the amount of Alcian blue extracted per gram of wet tissue was calculated from standard

curves and expressed as μg of dye/g. To evaluate gastric emptying, a solution of phenol red (0.6 g/l) was used as a liquid meal ⁸. Three ml of test meal were instilled into the gastric lumen, and 15 min later the gastric content was collected. The stomach was then rinsed with 3 ml of 154 mM NaCl and the washing solution was added to the recovered gastric content. Phenol red concentration was measured at 560 nm and the amount of dye marker recovered from gastric lumen was calculated. Gastric emptying was expressed as the volume of dye marker solution emptied over a period of 15 min, and expressed as ml/15 min.

Clinical investigation

Among patients with normal upper endoscopy, those with proven functional dyspepsia were admitted to the study. The inclusion criteria were: a) history of dyspeptic symptoms lasting at least 3 months; b) presence of at least one of the symptoms listed below; c) frequency and severity of the dominant dyspeptic symptom with a score of at least 3 (see below). Exclusion criteria were: presence of *Helicobacter pylori* infection; presence of esophagitis or gastroduodenal erosion/ulcer/scar at upper endoscopy; irritable bowel syndrome; other organic digestive or systemic diseases; diabetes; pregnancy or lactation; previous abdominal surgery; use of drugs able to interfere with digestive functions. Fully informed consent was obtained from each patient and the investigation was approved by the local University Hospital Ethics Committee.

At the time of enrolment (day 0), the intake of Uliveto[®] water (1.5 liter per day) was prescribed to all patients for 30 days. A further assessment of dyspeptic symptoms was carried out at the end of crenotherapy (day 30). The presence of the following symptoms was evaluated: (i) epigastric pain; (ii) epigastric burning; (iii) feeling of postprandial fullness; (iv) feeling of early satiety; (v) feeling of gastric distension; (vi)

nausea; (vii) vomiting; (viii) retrosternal pyrosis; (ix) regurgitation; (x) dysphagy. Symptom prevalence was estimated before crenotherapy (day 0) and expressed as percent values. Both before (day 0) and at the end (day 30) of crenotherapy, frequency and severity of symptoms were also evaluated and graded semiquantitatively. Frequency was scored as: 0 (never); 1 (rare; one day per week); 2 (occasional; 2-3 days per week); 3 (frequent; 4-6 days per week); 4 (extremely frequent; 7 days per week). Severity was scored as: 0 (none); 1 (mild; does not interfere with daily activities); 2 (moderate; daily activities are disturbed but not modified); 3 (severe; daily activities are markedly disturbed and affected); 4 (extremely severe; rest at bed is required).

Statistical analysis

Results are given as mean \pm standard error of mean. The significance of differences was evaluated by Student's t-test for paired data (clinical investigation) or one way analysis of variance followed by Student-Newman-Keuls test (preclinical investigation). P values lower than 0.05 were considered significant; 'n' indicates the number of patients (clinical investigation) or the number of animals (preclinical investigation).

Results

Preclinical investigation

In control animals (n=30), body weight at day 0 accounted for 188.4 \pm 13.7 g and increased up to 239.6 \pm 18.5 g at day 30. In animals treated with Uliveto[®] water (n=30), the body weight changed from 192.6 \pm 14.7 g (day 0) to 247.5 \pm 20.3 g (day 30). The daily water intake in controls accounted for 120.3 \pm 13.7 ml at day 1, and this value remained at a steady level up to day 30 (123.5 \pm 15.7 ml). In animals receiving Uliveto[®] water, the daily intake was similar to that recorded in controls and did not vary from

day 1 (126.9±14.6 ml) to day 30 (122.4±11.8 ml). In control rats (n=20), L-365,260 did not modify both acid and peptic secretory activities (Table I). In animals exposed for 30 days to Uliveto[®] water (n = 20), pylorus ligation was associated with a significant increase in both acid (+41.38%) and peptic gastric secretions (+43.24%). Such increments no longer occurred in animals treated with L-365,260 (Table I). In control rats, Alcian blue recovery from gastric mucus was not affected by L-365,260. In rats exposed to Uliveto[®] water, Alcian blue recovery was similar to that obtained in controls and was not modified by L-365,260 (Table I). Under control conditions (n=10), gastric emptying was insensitive to blockade of gastrin/CCK-2 receptors. In animals allowed to drink Uliveto[®] water (n=10) a significant enhancement of gastric emptying (+27.43%) was detected, and this effect was fully inhibited by L-365,260 (Table I).

Clinical investigation

Eight males and 10 females (age range: 25-75 years; median age: 52 years) were admitted to the study. At day 0 the prevalence of dyspeptic symptoms was as follows: epigastric pain, 9/18 (50%); epigastric burning, 9/18 (50%); postprandial fullness, 12/18 (67%); early satiety, 2/18 (11%); feeling of gastric distension, 8/18 (44%); nausea, 4/18 (22%); vomiting, 2/18 (11%); retrosternal pyrosis, 10/18 (55%); regurgitation, 3/18 (16%); dysphagia, 4/18 (22%). Symptoms with prevalence equal or higher than 40% were taken into account for subsequent efficacy analysis. Mean values obtained for frequency and severity of symptoms, are displayed in Table II. Evidence was obtained that Uliveto[®] water intake was associated with a relief of epigastric pain, retrosternal pyrosis, postprandial fullness and feeling of gastric distension (frequency and severity), as well as epigastric burning (severity).

Discussion

Mineral water might exert an adjuvant role in the management of dyspepsia, depending on its peculiar chemico-physical properties³. The present study examined the effects of Uliveto[®] water on patients with functional dyspepsia, and evidence was obtained that crenotherapy with this water may positively affect dyspeptic symptoms.

The observation that Uliveto[®] water promoted the relief of postprandial fullness and gastric distension suggests that this water might promote a positive modulation of gastric motility, leading to an improved pattern of gastric emptying. This hypothesis agrees previous clinical investigations on dyspeptic patients, where bicarbonate-alkaline mineral water enhanced gastric emptying, as measured by scintigraphic techniques⁵. Additional support to this view comes from the present preclinical experiments, where an increment of gastric emptying was observed in animals after one-month exposure to Uliveto[®] water.

The present preclinical study provided evidence that, animals treated with Uliveto[®] water displayed an enhancement of gastric acid and pepsinogen secretions, without concomitant changes in the pre-epithelial mucus layer. These findings support the view that Uliveto[®] water might promote a combined positive modulation of upper digestive processes. The peculiar electrolyte composition of Uliveto[®] water allows to hypothesize that its positive influence on dyspeptic symptoms might depend on the high concentrations of calcium or bicarbonate ions. Indeed, previous studies showed that intragastric calcium increases acid secretion through a local stimulation of gastric mucosa⁹, and that, conversely, calcium channel blockers inhibit gastric secretion¹⁰. Moreover, calcium ions can act on antral mucosa to evoke the release of gastrin⁹. Therefore, it is likely that calcium ions in Uliveto[®] water may contribute to its antidyspeptic actions.

Due to its high concentration in bicarbonate ions, Uliveto[®] water is expected to increase the pH in gastric lumen. This change might be relevant to the antidyspeptic effects of Uliveto[®] water in different ways: 1) exposure of duodenal mucosa to gastric acid results in a slowing of gastric emptying¹¹, and therefore lowering of gastric acidity may counteract this inhibitory reflex; 2) changes in gastric pH may affect the release of digestive hormones implicated in the regulation of gastric functions. For instance, maintaining intragastric pH above 3 increases the magnitude and duration of gastrin response after a meal⁹. Since gastrin regulates different gastric functions¹², we performed additional experiments with L-365,260, an antagonist of gastrin/CCK-B receptors⁶. Under these conditions, Uliveto[®] water was no longer able to stimulate gastric functions, suggesting that gastrin might be involved in its digestive effects.

In conclusion, the present investigation indicates that bicarbonate-alkaline water may promote the relief of digestive symptoms in patients with functional dyspepsia. The results obtained in preclinical experiments suggest that bicarbonate-alkaline water may exert positive influences on gastric functions, and that the release of endogenous gastrin might account for these actions.

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Table I. Effects of 30-day exposure to Uliveto[®] water, either alone or in the presence of L-365,260 (5 $\mu\text{mol/kg}$ i.p.), on gastric acid secretion, gastric pepsinogen secretion, Alcian blue recovery from gastric mucus, and gastric emptying in conscious rats.

Treatment	Acid ($\mu\text{EqH}^+/2\text{ h}$)	Pepsinogen ($\mu\text{g}/2\text{ h}$)	Mucus ($\mu\text{g}/\text{g}$)	Emptying (ml/15 min)
Control	131.3 \pm 11.6	59.1 \pm 9.6	96.5 \pm 5.2	2.3 \pm 0.2
L-365,260	138.1 \pm 14.8	62.3 \pm 7.1	93.2 \pm 9.5	2.1 \pm 0.3
Uliveto [®]	185.6 \pm 16.7 ^a	84.5 \pm 6.8 ^a	101.1 \pm 7.4	2.9 \pm 0.3 ^a
Uliveto [®] + L-365,260	117.7 \pm 10.8 ^b	53.2 \pm 5.3 ^b	95.5 \pm 8.3	2.1 \pm 0.1 ^b

Control animals were allowed to drink a commercially available oligomineral water for 30 days and were then subjected to experimental procedures either in the absence or in the presence of L-365,260. Each value indicates the mean value obtained from 10-20 animals \pm standard error of mean. Significance of differences from control values: ^aP<0.05. Significance of differences from values obtained in animals allowed to drink Uliveto water alone: ^bP<0.05.

Table II. Score values obtained for frequency and severity of dyspeptic symptoms both before (day 0) and after crenotherapy with Uliveto[®] water (day 30) in patients with functional dyspepsia.

Symptom	Frequency		Severity	
	Day 0	Day 30	Day 0	Day 30
Epigastric pain	2.11±0.31	1.67±0.47 ^a	2.67±0.29	1.78±0.49 ^b
Retrosternal pyrosis	2.60±0.34	1.70±0.42 ^b	2.60±0.31	1.60±0.40 ^b
Epigastric burning	2.33±0.24	1.78±0.40	2.67±0.17	1.44±0.34 ^b
Postprandial fullness	2.50±0.31	0.83±0.32 ^c	2.58±0.26	0.83±0.34 ^c
Gastric distension	2.88±0.30	1.25±0.41 ^c	2.38±0.26	1.25±0.41 ^a

Values reported for both frequency and severity are the mean ± standard error of mean obtained from 8-12 patients. Significance of differences between values obtained at day 30 and the respective control values obtained at day 0 (Student's t test for paired data):

^aP<0.05; ^bP<0.01; ^cP<0.001.

***Bacillus* species as probiotics for human use**

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Among bacteria used as probiotics for human use, members of the genus *Bacillus* display the unique feature of being delivered as spores. This trait makes them of particular industrial interest due to the long shelf life of the respective products and to the inexpensive conditions for storage and transport. In addition, the potential advantage of probiotic formulations containing bacterial spores is that spores can survive transit through the stomach intact.

The use of probiotic bacteria belonging to the genus *Bacillus* is not limited to a single species, but includes *B. clausii*, *B. subtilis*, *B. cereus*, *B. vietnami*, *B. polyfermenticus*, *B. pumilus*, and *B. licheniformis*. Nevertheless, little information is available on the effects of these spore preparations on the human well-being. It has been reported that *Bacillus* species could exert an immune stimulating activity, act as competitive exclusion agents, synthesize antimicrobial compounds that impair colonization of the gastrointestinal tract by a pathogen, or secrete useful substances such as vitamins. Although these claimed activities are based on the assumption that *Bacillus* species can germinate and grow in the human gut, no unequivocal data on these aspects have been reported.

The fate of spores of the four *B. clausii* strains contained in the formulation Enterogermina (Sanofi-Synthelabo OTC, Milan, Italy) following a single oral administration in twenty healthy volunteers has been recently investigated. The results demonstrate that i) bacterial elimination occurs in a defined time-dependent manner, with a survival in the intestinal environment for up to 15 days; ii) the four *B. clausii* strains are mainly present as spores in the stool samples; iii) the number of spores rescued from feces is, in some instances, greater than that administered, thus indicating germination of spores and bacterial multiplication in the intestinal tract. In addition, to evaluate whether probiotic *Bacillus* strains can actively secrete vitamin B₂, a new microbiological assay based on the use of a vitamin B₂ auxotroph was developed (Salvetti et al., J Appl Microbiol, 2003 in press). Most of the *Bacillus* strains analyzed are able to release substantial although different amounts of vitamin B₂ in the environment.

The capability of the *B. clausii* spores contained in Enterogermina to undergo germination, multiplication and sporification during the transit in the intestinal environment and to actively secrete vitamin B₂ can possibly contribute to their claimed probiotic activity.

Bacillus clausii probiotic strains: Antimicrobial and Immunomodulatory activities

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The concept of probiotics was proposed more than 100 years ago. However, the development of the fundamental scientific validation for the probiotics' efficiency and the understanding of their mechanisms of action are still in their infancies. In particular, the knowledge of *Bacillus* probiotics' activities remains significantly behind what is known about the probiotics based on Lactic Acid Bacteria. As the main described properties involved in the clinical benefits due to probiotics are the production of antimicrobial substances and the immunomodulation, we chose to investigate the possible actions of the *Bacillus clausii* strains contained in the probiotic Enterogermina® (Sanofi-Synthelabo OTC S.p.A., Italy) in these two fields.

The antimicrobial properties of these *Bacillus clausii* strains were evaluated using spot and agar diffusion methods. *Bacillus clausii* was inoculated as a spot on the surface of a starch agar plate. After 72 hours, the plates were overlaid with a medium containing a pathogenic strain. The antimicrobial activity was detected by the presence of a growth inhibition zone around the spot. The *Bacillus clausii* strains tested displayed inhibitory effects against *Staphylococcus aureus*, *Enterococcus faecium*, *Clostridium difficile* strains. Our results demonstrated that the culture conditions were crucial for this antimicrobial activity. The most determinant factors included pH and mineral supplements of the media. The activity, due to the release of antimicrobial substances into the culture medium, was associated with stationary growth phase and was concomitant with sporulation.

We also analyzed the effect of the vegetative cells of the *Bacillus clausii* probiotic strains on some parameters of host immune system.

Flow cytometric techniques were used to assess oxidative burst activity of lymphocytes and macrophages incubated with the probiotic bacteria. When compared to

the *Bacillus subtilis* 168 strain, the *Bacillus clausii* strains significantly stimulated the oxidative burst in both cell lines. In addition, the *Bacillus clausii* strains were able to induce the proliferation of C57 B1/6j mouse CD4⁺ T cells and also to stimulate the IFN- γ production by mouse spleen cells. The data show that these strains display immunomodulatory activity.

Our results put into evidence that at least two mechanisms could be involved in the healthy effects of the *Bacillus clausii* strains present in the probiotic Enterogermina®. Like some other known *Bacillus* species, they have the ability to produce antimicrobial substances which are active against some major gut pathogens. Moreover, they can also act through the stimulation of the immune system.

CLINICAL APPLICATION OF ORAL BACTERIOTHERAPY

Oral Bacteriotherapy

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Introduction

The integrity and the function of gastrointestinal tract depends on intestinal ecoflora, mucosal barrier and local immune system. Intestinal microflora is a complex ecosystem and modulates a number of factors, including proliferation and differentiation of mucosal epithelial cells, regulation of bowel motility, synthesis of substances such as vitamins and secondary biliary acids, and eventually functional regulation of immune system. Normal microbial ecoflora composition in the adult subject is a result of a balance among aerobic microorganisms, (*Streptococcus spp*, *Enterococcus spp*, *Staphylococcus spp*, *Enterobacteriae spp*), anaerobic microorganisms (*Peptostreptococcus spp*, *Bifidobacterium spp*, *Lactobacillus spp*, *Clostridium spp*, *Eubacterium spp*, *Bacteroides fragilis*), and yeasts (*Saccharomyces spp*) and among “good” bacteria (*Lactobacilli*, *Eubacteria* and *Bifidobacteria*), “bad” bacteria (*Staphylococci*, *Clostridia*, *Vibriones*, *Pseudomonaceae*), and several species of intermediate microorganisms, (*Enterobacteria*, *E. coli*, *Bacteroides* and methanogens).

This microenvironment can be modulated through oral bacteriotherapy with administration of prebiotics, probiotics or symbiotic. *Prebiotics* are defined as dietary indigestible ingredient which selectively stimulates growth and activity of one or multiple bacterial spp.. Substances used as prebiotics are: short chain fructo-oligosaccharides (sc-FOS) or medium-long chain (Inulin) and other non digestible oligosaccharides (galacto and soy-oligosaccharides).

Probiotics are non pathogenetic microorganisms that, when ingested, exert a positive influence on host by altering his microbial balance. Used probiotics in clinical practice include *Lactobacillus spp* (*casei*; *salivarius*; *acidophilus*; *bulgaricus*); *Bifidobacterium spp* (*bifidum*; *longum*; *breve*; *lactis*...); *Bacillus clausii* and other species such as *E.coli*; *S. boulardii*; *S. thermophilus*.

Symbiotics are a mix of probiotics and prebiotics that increase survival of the probiotic, making immediately available its substrate for fermentation.

Proposed mechanisms of action of oral bacteriotherapy are different, and include the synthesis of antimicrobial substances as organic fatty acids, ammonia, hydrogen peroxide and bacteriocines; a competitive interaction with pathogens for “space and food”, through the use of available nutrients and occupation of microbial adhesion sites; modification of toxins or toxin receptors, partial sugar

digestion, and finally immunomodulation. Immunomodulation is achieved through adjuvant like effects on intestinal and systemic immunity, the enhancement of specific serologic antibody response, and a balance in the generation of pro- and anti-inflammatory cytokines¹.

Potential clinical applications are currently in both gastrointestinal and extraintestinal diseases.

Among the different microorganisms used *B. clausii* presents several unique properties and a long history of use in many gastrointestinal and extraintestinal diseases so that his safety is definite. Several studies show that the administration of *B. clausii* spores during antibiotic therapy can significantly reduce therapy-associated gastrointestinal symptoms normalizing intestinal microflora during antibiotic therapies because of their resistance to commonly used antibacterial drugs.

An important advantage of *B. clausii* compared to other probiotics is the ability to form spores. Bacterial spores are metabolically dormant and this state is particularly suitable for cell survival in adverse nutritional conditions. Spores may survive for many years, thus yielding a stable pharmaceutical preparation wich can be stored without loss of viability during time. Suitable enviromental conditions can cause rapid activation and synchronous germination of a spore population. In adition the spore can cross undamaged the gastric barrier, where the low pH allowed their activation and reach the intestinal tract where they germinate to vegetative forms. The actual mechanism of action of *B. clausii* spores in the restoration of destroyed or altered intestinal flora in small bowell disorders has not yet fully clarified and this is probably due to difficulties in gastrointestinal flora studies. The experimental data permit us to speculate that both *B. clausii* spores and cells have the ability to adhere to the intestinal tract allowing colonization of the mucosa.

Gastrointestinal diseases

Lactose intolerance

Probiotics exert their main effect stabilizing gut microflora and improving specific physiologic functions in gastrointestinal tract. In the last decade there has been increasing interest on the benefits

of modifying gut ecoflora in high-risk groups of patients as premature infants, travellers, children and adults receiving antibiotics.

Living bacteria added in yoghurts, including several strains of *Lactobacillus* and *Streptococcus thermophilus*, improve lactose digestion and alleviate intolerance related symptoms exerting their lactase activity in vivo in the gut lumen^{2,3}.

Infectious diarrhoea

The treatment of acute pediatric infectious diarrhoea is the best documented clinical application of probiotics⁴. Several large and well-controlled clinical studies have shown that specific strains of probiotics (eg *Bacillus clausii*, *Bifidobacterium bifidum*, *Streptococcus thermophilus*, *Lactobacillus reuteri*, *L. rhamnosus* GG and *L. casei*), administered either as dietary supplements, in fermented milk or added to a rehydration solution, are able to reduce significantly incidence, frequency and duration of acute diarrhea in children^{5,10}.

Different mechanisms of action have been taken into account to explain beneficial effects of probiotics in infectious diarrhea: exclusion of pathogens by means of competition for binding sites and available substrates, lowering of luminal pH, production of bacteriocins and promotion of the production of mucus, enhancement of intestinal motility, production of short chain fatty acids which regulate cell growth and differentiation, trophic effects on intestinal epithelium, balance in the generation of pro- and anti-inflammatory cytokines⁴. All these specific actions result in the normalization of the increased intestinal permeability and altered gut microecology, in the promotion of intestinal barrier functions and in the reduction of the intestinal inflammatory response.

Colorectal cancer

It is well established that probiotics and prebiotics can be used to prevent colon cancer in several animal models, but there isn't the same evidence for a chemopreventive effect in humans. Probiotics seem to reduce the production of genotoxic compounds that act as tumour initiators in human beings.

Studies of the composition of faecal flora of subjects with different risks of colon cancer have shown respectively the association of *Bacteroides vulgatus* and *Bacteroides stercoris* with high risk of colon cancer and of *Lactobacillus acidophilus*, *Lactobacillus S06* and *Eubacterium aerofaciens* with a low risk^{11,12}.

***Helicobacter pylori* infection**

Probiotics improve *Helicobacter pylori* eradication rate through two principal mechanisms: directly, lowering bacterial load with the inhibition of adherence and production of metabolites, bacteriocins or antibiotics; indirectly, decreasing antibiotics side effects and improving patient compliance. Lactobacilli are predominant bacteria in the normal stomach of fasting subjects due to their ability to adhere and even transiently reside in the stomach, and to enhance the immune response. Several well-conducted studies demonstrated that specific strains of lactobacilli (eg, *L. salivarius*, *L. gasseri* and *L. acidophilus*) are able to inhibit bacterial growth in vitro, and to reduce *H. pylori* attachment to gastric cells and bacterial urease activity in vivo^{13,16}.

L. acidophilus LB have been shown to increase significantly *Helicobacter pylori* eradication rate of a triple standard therapy (amoxicillin, clarithromycin and omeprazole)¹⁷.

Moreover, in such studies probiotics were able to ameliorate the tolerability of eradicating treatments. Actually, *H. pylori* eradication therapy has the potential burden of antibiotic-associated gastrointestinal side effects: bloating, taste disturbance, epigastric pain, nausea, diarrhoea, constipation, loss of appetite, vomiting. Side effects occurrence is among major drawbacks of such antibacterial regimens, and gastrointestinal manifestations may be related to quali/quantitative alterations in the intestinal microflora.

The preparations used in such trials are based on supplementation of a single strain of probiotic or combinations of different bacteria. The use of *Lactobacillus GG* or multi-strain combination (*Lactobacillus ssp* and *Biphidobacteria*) in asymptomatic patients who undergo *H. pylori* eradication, induces a significant reduction of frequency and intensity of taste disturbance, nausea and diarrhoea^{18,19}. In a randomised double blind – placebo controlled study, supplementation with

Bacillus clausii during a standard triple eradication therapy in asymptomatic patients has been shown to be effective in reducing the occurrence of epigastric pain, diarrhoea and nausea²⁰.

Bacillus clausii represents a valuable option to reduce side effects associated to the currently recommended anti *H. pylori* regimen.

Irritable bowel syndrome

Irritable bowel syndrome (IBS) pharmacologic management is still unsatisfactory. Functional gut diseases seem to be related to an altered neuro-hormonal control of gut motility and perception due to central and peripheral factors, probably associated to a dysbiosis. Very few controlled studies have been carried out, investigating the effect of probiotics in irritable bowel syndrome.

There are in literature only two controlled studies in which the efficacy of oral administrations of *L. plantarum* DSM 9843 and VSL#3 respectively has been evaluated: such supplementations seem to be effective in the reduction of some of the symptoms of IBS, especially abdominal bloating. Potential mechanism of action is still unknown^{21,22}. The observations that colonic gas production (especially hydrogen) is higher in patients with IBS than in control subjects and that both symptoms and gas production decrease after an exclusion diet, suggest a role of gut ecoflora in IBS.

Inflammatory bowel disease (IBD)

Intestinal microflora has been suggested to be an important factor in the development of many inflammatory disorders in the gastrointestinal tract. The hypothesis that resident bacteria of the normal flora are involved in intestinal mucosal inflammation is supported by data from animal models. Strains of *Lactobacillus reuteri* and *Lactobacillus plantarum* have been used to prevent inflammatory changes associated with methotrexate-induced enterocolitis in rats²³.

Animal IBD models show that colitis does not develop in a germ-free environment and that resident bacteria are required for development of spontaneous colitis in interleukin 10 deficient mice and in HLA-B27 transgenic rats²⁴. Treatment with wide-spectrum antibiotics has been shown to mitigate mucosal inflammation in rats and mice with IBD.

IBD inflammation is mainly located in bowel traits that contain the higher bacterial loads and in areas of relative stasis (terminal ileum, cecum, rectum) where a prolonged exposition of the mucosa is favoured, to bacteria or their products: these observations suggest that in IBD pathogenesis a dysbiosis might be implicated²⁵.

In IBD patients an alteration in microflora composition has been shown, with respect to healthy subjects: patients have a relative diminution a *Lactobacilli* species, with increases in *Bacteroides*, *Eubacteria* and *Peptostreptococci* representation^{26,27}. In ulcerative colitis (UC) patients, supplementation with *E. coli* and *Lactobacillus GG* reduce the recurrence rate, with respect to mesalazine treatment^{28,29}. *S. boulardii* and *Lactobacillus GG* have been shown to be useful in the prevention of recurrence in Crohn disease (CD) patients, although these results are still controversial^{30,31}. Multistrain *VSL#3* has been proven to be effective in pouchitis prevention and reduction of recurrence rate in UC patients^{32,33}. Although further and wider studies are wanted, current perspectives seem to encourage the use of probiotics in the clinical management of IBD patients, also as a substitute for standard therapies, to antagonise bacteria for therapeutic purposes.

Extraintestinal diseases

Oral bacteriotherapy has been used in the prevention and treatment of bacterial vaginitis and urinary tract infections, and it seems to be beneficial through arginine deaminase activity and the ability of the bacteria to restore and maintain normal urogenital flora³⁴.

One of the most important clinical application of oral bacteriotherapy is primary prevention of atopic disease. The ability of probiotics to reverse increased intestinal permeability- characteristic of children with atopic eczema and food allergy-, to enhance gut-specific IgA responses, to promote gut barrier function and restore normal gut microecology and to stimulate anti-inflammatory cytokines production such as interleukine-10 and TGF beta, makes oral bacteriotherapy a new therapeutic approach for the management of hypersensitive disorders. Perspective studies show a significant improvement of atopic dermatitis and markers of allergy response in children³⁵ and adults with the use of bacilli, lactobacilli and bifidobacteria^{36,37}. In particular, *B. clausii* has been proved useful in the

treatment of various allergic diseases: a sensible amelioration in the clinical picture has been shown, following treatment with this probiotic, in children with eczema, atopic dermatitis, food allergy and in prevention of sensitizations³⁸⁻⁴⁰. *B. clausii* supplementation proves efficacious, also, in the prevention of recurrent urinary tract infections, otitis and respiratory tract infections, possibly because of its documented immunological effects (eg, secretory IgA synthesis, stimulation and INFgamma production)⁴¹⁻⁴⁴.

Conclusions

Since the immune system is located mainly in the gut, oral bacteriotherapy, modifying the intestinal bacterial flora and affecting the immune system, could be an effective biological therapy for the treatment of GI and systemic pathologies. The efficacy of oral bacteriotherapy in prevention and treatment of several diseases is well established.

The best documented clinical applications of oral bacteriotherapy show as probiotics are able to alleviate lactose intolerance related symptoms, to reduce incidence, frequency and duration of acute diarrhea, to improve *Helicobacter pylori* eradication rate and to improve atopic dermatitis and markers of allergy response.

However, double-blind placebo controlled studies to document the individual efficacy of each specific microorganism for each potential clinical application are still lacking, and issues to be established include the choice of microorganism strain, the dose to be administered and the route of administration (lyophilized suspension, milk, yoghurt or others).

In particular *B. clausii*, probably because of its peculiar properties and its documented immunological effects, has been proven to be efficacious in the management of atopic disease and infectious diarrhea, in the prevention of recurrent infectious diseases and in the reduction of antibiotics-associated side effects during *Helicobacter pylori* eradication. The ability of orally administered spores of *B. clausii*, to potentiate immune defences by stimulating IgA synthesis and consequently increasing

immunological defence mechanisms, makes this probiotic useful and potentially effective in a large number of intestinal and extraintestinal diseases.

The field of probiotics is very open to further studies in several branches of human pathology, and new applications are continuously proposed. Future directions include the use of probiotics as live vectors for oral immunization (vaccine) and immunomodulation in inflammatory and allergic condition, possibly in the prevention of transmission of AIDS and sexually transmitted diseases, in infection control programs, and in antibacterial treatments against *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli* and *Helicobacter pylori*⁴⁵.

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IMMUNOLOGICAL PERSPECTIVES OF *BACILLUS CLAUSII*: NEW EVIDENCE IN ALLERGIC CHILDREN.

Running title: Immunomodulatory activity of *B clausii*

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SUMMARY

Background *Bacillus clausii* has been demonstrated to exert some immunomodulatory activities and to be safe.

Epidemiological data demonstrate that children attending day care centers are at elevated risk of respiratory infections.

Aims We conducted a study to investigate whether *Bacillus clausii* administration in allergic children with recurrent respiratory infections might modulate cytokine pattern.

Patients Ten children (mean age 4.4 years) attending the nursery-school were enrolled at the end of school year (i.e. in the summer).

Methods *Bacillus clausii* spores (Enterogermina: 2 billions spores/vial) were administered at the dosage schedule of 2 vials a day for 4 weeks. Nasal lavage was performed in all subjects before and after the treatment. A panel of cytokines, including IL1, IL3, IL4, IL6, IL8, IL10, IL12, IFN γ , TGF β , and TNF α , was measured by immunoassay in the fluid recovered from nasal lavage, before and after the treatment.

Results *Bacillus clausii* treatment showed a significant decrease of IL4 levels ($p < 0.01$) and a significant increase of IFN γ ($p < 0.05$), IL12 ($p < 0.001$), TGF β ($p < 0.05$), and IL10 ($p < 0.05$) levels. Other cytokines were not significantly modified.

Conclusions This study shows that the *Bacillus clausii* may exert immunomodulating activity by affecting cytokine pattern in allergic children with recurrent respiratory infections.

Key words: *Bacillus clausii*, cytokines, allergic children, recurrent respiratory infections

INTRODUCTION

Probiotics are viable microorganisms that exhibit a beneficial effect on the health of the host by improving its intestinal microbial balance (1). Probiotic consumption is reported to exert a myriad of positive effects including: enhanced immune response, balancing of colonic microbiota, vaccine adjuvant effects, treatment of diarrhea associated with travel and antibiotic therapy, and control of rotavirus- and *Clostridium difficile*-induced colitis (2).

To be effective a probiotic must be able to survive passage through the acidic environment of the stomach and grow in and colonise the intestine, even in the presence of antibiotics (3).

Moreover, to be widely used a probiotic must also be safe, as importantly demonstrated in numerous studies (4).

Probiotics are presumed to promote healing of the enteric mucosa by reducing gut permeability and by enhancing local intestinal immune responses, particularly the IgA synthesis, as well as by reconstituting the intestinal flora (5).

Recurrent respiratory infections (RRI) in children constitute a demanding challenge for the paediatrician, mainly concerning the diagnosis, the therapeutic strategies, and the preventive approach.

Children attending day care centers show a 1.5-3 times higher risk of respiratory and gastroenteric infections than children cared for at home or in small family care groups (6). This increased risk obviously involves

pharmacoeconomic issues concerning direct medical costs and indirect costs for parents (7). Moreover, antibiotic resistance have lead to an increased interest in alternative approaches for controlling common childhood infections (5).

As prevention should reduce the need for treatment, the prophylactic use of probiotics to prevent infections has been proposed, as recently demonstrated by a study investigating the effect of a probiotic milk on respiratory and diarrheal infections in children attending day care centers in Finland (8).

As prevention of infections in day care is of major importance, the Finnish study reported that *Lactobacillus GG*, added in the milk and administered three times a day, five days a week, for seven months, reduced respiratory infections and their severity among children in day care (8). Thus, this study provided evidence that probiotics may be useful in preventing respiratory infections in children at increased risk.

As airway mucosa is a site not in direct contact with the site of colonisation by the probiotic, this finding requires a hypothesis to explain these systemic effects.

Probiotics may stimulate immune system at all mucosal surfaces (9). In this regard, it has been demonstrated that probiotics exert a primary prevention of atopic diseases (10).

Gastroenteric microflora promote potentially antiallergenic processes: T helper-1 (Th1) immunity (11), generation of TGF β (12), and IgA synthesis (13). In addition, commensal gastrointestinal microbes are the earlier and biggest stimulus for development of gut-associated lymphoid tissue (GALT). Thus, the gut microflora may be a major postnatal counterregulator of the universal Th2-skewed immune system in infants. In fact, allergic children are characterised by a Th2 polarization and are more susceptible than normal children to have infections.

Allergic children show low levels of IFN γ , key cytokine in fighting viral infections, and upregulation of adhesion molecules, such as ICAM-1 that is the main receptor for rhinovirus (14).

Therefore, probiotics could represent an important therapeutic advance concerning the prevention of infections and atopy (15).

In this context, *Bacillus clausii* is a safe and frequently prescribed probiotic. There are some studies showing its effects in preventing gastroenteric and respiratory infections in children (16). In addition, its effects on immune response have been reported in *in vitro* and *in vivo* studies (16).

Therefore, we aimed at investigating the potential effects exerted by *B. clausii* on cytokine pattern, including IL1, IL3, IL4, IL6, IL8, IL10, IL12, IFN γ , TGF β , and TNF α , in allergic children, attending the nursery-school, with recurrent respiratory infections.

MATERIAL AND METHODS

Subjects: Ten allergic children, 6 males and 4 females, with an average age of 4.4 years (range 3-6), attending the nursery-school were evaluated consecutively. A detailed clinical history and a complete physical examination, including allergy evaluation, were carried out for each patient.

The diagnosis of allergy was made on the basis of skin prick test for a panel of common allergens described elsewhere (17).

Study design: To evaluate the cytokine pattern in a group of allergic children with recurrent respiratory infections, we excluded all the children who met the following exclusion criteria: use of antihistamines, nasal, inhaled or oral corticosteroids within the previous 4 weeks.

A placebo-group was not considered, since the main scope of this study was the evaluation of the immunopathological phenomena appearing after the *B. clausii* administration. Anyway, the study was blind to the investigator who performed the cytokine dosages.

All children were treated with oral *B. clausii* (Enterogermina, Sanofi-Synthelabo OTC, Milan, Italy) spores (2 billions spores/vial) at the dosage schedule of 2 vials a day for 4 weeks.

The study was performed at the end of the school year, i.e. in the summer: period with very low incidence of respiratory infections in our area.

Nasal lavage was performed in all subjects before and after *B. clausii* administration.

The study was approved by the Institutional Review Board and an informed oral consent was obtained from the parents of the children.

Nasal lavage: A lavage of the nasal cavity was done using 5 mL of physiologic saline, according to standard methods described

elsewhere (18). This was performed with the patient's head bent backwards during closure of the soft palate. The recovered fluid was comparable in all groups, it was collected to dose cytokine levels and stored at -20° (18).

Cytokine assessment: The cytokines were measured with ELISA method (R&D Systems, U.S.A.). This assay employed the quantitative sandwich enzyme immunoassay technique. The minimum detectable dose was less than 1 pg/mL for all cytokines except for IL8 (less than 10 pg/mL). A monoclonal antibody specific for each cytokine was pre-coated onto a microplate. Standards and samples were pipetted into the wells and each cytokine present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for cytokine was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of cytokine bound in the initial step. The color development was stopped and the intensity of the color measured.

Statistical analysis: The data collected were subjected to statistical analysis by M-ANOVA and post hoc comparison by Duncan test. All results with p values less than .05 obtained were considered statistically significant.

RESULTS

All children assumed the prescribed treatment with *B. clausii* spores without any side effect. IL4, typical Th2 cytokine, levels significantly diminished after treatment ($p < 0.001$).

B. clausii significantly increased IFN γ , Th1 cytokine, levels ($p < 0.05$). Moreover, the treatment induced a significant increase of IL12, cytokine produced by antigen presenting cells and inducing Th1 polarization ($p < 0.001$).

TGF β , whose source is the putative Th3 lymphocytes, levels were significantly augmented by *B. clausii* ($p < 0.05$).

Finally, IL10 cytokine, released by Tr1 cells, were significantly increased by the treatment ($p < 0.05$).

The other evaluated cytokines (IL1, IL3, IL6 and IL8) were not significantly modified by *Bacillus clausii* (data not shown).

DISCUSSION

Respiratory and gastrointestinal infections in children constitute a significant burden for society in general and exert a critical impact on families. This issue is particularly crucial for children attending day care centers as they are at elevated risk of having infections. In addition, allergic children are some more prone to have infections because of a reduced immune response to fight microorganisms, mainly viruses, because of Th2 polarization.

The prevalence of allergic diseases has been increasing progressively in western societies (14). Altered hygiene and nutrition may be candidate as possible factors causing this "allergic epidemic" (14). The hygiene hypothesis conceives the quick increase in allergy to be related to reduced exposure to

microbes at an early age (19). There is evidence suggesting that delayed maturation of humoral immune defence mechanisms, particularly of circulating IgA- and IgM-producing cells, is a consequence of delayed compositional development of the gut microflora (20). In addition, antibiotic use during infancy may quantitatively and qualitatively disturb the intestinal microflora and thereby prevent postnatal Th1 cell maturation, thus resulting in a Th2-polarized immune deviation (21).

In fact, the gastrointestinal tract is colonized with more than 10¹⁴ microorganisms that weigh more than 1 Kg, which thus suggests that the intestinal microflora is quantitatively the most important source of microbial stimulation and may provide a primary signal for inducing the postnatal maturation of Th1-immunity (22).

Th2 polarization is associated with abundant release of some cytokines, including IL4, IL5, and IL13, that enhance the production of specific IgE and the eosinophil accumulation and activation: consequently constitutes a hallmark of allergic disease (23).

Particularly, IL4 is a prominent cytokine in allergic rhinitis and asthma not only on account of its proinflammatory role, but also due to its effects on mucus hypersecretion and airway of inflammatory cells such as mast cells and eosinophils (24). In vitro studies have confirmed that IL4 has direct effects on

epithelial and fibroblast function. IL4 enhances mucin expression by epithelial cells and promotes release of GM-CSF and IL8 from epithelial cell lines; IL4 also stimulates eotaxin release from fibroblasts (24).

As Th2 cytokines have a pivotal role on development of allergic inflammation, there is accumulating evidence that Th1 cytokines, such as IFN γ and IL12, suppress and counteract this Th2 response and vice versa (25). Thus an imbalance between Th1 and Th2 cytokines is thought to underlie various allergic disorders. The suppressive effects of IFN γ have been shown to be mediated by various mechanisms, such as inhibiting Th2 cytokine production and skewing the differentiation of naive T cells toward Th1 subtype preference (26). A recent study demonstrated persistent hyperresponsiveness and inflammation of airways in sensitised IFN γ -KO mice after allergen challenge, whereas these responses were transient in normal animals (27). In addition, exogenous IFN γ protein could reverse the ongoing reactions (27). Thus, there is considerable evidence supporting mechanisms whereby exogenous IFN γ may play a regulatory role in the extent of airway responses to allergen. Some of the mechanisms that may be relevant include suppressing the release of Th2-type cytokines from activated T cells, suppressing differentiation of naive cells to Th2 subtypes, facilitating apoptosis of T cells and eosinophils, suppressing

local recruitment of eosinophils, and inducing nitric oxide production, which might suppress contraction of airway smooth muscle (27). Therefore, IFN γ clearly exerts a crucial role to reverse ongoing allergic reaction.

Probiotics may provide a microbial stimulus for the physiologic maturation of the GALT. The probiotic effects are attributed to restoration to normal of increased enteric permeability and unbalanced intestinal microecology, improvement of the gut immunological barrier functions, control of the intestinal inflammation, and reduced production of proinflammatory cytokines (10).

Specific strains of the gut microflora have been demonstrated to contribute to the generation of counter-regulatory Th1- and Th3- type immune responses, thus creating an optimal situation to reverse the polarised immunologic memory to a healthy balance (28).

Even though the exact mechanisms by which probiotics may affect immune response remain speculative, there is increasing evidence that specific input from faecal flora to the innate immune system is essential for the establishment and maintenance of mucosal immune tolerance (29). Particularly, the Toll-related proteins recognise specific microbial components and induce production of Th1 cytokines through a process dependent on nuclear factor- κ B (NF- κ B) (30). In this regard, probiotics have been demonstrated to induce such an NF- κ B-mediated response (31).

Although this effect alone might suggest a direct link with reduced Th2-response, it has become evident that the Th1/Th2 paradigm seems to be not completely exhaustive to explain mucosal immune responses. Specific oral tolerance is critically dependent on inhibition of potential lymphocyte reactivity, and two recently recognised suppressor-cell populations are pivotal in this process. Th3 and T regulatory (Tr1) cells, which produce TGF β and IL10, respectively, have been shown to downregulate mucosal inflammatory responses (29). An obligatory input from the normal flora

controls the generation of regulatory lymphocytes, maintaining oral tolerance, through IL10 and TGF β release (32).

Such specific links between innate and adaptive immunity in the gut suggests the important role exerted by initial gut colonisation in maturing immune function (15).

IL10 is a cytokine critical for the maintenance of immune tolerance. It inhibits pro-inflammatory cytokines, including IL1, IL4, IL6, and TNF α , growth factors, adhesion molecule expression, and chemokine production by inflammatory cells (33). Thus, this regulatory activity exerted by IL10 has led to the concept that IL10 might be beneficial in mitigating allergic inflammation.

Similar to IL10, TGF β is an inhibitory cytokine that exerts a wide range of biological functions, mainly concerning the suppression of the immune system in vivo and the determination of immune tolerance (34). TGF β orchestrates events vital to the initiation, progression, and resolution of inflammatory responses. The termination of the inflammatory response occurs by inhibiting proliferation of many cell types, including lymphocytes (35).

Particularly, TGF β blocks T cell activation by modulating antigen presenting cell function, inhibiting antigen-induced eosinophilic inflammation, and down-regulating the switch to IgE synthesis (35).

In this regard, it has been reported that the systemic administration of recombinant TGF β 1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice associated with reduced serum IgE levels and down-regulation of IFN γ production from the splenocytes (36). Moreover, TGF β stimulates the synthesis of secretory IgA by gut epithelial cells (37). This evidence is very important as it allows of explaining previous findings showing the ability exerted by *B clausii* of increasing IgA production (38).

Bacillus clausii spores have been demonstrated safe at any age and effective in the treatment of several disorders (16). Particularly, Novelli and coll., in an open study, showed prevention of recurrent respiratory infections in children (39).

Galli and coll. reported a beneficial effect in children with recurrent otitis (40).

Moreover, *B. clausii* has been shown to exert widely documented health effects especially on immune system. Its immuno modulating activity concerns both specific stimulation of production of secretory IgA in humans (38) and non-specific enhancement of IFN γ synthesis in animals (41). In addition, normal subjects treated with spores of *B. clausii* showed an increased percentage of T lymphocytes positive for HLA-DR as a marker of T cell activation (42).

Our findings suggest that *B. clausii* administration in allergic children with recurrent respiratory infections may exert some immuno-modulating effects.

Firstly, there is a reverse of Th1/Th2 ratio: IL12 increased production stimulates Th1 function as evidenced by the augmented IFN γ levels in nasal lavage fluids. Moreover, the stimulated Th1 response causes a reduction of IL4 synthesis as documented in children after *B. clausii* administration. In addition, *B. clausii* should affect the putative Th3 and Tr1 cells as provided by increased levels of TGF β and IL10, respectively.

Of course, this study was performed on a very small cohort of children and the scheduled time of administration and observation was limited. On the other hand, we investigated the *B. clausii* effects at the level of the target organ, such as the upper airways, in children with a history of recurrent respiratory infections. These data are supported by the concept of the recirculation of immunocompetent cells as the *B. clausii* administration occurred per os.

Anyway, our findings provide possible mechanisms of action exerted by *B. clausii* in stimulating immune functions and suggest that this microorganism might help to prevent respiratory infections, supporting the outcomes reported by previous studies (39,40,43).

Further studies are necessarily required to confirm this hypothesis, enrolling wide cohorts of children and using long term administration schedules.

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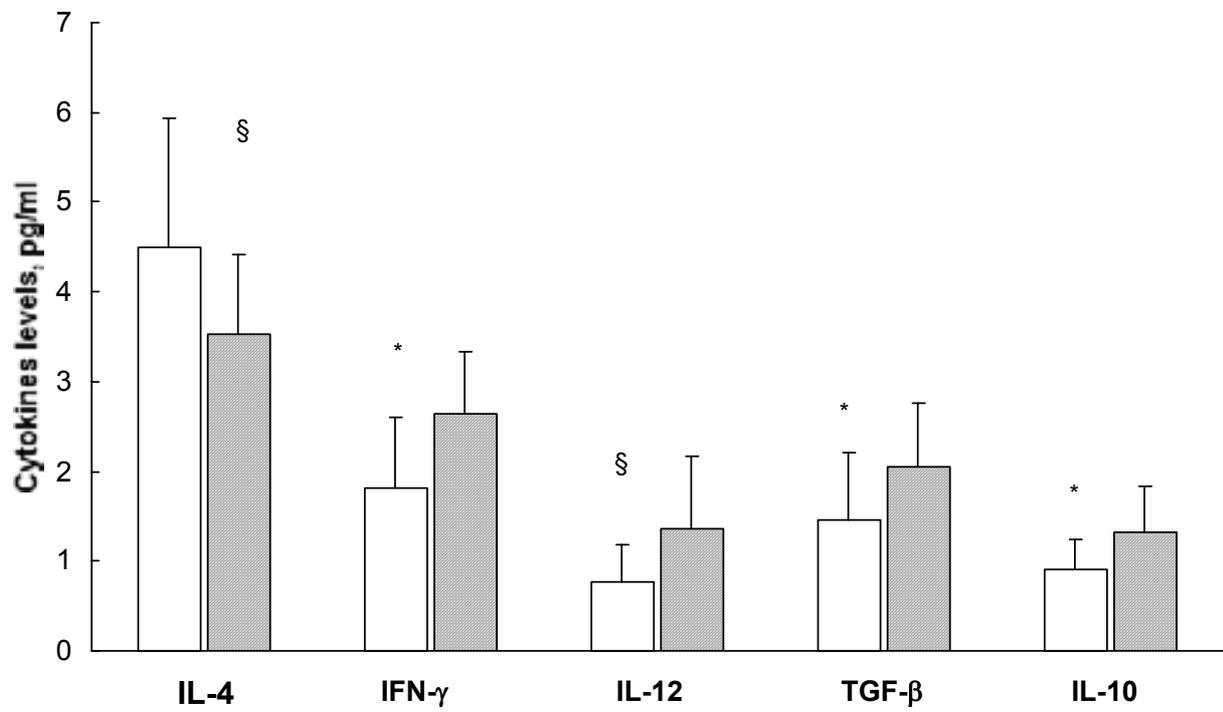
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FIGURE LEGEND

Figure 1: Cytokines at baseline and after treatment. Data are expressed as means + SD.

§ = p<0.01; * = p<0.05



INTERNATIONAL EVALUATION OF PROBIOTICS IN FOOD

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The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have recently collaborated to establish guidelines for the use of the term 'probiotic' in foods and levels of evidence necessary to make a health claim. Their recommendations are being considered by Codex Alimentarius, the international food standard setting body, at its specific committees, in terms of labelling and claims for foods.

The beneficial effects of food with added live microbes (probiotics) on human health, and in particular of milk products on children and other high-risk populations, are being increasingly promoted by health professionals. It has been reported that these probiotics can play an important role in immunological, digestive and respiratory functions and could have a significant effect in alleviating infectious disease in children.

As there were no international consensus on the methodology to assess the efficacy and the safety of these products, it was considered necessary to hold a consultation to evaluate and suggest general guidelines for such assessments. FAO and WHO convened a Joint Expert *Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food*, in October 1-4, 2001, Cordoba, Argentina. The Consultation evaluated the latest information and scientific evidence available on the functional and safety aspects of probiotics, as well as the methodology to assess such aspects, by bringing together worldwide scientific experts in the field.

The Consultation agreed that the scope of the meeting would include probiotics and prebiotics in food, and exclude reference to the term biotherapeutic agents, and beneficial micro organisms not used in food. The Consultation redefined probiotics for the purpose of the meeting as 'Live micro organisms which when administered in adequate amounts confer a health benefit on the host' but restricted its scope to discussion of 'Live micro organisms which when consumed in adequate amounts as part of food confer a health benefit on the host'.

Main topics addressed were:

- Properties of probiotic strains and their assessment
- Probiotic product specifications, quality assurance and regulatory issues
- Safety and beneficial human health effects

The experts agreed that adequate scientific evidence exists to indicate that there is potential for the derivation of health benefits from consuming food containing probiotics. However, it was felt that additional research data are needed to confirm a number of these health benefits in humans, applying a systematic approach and following recommendations for the assessment of probiotics suggested in the report.

There is good evidence that specific strains of probiotics are safe for human use and able to confer some health benefits on the host, but such benefits cannot be extrapolated to other strains without experimentation.

The health benefits for which probiotics can be applied include conditions such as gastrointestinal infections, certain bowel disorders, allergy, and urogenital infections, which afflict a large portion of the world's population. The application of probiotics to prevent and treat these disorders should be more widely considered by the medical community.

In addition, there is emerging evidence to indicate that probiotics can be taken by otherwise healthy people as a means to prevent certain diseases and modulate host immunity.

The regulatory status of probiotics as a component in food is currently not established on an international basis. In only a few countries, regulatory procedures are in place or sufficiently developed to enable probiotic products to be allowed to describe specific health benefits.

Specific recommendations were:

1. Potential probiotic strains must be identified by methods including internationally accepted molecular techniques and named according to the International Code of Nomenclature, and strains should preferably be deposited in a reputable internationally recognized culture collection.

2. In order to be termed a probiotic, the probiotic micro organism must be able to confer defined health benefits on the host, as outlined in the Report of the Consultation, in the actual product vehicle that will be made available to humans.
3. There is a need for refinement of *in vitro* and *in vivo* tests to better predict the ability of probiotic micro organisms to function in humans.
4. There is a need for more statistically significant efficacy data in humans.
5. Good manufacturing practices must be applied with quality assurance, and shelf-life conditions established, and labelling made clear to include minimum dosage and verifiable health claims.
6. The regulatory status of probiotics as a component in food has to be established on an international level.
7. The Consultation recommends that a regulatory framework be established to better address issues related to probiotics including efficacy, safety, labelling, fraud and claims.
8. Probiotic products shown to confer defined health benefits on the host should be permitted to describe these specific health benefits.
9. Surveillance systems, including trace-back and post marketing surveillance should be put in place to record and analyze any adverse events associated with probiotics in food. Such systems could also be used to monitor the long-term health benefits of probiotic strains.
10. Efforts should be made to make probiotic products more widely available, especially for relief work and populations at high risk of morbidity and mortality.
11. Further work is needed to address criteria and methodologies for probiotics.

The full Report of the Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food may be found at: ftp://ftp.fao.org/es/esn/food/probio_report_en.pdf

In addition, the Consultation recognized that there was a need for guidelines to set out a systematic approach for the evaluation of probiotics in food leading to the substantiation of health claims. Consequently, a Working Group was convened by FAO/WHO to generate guidelines and recommend criteria and methodology for the evaluation of probiotics, and to identify and define what data need to be available to accurately substantiate health claims. The aims of the Working Group were to identify and outline the minimum requirements needed for probiotic status. Guidelines were prepared to meet this objective.

II) Guidelines for the Evaluation of Probiotics in Food

As a follow up to the Expert consultation, Guidelines were prepared by a Joint FAO/WHO Working Group in 2002. An assessment scheme for probiotics was developed. The Guidelines addressed the following aspects: genus/species/strain identification; *in vitro* tests to screen potential probiotics; safety considerations; *in vivo* studies for substantiation of effects; health claims and labelling. The FAO/WHO Working Group report recommended use of the Guidelines as a prerequisite for calling bacterial strains “probiotic” and also recommended allowing specific health claims on probiotic food in cases where scientific evidence existed, as per the Guidelines. The Guidelines are a possible model for scientific criteria for the evaluation of health claims, as part of the science-based risk assessment process and not a management recommendation.

In order to claim that a food has a probiotic effect, the Guidelines set forth call for:

1. Speciation of the bacteria must be established using the most current, valid methodology. It is recommended that a combination of phenotypic and genetic tests be used.
2. Nomenclature of the bacteria must conform to the current, scientifically recognized names
3. It is recommended that all strains be deposited in an internationally recognized culture collection.
4. *In vitro* tests are critical to assess the safety of probiotic microbes

In addition, requirements for proof that a probiotic strain is safe and without contamination in its delivery form should be met. In recognition of the importance of assuring safety, even among a group of bacteria that is Generally Recognized as Safe (GRAS), the Working Group recommends that probiotic strains be characterized at a minimum with the following tests:

1. Assessment of lack of infectivity by a probiotic strain in immunocompromized animals would add a measure of confidence in the safety of the probiotic
2. In some cases, animal models exist to provide substantiation of *in vitro* effects and determination of probiotic mechanism. Where appropriate, the Working Group encourages use of these prior to human trials.
3. No adverse effects related to probiotic administration should be experienced when food is considered. Adverse effects should be monitored and incidents reported.

The Working Group recommends that information accumulated to show that a strain(s) is a probiotic, including clinical trial evidence be published in peer-reviewed scientific or medical journals. Furthermore, publication of negative results is encouraged as these contribute to the totality of the evidence to support probiotic efficacy.

Specific Recommendations were:

- Adoption of the definition of probiotics as ‘Live micro organisms which when administered in adequate amounts confer a health benefit on the host’.
- Use and adoption of the guidelines in this report should be a prerequisite for calling a bacterial strain ‘probiotic’.
- Regulatory framework to allow specific health claims on probiotic food labels, in cases where scientific evidence exists, as per the guidelines set forth in this report.
- Promotion of these guidelines at an international level.
- Good manufacturing practices (GMP) must be applied in the manufacture of probiotic foods with quality assurance, and shelf-life conditions established.

- Further development of methods (*in vitro* and *in vivo*) to evaluate the functionality and safety of probiotics.

Currently in most countries, only general health claims are allowed on foods containing probiotics. The Working Group recommends that specific health claims on foods be allowed relating to the use of probiotics, where sufficient scientific evidence is available, as per the Guidelines set forth in this report.

When a claim is made for a probiotic altering a disease state, the claim should be made based on sound scientific evidence in human subjects.

The Guidelines for the Evaluation of Probiotics in Food may be found at:

<ftp://ftp.fao.org/es/esn/food/wgreport2.pdf>

III) International Perspectives

The Consultation considered the need for specific and substantiated health claims for probiotics, and that its recommendations were especially relevant in relation to the Draft Guidelines for Use of Health and Nutrition Claims, at present being elaborated in Codex Alimentarius, by providing a framework for the evaluation of probiotics from the scientific point of view. Management and regulatory aspects would be dealt by individual countries, and at the international level, within the relevant Codex Alimentarius Committees. The recommendations should be discussed in detail at the national level and that their possible use in the framework of the criteria for the scientific basis of health claims be developed at international level, such as Codex Alimentarius.

The Problem of Bacterial Vaginosis and its Complications including HIV.

Running title: Bacterial vaginosis is associated to vaginal lactobacilli depletion and impairment of host mucosal defence.

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Introduction.

Bacterial vaginosis (BV) is an extremely common problem for women and is associated with adverse pregnancy outcomes and HIV infection. BV is an alteration of the human vaginal flora, the main hallmark of this disorder is the destruction of lactobacilli colonization and overgrowth of many facultative and/or anaerobic micro-organisms, especially *Gardnerella vaginalis* (1). BV affects approximately 10-20% of European, 10-40% of US and 20-60% of African women. BV is associated with several adverse pregnancy outcomes including low birth weight (LBW), and preterm delivery (PTD). PTD and LBW are the main risk factors for neonate morbidity and mortality. In addition BV is associated with increased risk of upper genital tract infections (PID), post surgical infections, and urinary infections. Presently the most concerning BV consequence is its association with HIV sexual transmission (2-7).

The pathogenesis of BV is obscure; most women do not show inflammatory signs in spite of a massive alteration in the microbial colonization of the vaginal mucosa. It is not known what provokes the shift from the normal lactobacilli flora to the anaerobic colonization, and it is not yet established if the decrease of lactobacilli colonization is a cause or a consequence of the anaerobic overgrowth. The only so far characterized adaptive immunity response in BV is vaginal IgA against the *G. vaginalis* hemolysin (a pore forming toxin) (anti-Gvh IgA) (8). Our goal is to disclose the relationships of microbial released virulence factors and local immunity with severity of BV.

Patients and Methods.

We analysed pregnant and non pregnant women with BV and evaluated the levels of anti-Gvh IgA, interleukins, and microbial enzyme activities in vaginal fluid as described (9-11).

Results.

In the white European population BV is a low risk factor for LBW (OR 1.5; 0.9-2.6) and is not a risk factor for PTD (OR 0.9; 0.4-1.7). No one of the women with BV having a strong anti-Gvh IgA response (9% of all BV) had LWB or PTD. However sialidase activity, which is a BV-associated microbial enzyme, is associated with adverse outcomes: the OR is 2.2 (1.4-3.6) for LBW and 1.4 (0.8-2.5) for PTD. At the highest level of sialidase activity the risk increases: the OR is 3.7 (0.9-15.1) for LBW and 2.5 (0.4-13.8). All women with the highest level of sialidase activity are BV positive; in addition they are not able to elicit a strong anti-Gvh IgA (9).

Conclusions.

Our data indicate that the ability of the host to elicit a strong specific IgA at vaginal levels is protective against colonization of the upper genital tract. It is the balance between the ability of the microorganisms to produce specific enzymes and/or toxic factors and the capability of the host to neutralize them that determine the risk of adverse pregnancy outcomes more than the amount of vaginal microbial colonization *per se*. BV positive women can be subdivided in two extremes: 1) women with high levels of sialidase activity which are at high risk of adverse pregnancy outcomes and should be considered for therapeutic treatment, 2) women able to elicit a strong vaginal anti-Gvh IgA response, which are fully protected by their mucosal immune system and are not at risk.

The vaginal anti-Gvh IgA response is associated with vaginal innate immune factors, specifically neutrophils, interleukin (IL)-8 and IL-1beta concentrations in the vaginal fluid (11, 12). However vaginal IL-1beta levels are higher in the BV positive women, whereas IL-8 and neutrophils are not statistically more elevated than in healthy women. Such findings suggest that in BV positive women the vaginal mucosa strongly reacts to fight anaerobic micro-organisms colonization (by high IL-1beta rise), however BV-associated factors dampen IL-8 rise, this may explain the absence of neutrophils recruitment in most women with BV (13, 14). This finding supports the notion that an impairment of the inflammatory response and innate defence system occurs in a subgroup of women with BV showing low anti-Gvh IgA. The failure of innate immunity associated with the impairment of local immunoglobulin response may be responsible for the higher susceptibility to viral and upper genital tract infections. Indeed a recent study demonstrated that women infected with HIV type 1 and positive for BV have 5-fold lower anti-gp 120 IgG titer and 5-fold lower total IgG concentration (15).

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Probiotics for the mother and child

Running title: Probiotics for the mother and child

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Summary

For the survival of humankind, nothing can be as important as the health of a mother and a child. As the world's population grows to more than 6 billion, it might seem ridiculous to suggest that any real threat exists to the human species. Diseases have long ravaged populations as have wars, poverty and malnutrition. Life today is no different with new and emerging diseases such as SARS and Mad Cow Disease leaving a trail of concern around the planet. All that being said, the AIDS crisis is threatening humans like no other. In countries such as Botswana, close to half the population is infected. Of great concern, the disease is now prevalent amongst women and teenage girls threatening not only their lives but those of their offspring. Efforts to control this spread are quite abysmal, albeit well-intentioned. Likewise, the death of a child every fifteen seconds from diarrheal disease is not being addressed with the same vigour as SARS, even although the risk of dying from the latter for most people is similar to being struck by lightning. In the end, it is the economy and politics that dictate health spending. Image and perception are everything. While deaths mount amongst women and children from AIDS and other infections, the potential to intervene with a low-risk natural concept of probiotics seemed all too distant. While evidence mounts of the attributes of certain probiotic strains to treat diarrhea and reduce the risk of urogenital and other diseases, the developing world has failed to embrace it, support its evaluation and implementation, and take it to where it is needed the most. In this review, the case for and against probiotics for urogenital and intestinal infections will be presented based upon current literature. The story is far from complete, but the potential for improving the health of the mother and child is significant. United Nations and World Health Organization Guidelines have now been developed to vanquish the unproven marketing hype products that have given probiotics a bad name. It is now up to science to deliver the remedies and society to make sure that only products reach the marketplace and the people in most need.

Introduction

Each year, an estimated one billion episodes of urogenital infection occur. This epidemic has largely gone unnoticed by governmental, pharmaceutical and scientific sectors of society. While figures for urinary tract infection (UTI), bacterial vaginosis (BV) and yeast vaginitis are difficult to acquire, rates of these and sexually transmitted

diseases show a serious upward trend. Not only are cases of chlamydia a concern but HIV infections are especially increasing amongst women (1-3).

In terms of child health, two factors transfer from the mother to the infant which have an impact of health. During pregnancy, the presence of BV in the mother corresponds to a higher risk of preterm birth (4) which brings with it an increased risk of fatal necrotizing enterocolitis in the newborn (5). If pregnancy runs to term, an abnormal vaginal microflora that is colonized by group B streptococci, HIV or other virulent organisms, will have implications for the baby's well-being (6,7), while depletion of lactobacilli may increase the risk of allergy development (8).

These problems are placing in jeopardy the very existence of some developing countries. For example, over 30% of the population of Botswana and 25% of young adult women in South Africa are infected with HIV (9,10). The death rate of infants within x weeks of birth is still high (over 100 per 1,000 births) in some parts of the world (<http://www.un.org/Depts/eca/divis/fssd/worldmor.htm>). Such figures clearly indicate that drastic measures need to be taken. The growing antibiotic resistance, the expense and lack of availability of drugs in many communities, and the failure or side effects of drugs make it necessary to consider other management approaches (11).

Urogenital infections

Bacterial vaginosis

BV is currently regarded as a depletion or loss of lactobacilli in the vagina, with colonization by Gram negative anaerobes, or in rare cases aerobes (12). BV is common in women of all age groups, often without any symptoms. When symptomatic BV occurs, it is associated with odor, discharge and an alkaline pH. Diagnosis can be achieved by detection of at least 20% of "Clue" cells (vaginal cells heavily colonized by Gram negative rods) in the squamous cell population on microscopic examination of a saline suspension of vaginal discharge, associated with two of the following;

- (1) anterior fornix vaginal pH equal or greater than 4.7
- (2) release of a fishy odour on addition of 10% KOH to the vaginal discharge
(positive "whiff test"), or
- (3) presence of an increased thin homogenous white vaginal discharge.

Another screening option uses a Gram stain of a vaginal swab specimen to generate a “Nugent” score, in which the presence of mainly gram positive rods (indicative of lactobacilli) is scored ‘normal’ and the presence of clue cells, gram negative rods and absence of lactobacilli is scored as ‘BV’ (13). Metronidazole is the agent of choice to treat BV, but recurrences are common (14). Often, BV is missed during diagnosis and patients use anti-fungal therapies believing the problem is caused by yeast (15).

Yeast vaginitis

Yeast vaginitis is common especially amongst black Americans. Diagnosis can be visual findings of white yeast mucus-like colonization, and dense presence of yeast cells in a wet microscope preparation. The discharge is characteristically white, and symptoms include vaginal and introital itching and irritation. *Candida albicans* is the major pathogen (around 85%) followed by *C. glabrata*, *C. krusei* and *C. tropicalis*.

Urinary tract infection

Between 25-50% females will suffer from UTI at some point in their life, and recurrences can be as frequent as 48% (16). Diagnosis usually requires 10^5 or more colony forming organisms per millilitre of urine but 10^3 organisms/ml can signal an infection if present with symptoms of dysuria, frequency of micturition, and occasionally haematuria (particularly terminal). Asymptomatic bacteriuria is very common. Gram-ve organisms, particularly *E. coli* (up to 85%) are the causative agents in most cases of UTI, followed by *Enterococcus faecalis* and *Staphylococcus saprophyticus*.

Gastrointestinal infections

The American Academy of Microbiology reports that around one child dies every 15 seconds in the world from diarrheal diseases, and there are an astounding 60 billion cases of these ailments each year. There are many causes of diarrhea, with rotavirus being the most common agent in children (17). Viruses such as Norwalk virus, astrovirus, calicivirus and enteric adenovirus are also important etiologic agents as well as toroviruses, coronaviruses, picobirnaviruses and pestiviruses (17, 18). Parasites such as *Strongyloides*, *Giardia* and *Cryptosporidium* are quite common especially in undernourished children (19), but *Campylobacter jejuni* and *C.*

coli are the leading causes of bacterial foodborne diarrhoeal disease throughout the developed world (19), and *Clostridium difficile* is the most common cause of antibiotic associated diarrhea (20). An estimated 20-50% of the 35 million people said to travel to developing countries each year suffer from traveller's diarrhoea caused by *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Vibrio parahaemolyticus* (in Asia), rotavirus (in Latin America), and protozoa (*Giardia*, *Cryptosporidium* and *Cyclospora* spp., and *Entamoeba histolytica*)(21). Such is the lethality of some intestinal pathogens that some of their virulence factors are being targeted for biological warfare and terrorism (22).

While diarrheal pathogens rarely cause urogenital infections, the impact of the disease can disrupt the mucosal immune system, thereby affecting the bladder and vaginal mucosa, and the rapid defecation of billions of indigenous organisms could increase the risk of the vaginal microflora being at least transiently imbalanced.

Probiotics

For the past 21 years and more, our group has been studying the role of indigenous bacteria in urogenital health. This has led to the development, testing and validation of lactobacilli strains to confer health benefits to women. Such therapy is regarded as probiotics, defined as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (23). In this mini-review, the potential role of probiotics in urogenital health and intestinal will be discussed.

Evidence For and Against Probiotics to reduce the risk of Urinary Tract Infections

A quality of life assessment has shown that women with urinary tract infections suffer from a significantly reduced quality of life, including reduced general perception of wellbeing, physical functioning, role limitation vitality, social functioning and increased pain (24).

Evidence For:

The evidence indicating a role for probiotics in reducing the risk of UTI originates from studies in 1915 by Newman (25), who showed that instillation of bacilli into the bladder could actually resolve some active infections. The concept, like much of the study of the urogenital microflora, was ignored during the antibiotic era when use of

pharmaceutical drugs was viewed as the ultimate solution for treating and preventing UTI. However, as time passed, antibiotic resistance (26), drug side effects including fixed drug eruption dermatitis (27), serious life threatening ones such as Steven's Johnson syndrome caused by sulfonamides (28), and failures of drug therapy (29) led to a re-examination of the disease starting in the late 1970s. While some people still recommend TMP/SMX and other agents at a daily dose for up to five years to reduce the risk of recurrences (30) others who have more closely examined the adverse effects strongly recommend its restricted use only for diseases such as *Pneumocystis carinii* pneumonia (31). This is particularly the case in HIV infected children (32), a problem in itself that is poorly managed in most developing countries. The dropping of sulfonamides has not solved the problem and trimethoprim resistance alone is increasing (33). In short, problems with drug therapy have led to a revised interest in alternatives such as probiotics.

The first evidence of indigenous bacteria such as lactobacilli protecting the host from ascension of urinary pathogens into the bladder came from studies in the 1970s which showed the predominant organism in the vagina of healthy women was lactobacilli, while those of women with recurrent UTI was the pathogen itself (34). More recent studies support this observation and hypothesis. An inverse correlation between the presence of hydrogen peroxide producing lactobacilli in a study of 140 women, indicated provides indirect evidence that the lactobacilli could protect against *E. coli* UTI (35). Studies on the lactobacilli strains that might be particularly effective were done in the early 1980s, and certain strains were shown to inhibit the growth and adhesion of uropathogens *in vitro* (36, 37). At the time, this was believed to be a critical step in identifying probiotic strains. However, more recently it has been recognized that no proven correlation exists, as yet, between *in vitro* properties and clinical benefits. Nevertheless, strains such as *Lactobacillus rhamnosus* GR-1, were selected and tested quite successfully in animal models of chronic and acute UTI (38,39). The protocols essentially had lactobacilli instilled into the bladder followed by direct intravesical challenge by *E. coli*. This is not the system that would be used in humans, whereby the lactobacilli would be inserted vaginally to prevent ascension of the pathogens into the bladder, but no such animal model exists for testing. These findings have quite recently been verified by a Japanese study using *L. casei* Shirota, in which a single intraurethral instillation of the lactobacilli 'dramatically' inhibited *E. coli* growth, inflammation and infectivity (40) The mechanism of action was reported to be due to a growth inhibiting product of lactobacilli, which administered intraurethrally at 100ug/mouse) gave a protective effect. Human studies followed in the late 1980s, in which *L. rhamnosus* GR-1 in a douche suspension was instilled into the vagina (41). This was followed by studies

using a gelatin capsule containing freeze dried lactobacilli instilled into the vagina (42). In both cases, the process did not result in any adverse events and did show an effect of reducing the risk of recurrence of UTI.

Oral use of probiotic lactobacilli has also resulted in reducing the risk of UTI as well as BV and yeast infections (43-46). The mechanism of action is likely multi-factorial and could include the ingested lactobacilli ascending from the rectal skin to the vagina, or causing a reduced pathogen ascension, or influencing the immune or host system in some other ways that reduces infectivity.

In post-menopausal women, UTI is common and coincidentally lactobacilli are only present in about one third of subjects (47, 48). The incidence of UTI is significantly reduced when vaginal estriol cream is applied (47) but only after oral premarin (49) not oral estriol (50). In the latter study, the authors noted that unless the lactobacilli population increased no reduction in UTI prevalence occurred. The reduction in UTI cases with premarin use is further supported by a Chinese study of 45 women, in which UTI incidence was significantly lower (2/27) than oral antibiotic (12/15), particularly when lactobacilli reappeared (from 0 to 59% reappearance)(51). Animal experiments have also shown that beta-estradiol therapy combined with *Lactobacillus fermentum* administration induced protection against uropathogenic *E. coli* challenge (52), supporting the conclusion of Raz et al. (50) that lactobacilli are needed for protection from UTI in post-menopausal women.

Evidence Against:

One randomized, placebo-controlled, double-blind Norwegian study of 47 women with recurrent UTI using twice weekly vaginal instillation of lactobacilli showed a six month infection rate of 1.41 in each group (53). However, given that the lactobacilli strain did not colonize the vagina, it is no surprise that no clinical impact occurred. A better study design would have been to follow women with low or absent lactobacilli presence and determine their incidence of UTI. If this was significantly higher than 1.41 over six months, it would imply that the indigenous lactobacilli presence in the group studied here had indeed reduced the recurrences of UTI.

The only other negative study was by the same group from Finland who subsequently achieved success in preventing UTI. In their earlier study, they found that 100ml of a drink containing *L. rhamnosus* GG five days a week did not reduce the rate of UTI versus controls (54). Here a specific probiotic drink was tested, while in the recent paper the findings were based upon 139 women without a history of UTI where they took a fermented milk

drink more than three times weekly versus 185 with acute UTI who took less than one such drink per week on average.

In summary, there is significantly more evidence for than against an effect of probiotics in reducing the risk of UTI.

Evidence For and Against Probiotics to reduce the risk of Vaginal Infections

A study from in Sweden in 1996 described “The silent suffering women”, referring to their finding that one quarter of 611 healthy young women (aged 19-25) studied reported symptoms of lower genital tract illness particularly itching, discharge and soreness associated with a disturbed vaginal flora depleted in lactobacilli, and in some cases to past *Chlamydia* infection (55).

Evidence For:

In vitro studies have indicated that certain lactobacilli strains can inhibit the growth and adhesion of candida (56, 57) yet lactobacilli were present in the vagina or most (72-88%) patients with candidiasis. Other *in vitro* studies have shown that certain lactobacilli strains produce H₂O₂, lactic acid and bacteriocins active against a primary cause of BV, namely *G. vaginalis* (58), implying an anti-infective role in BV. However, as stated above, this does not confirm clinical efficacy.

A strong link exists between absence of lactobacilli in the vagina and risk of sexually transmitted diseases. This has led to one call for promotion of ‘women-controlled prevention methods’ in addition to proactive detection kits for pathogens such as HIV (59). Convincing data comes from a recent study of 255 US women who had sex with an infected partner, where the absence of lactobacilli in the vagina correlated with a positive test for *Neisseria gonorrhoeae* (odds ratio 4.1) and *Chlamydia trachomatis* (OR 3.4) (60).

As stated above, evidence indicates that daily oral use of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 can benefit some patients with BV (43-45). Use of these strains also appears to reduce the risk of yeast vaginitis. Data on *L. crispatus* CTV05 is sparse, but web based reports suggest that if given post-metronidazole to prevent one month recurrence of BV, it can do so successfully when it colonizes the host.

The absence of lactobacilli is a predictor of preterm delivery at <33 weeks (61), but no studies have tested whether probiotic use can delay or prevent preterm birth.

Evidence Against:

In patients with candida infections in the vagina, there is little evidence to suggest that lactobacilli can be effective in eradicating the yeast. In one study of 1110 cervico-vaginal smears from premenopausal women, the prevalence of lactobacilli (85%), but this did not correspond to any evidence of inhibition of candida or reduced presence of candida (62).

One mouse study showed that commensal lactobacilli supported the growth of *Neisseria gonorrhoeae*, by promoting solubilization of iron on mucosal surfaces (63). This is contrary to findings showing a potential role of lactobacilli in protecting the host against this pathogen, but emphasizes that further studies are necessary.

Evidence For and Against Probiotics to treat and prevent Diarrhea

Evidence For:

The Food and Agriculture Organization of the United Nations and the World Health Organization have concluded that there is sufficient data to indicate that certain probiotic organisms are able to provide significant clinical benefits to patients at risk or, or suffering from, diarrhea (23). Without repeating the extensive data on this subject, reviewed in depth elsewhere, some examples of studies are worthy of citation.

While it is difficult to verify that all subjects are equally exposed to pathogens, one such study of 204 undernourished children 6 to 24 months old in Peru, showed that once daily intake of *L. rhamnosus* GG six days a week for 15 months led to significantly fewer episodes of diarrhea (5.21 episodes diarrhea/child/year v 6.02 in placebo group; P = .028)(64).

The strongest evidence that probiotics can alleviate acute diarrhea comes from studies primarily using three strains, *L. rhamnosus* GG, *B. bifidum* and *L. reuteri* SD2222 (65-67). The strength of the evidence comes from the randomized, double blind, placebo control design of the studies. The statistically significant reduction in duration of diarrhea is consistent, especially for the *L. rhamnosus* GG strain (68,69). Even in low birth weight premature infants, the use of *Lactobacillus acidophilus* and *Bifidobacterium infantis* has been found to reduce not only cases of necrotizing enterocolitis but also death (70). Additional studies in patients with inflammatory bowel diseases such as pouchitis, have shown that a combination of 8 strains of lactobacilli, streptococci and bifidobacteria can reduce

recurrences (71). In some cases a yeast strain of *Saccharomyces boulardii* has been used with some effects in patients with *C. difficile* infections (72).

Evidence Against:

The main source of criticism of probiotics has been a gradually decreasing number of physicians, some of whom prefer pharmaceutical interventions and are reluctant to consider a food or dietary supplement alternative. As more and more evidence about probiotic clinical benefits is generated, this innate bias may dissipate. Having said that, the medical community is right to question the methodologies of clinical studies, and to that end more trials are needed using probiotics to show specific mechanisms of action for specific strains against infecting agents.

A prospective, randomized, double-blind, placebo-controlled trial of twenty patients who had a previous history of pouchitis and endoscopic inflammation, showed that *L. rhamnosus* GG b.d. for 3 months increased the ratio of total faecal lactobacilli to total faecal anaerobes ($P = 0.03$), but did not alter the mean pouchitis disease activity index (73).

The GG strain was shown to colonize a portion of preterm infants of less than 2000 g birth weight neonates. Infants received the lactobacilli twice daily for up to 21 days, and while colonization occurred in 5/24 cases (21%) of babies under 1500g and 11/23 (47%) of larger infants (74), implying the potential to prevent necrotizing enterocolitis. However, while one week of GG therapy decreased the incidence of NEC (1.4 vs. 2.7%) in another study, the level did not reach statistical significance (75).

A study of 45 Crohn's patients treated with strain GG or placebo for 12 months showed no differences in endoscopic and clinical remission (76).

A controlled pilot study showed that 3 weeks supplementation of elderly people with milk fermented with yoghurt cultures and *L. casei* DN_114 001 did not significantly reduce the incidence of winter infections (gastrointestinal and respiratory), but duration of disease was significantly lower in the treatment group (7.0 3.2 days, n=180) than in the control group (8.7 3.7 days; n=180) ($p=0.024$)(77).

A double-blind, placebo-controlled, cross-over, four-week trial of *L. plantarum* 299V in 12 previously untreated patients with irritable bowel syndrome, showed no effect on symptom score or median hydrogen production (78).

In short, there have been clinical failures of probiotic therapy. This is not necessarily unexpected or restricted to the strains referenced here. Rather, it illustrates the need to carefully select strains and product formulations for specific health targets, and understand how they work or fail. In this way, scientific progress will continue and untested or unproven products will hopefully either get out of the market or undertake the research necessary to fulfill the FAO/WHO Guidelines discussed below.

Is there a Role for Oral and Vaginal Probiotics in Pregnancy?

Figure 1 is a rather complicated diagram designed to illustrate the potential for probiotics to reduce the risk of preterm labour. Studies have shown that oral administration of *L. rhamnosus* GG is safe during pregnancy. The question is can oral and/or vaginal probiotics alter the process which appears to involve inflammatory mediators perhaps induced by pathogens such as *Gardnerella*, *Prevotella*, *E. coli* and others at the mucosal surfaces of the vagina and cervix (80-82). This inflammatory process can then stimulate cyclooxygenase expression leading to prostaglandin release and preterm labour (83). Arachidonic acid production by anaerobes such as *Fusobacterium* also can stimulate the prostaglandin release through liberation of phospholipase (84).

The potential for remediation of this process by probiotics is illustrated in several ways. Studies have shown that lactobacilli can stimulate IL-10 and IL-12 production in the gut (85), a process that can inhibit 1₂-matrix metalloproteinase (MMP) synthesis (86), while MMP-2 stimulated by IL-8 can be inhibited by modulation of IL-10 (87). Studies with *L. paracasei* NCC2461 have shown that it can induce the development of a population of CD4(+) T cells with low proliferative capacity that produced TGF β and IL $_{10}$ (88), while *L. rhamnosus* GG has been shown to induce NO production through the iNOS pathway (89), potentially influencing the Cox-2 pathway. An experimental colitis study has shown that *L. reuteri* R2LC reduced the protein content of inducible nitric oxide synthase by 50% and the median of the protein content of inducible cyclooxygenase by 30%, while *L. rhamnosus* GG reduced the median of inducible nitric oxide protein content by 40% and actually increased the median of inducible cyclooxygenase protein content by 30% (90). This illustrates the potential to block the Cox-2 pathway, but also suggests that a cocktail or strains may be required.

Three other steps might interfere with the preterm labour cascade. The production of collagen binding proteins by *L. fermentum* RC-14 (91) and stimulation of mucus production (92) could theoretically reduce MMP degradation. The displacement of pathogens on the vaginal surface by oral or vaginal lactobacilli use (15, 42-46, 93) could then reduce the adverse effects of the phospholipase production that stimulates prostaglandin release (94). Lastly, the increased production of sIgA by lactobacilli (56) and the bioconversion of linoleic acid into conjugated linoleic acid by adherent lactobacilli (95) potentially reducing the impact of the pathogen phospholipases completes the hypothetical illustration related to pregnancy. While no single study will determine the feasibility of the Figure 1 proposal, the potential to use probiotic strains to improve the health of the mother and the outcome of pregnancy is certainly worthy of further pursuit.

FAO/WHO Guidelines

The history of probiotics had a laudable beginning with Nobel Laureate Elie Metchnikoff, but sadly the legislative framework has not been created in most countries to ensure that only proven probiotic products reach the consumer. Thus, ridiculous claims are made by some companies selling so-called ‘probiotics’, others misuse the term and produce ‘probiotic aftershave’, while still others sell products containing dead organisms or contaminated contents. In an effort to ‘clean up’ the mess, and ensure that companies follow guidelines for creation and manufacture of probiotics, the FAO/WHO established a set of Guidelines for probiotics in food (96). If implemented properly by member nations, these guidelines will ensure that probiotic organisms are speciated properly, named and numbered so that evidence for their use in humans and animals, and their mechanisms of action can be followed in the scientific literature, and that consumers can be reassured of their quality at time of use in any given part of the world. One end result of such efforts will be to provide the medical community with reliable probiotics for evaluation in appropriate clinical settings. This will advance the field of science, nutrition and medicine and lead to a broader based use of these remedies, thereby resulting in larger profit margins for companies meeting the guidelines.

In Summary

There is growing evidence that the indigenous vaginal microbiota provides a protective barrier against various vaginal and bladder infections. Furthermore, the application of some strains of probiotic lactobacilli indicates that this protective effect can be delivered to women whose flora has been depleted of these organisms. After menopause, estrogen therapy will likely be needed to support dense lactobacilli colonization, although the reason why some women still harbour these strains needs to be investigated.

Future studies are not only needed to identify mechanisms of protection of lactobacilli, but also to determine if any indigenous organisms other than lactobacilli, have a protective function in the urogenital tract of pre-pubertal children and elderly women. Nevertheless, such is the enormity of the crisis facing many mothers, potential mothers and children especially but not exclusively in developing countries, that serious consideration must now be given to probiotics. The evidence of efficacy may not be as solid as would be preferred, but as HIV vaccines fail, as relief to communities is inadequate, and as people die unnecessarily, any contribution to human well-being surely is worthy of pursuit?

Probiotics have significantly more benefits than risks. Indeed, the risk of adverse effects is extremely small. The technology exists to deliver reliable dried and food preparations including as a dairy, nutritional product line able to reach outlying communities, making it feasible to improve the health of women and children in developing countries and poorer districts of developed countries. Access to oral and vaginal probiotics could provide a means of self-empowerment whereby individuals use them without requiring hospitalization, high costs or approval from sexual partners. If colonization of the intestine of a newborn by lactobacilli and bifidobacteria were to save one life, and colonization of the vagina of a woman by lactobacilli were to prevent one case of HIV, our contribution to humanity would indeed be great.

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Probiotics for Pregnancy – health of the mother and baby

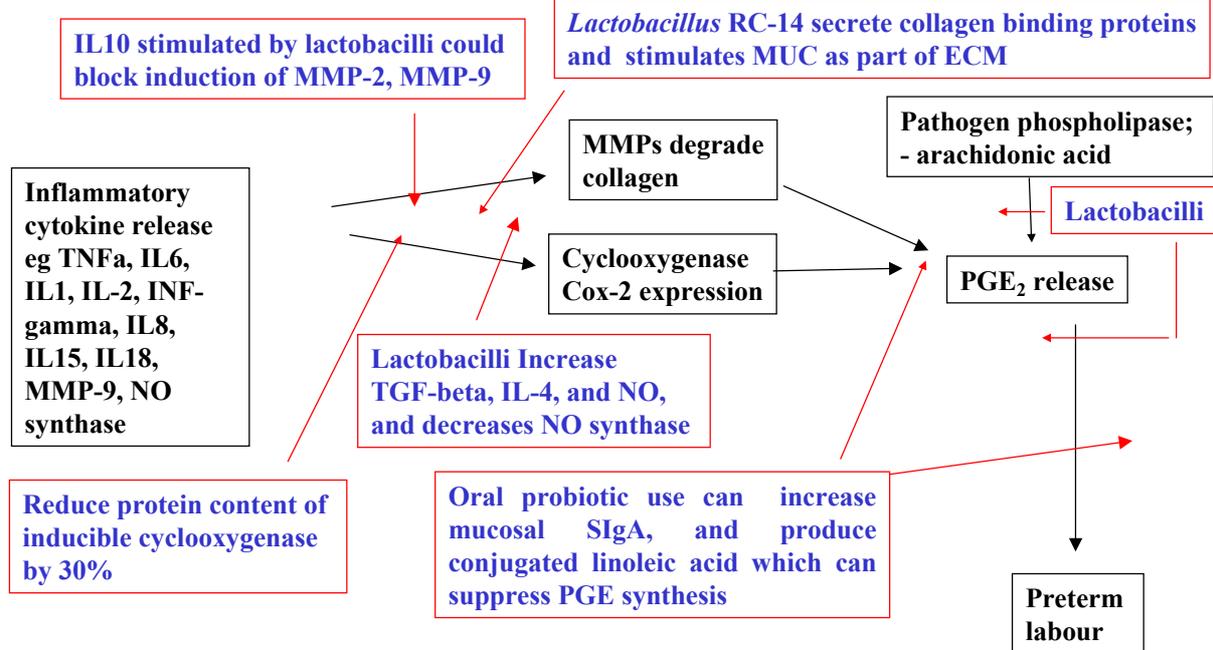


Figure 1. An illustration of the types of reactions that could lead to preterm labour via infection of the vaginal and cervical mucosa (black boxes and font), and the means by which probiotics could potentially interfere with this process (red boxes, blue font).

Diet and colon cancer - mechanistic studies with Min mice
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Epidemiological evidence strongly suggests that diets rich in plant derived foods, including grains, fruits, and vegetables, and low in red meat and fat are associated with a reduced risk for colon cancer. The mechanisms by which foods, nutrients or bioactive compounds promote or inhibit colon carcinogenesis are still poorly understood. Min mouse offers an excellent model to study the mechanisms of colon cancer *in vivo* because the genetic events initiating and promoting the tumourigenic process so closely mimic those in humans. Particularly the mutation in the *adenomatous polyposis coli (APC)* tumour suppressor gene is important in this regard because it is found in the majority of human sporadic colorectal tumours.

The tumour suppressing activity of the APC protein is largely due to its ability to regulate intracellular levels of β -catenin, an oncogenic transcriptional activator. Mutations in *APC* cause aberrant accumulation of cytoplasmic β -catenin and its translocation to the nucleus, where it induces expression of several genes critical for cell growth and cancer development, such as *cyclin D1* and *c-MYC*. Colon tumourigenesis also involves epigenetic changes in several other cell signalling pathways regulating cell proliferation, differentiation and apoptosis. These include p53, NF κ B, epidermal growth factor, cyclooxygenase-2 and many others. Dietary factors may affect cancer development either at the level of mutations or, more likely, at the different steps of these cell signalling pathways.

Due to the germline *Apc* mutation, Min mice develop dozens of intestinal polyps along the entire intestine within a few weeks. A number of dietary factors and pharmaceutical agents have already been shown to modulate tumour number and size in Min mice. Our studies with Min mice focus to understand how the above-mentioned cell signalling pathways are regulated by diet and, furthermore, how changes in these pathways are related to tumor formation in this animal model. As an example, results of the effects of dietary inulin on tumor development and cell signalling proteins in Min mice will be presented.

Short chain fatty acids and faecal bile acids in the characterization of intestinal ecosystem

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1. Introduction

In view of the steady increase in the proportion of Europeans over 60 years old the research community is challenged to contribute to improving the quality of life for this age group. An optimal nutrition for the specific needs of the elderly is an important factor in achieving this objective. In elderly individuals, faecal bifidobacterial counts are thought to show a marked decrease in comparison to those of younger people (1, 2, 3, 4). This may be relevant in the susceptibility of these individuals to pathogenic infection. Confirmation of a decline in bifidobacteria and other lactic acid bacteria (LAB) in the gut of ageing subjects open up the possibility of reversing such trends by administration of probiotic (bifidobacteria or lactobacilli), prebiotics that selectively encourage the growth of LAB in the intestine, or a combination thereof called a symbiotic.

The EU-funded project “CROWNALIFE-Functional foods, gut microflora and healthy ageing” aims to provide information on the structural and functional alterations of the microflora with ageing in Europe and to improve the health status of the ageing population via specific nutritional recommendations that will necessitate the design and provision of functional food ingredients that positively affect the intestinal microbiota.

The human intestinal microflora form an extremely complex ecosystem. Several approaches are possible for studying the intestinal microflora and its effects on and the relationship to the host. One of the approach to identifying and enumerating the bacteria is to study the metabolism of the microbial ecosystem. The metabolic activity of intestinal microbes is complex and the biochemical activities of the gastrointestinal microbiota may be more important to the host organism than their numbers in any particular site in the intestine (5).

Short-chain fatty acids (SCFAs) and secondary faecal bile acids constitute two groups of metabolites of the intestinal microbial activity which could be considered indicator of the status of intestinal microbiota.

1.1 Short-chain fatty acids

SCFAs are the main end-products of intestinal microbial metabolism in the gut and they are the major anions in human faeces (6). They are produced in the large bowel of non-ruminant mammals by bacterial anaerobic fermentation of substrates from the diet and substrates of endogenous origin. The major substrates from the diet are polysaccharides, of which resistant starch is the most important in terms of quantity. During the past 15 years, SCFAs in the large intestine have attracted considerable attention in studies of human nutrition, physiology and pathophysiology following the realization that the microbial production of these acids is an important mechanism for conserving carbohydrates and calories, and that they may play a role in various colonic diseases (7). Quantitatively the main SCFAs in the intestinal tract are acetic, propionic and butyric acids. All of these fatty acids have important functions in host physiology. Butyrate is almost completely consumed by the colonic epithelium, and it is a major source of energy for colonocytes (6). Acetate and propionate are found in portal blood and are eventually metabolised by the liver (propionate) or peripheral tissues, particularly muscle (acetate) (6,8). All three major SCFAs stimulate epithelial cell proliferation and differentiation in the large and small bowel in vivo (9). However, butyrate inhibits cell proliferation and stimulates cell differentiation in epithelial cell lines of neoplastic origin in vitro (10). Moreover, butyrate promotes reversion of cells from neoplastic to non-neoplastic phenotypes (11). Another important role of SCFAs is the regulation of the microflora. SCFAs are known to have antimicrobial effects. They may therefore be an important factor in establishing a balanced ecosystem in the gut, and may prevent colonization of pathogenic microbes, such as salmonella and shigella (12, 13). On the other hand, they promote the growth of some bacteria.

1.2 Faecal bile acids

Bile acids are the major end products of cholesterol metabolism and are synthesised in the liver. The primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are derived via several intermediate steps from cholesterol and secreted in bile as glycine or taurine conjugates. They serve as cholesterol solubilising agents by the formation of micelles, and play an important role in the digestion and absorption of lipids in the small intestine. The primary bile acids are subject to extensive metabolism by the intestinal microflora (14), predominantly 7- α -dehydroxylation, which converts cholic to deoxycholic acid (DCA) and chenodeoxycholic to lithocholic acid (LCA), thereby increasing their hydrophilicity. DCA is partly absorbed in the colon and enters the enterohepatic circulation where it is conjugated in the liver and secreted in bile; LCA is almost insoluble very little is reabsorbed. Both secondary bile acids are excreted in the stool and

make up to 95% of the total amount of excreted bile acids. These secondary bile acids are postulated to play an important role in the aetiology of colon cancer by acting as promoters of the tumourigenic process (15). It is also postulated that a high DCA concentration and DCA to LCA ratio may be a risk indicator of colorectal cancer (CRC) (16). However a recent study by the Kok found no significant correlations with either bile acid concentrations or ratios (17). Although there is no definite proof that bile acids are the cause of CRC, there is considerable evidence to indicate that acid steroids, in particular secondary bile acids, can exert a range of biological and metabolic effects. They induce cell necrosis, hyperplasia, metabolic alteration and DNA synthesis in intestinal mucosal cells, enhance the genotoxicity of a number of mutagens in *in vitro* assays, and exhibit tumor-promoting activity in the colon (18). Secondary bile acids can also induce DNA damage in colon cells, leading to apoptosis, as DCA induced DNA damage triggers calcium ion dependent apoptosis, in a manner independent of p53 (19). It has also be suggested that secondary bile acids influence CRC by selecting for apoptosis-resistant cells or potentially through bile acid interactions with important secondary messenger signalling systems know to be activated in CRC (arachidonic acid-prostaglandin E2 and PKC) (20). Serum levels of DCA are correlated with increased rates of mucosal proliferation, which is a know factor in CRC causation (21). Diet has an obvious and pronounced effect on bile acids as high levels of animal fat and protein increase both secretion and flow (22), but by itself diet cannot be considered harmful, unless in the absence of balancing amounts of carbohydrate. Fibre (ispaghula husk) has been shown to lower faecal levels of LCA and decrease DCA to LCA ratio (23).

2. Materials and methods

Fresh faecal samples were taken from 98 subjects (adults and elderly). Each samples was used to prepare the faecal water for the determination of SCFA and FBA concentrations.

FBA were analysed by mass spectrometry and SCFA by gas chromatography as reported by Dolara *et al.*(24).

3. Results

Fig. 1 shows the mean values of the concentration of the single acids and of the total SCFA in elderly and adult Italian subjects. For the three principle acids (acetic, propionic and butyric) and for the total SCFA the concentration values are always higher in adults than in elderly even if there isn't a significantly difference between the two groups.

The mean value of faecal pH was 6.73 for the elderly and 6.51 for the adults group.

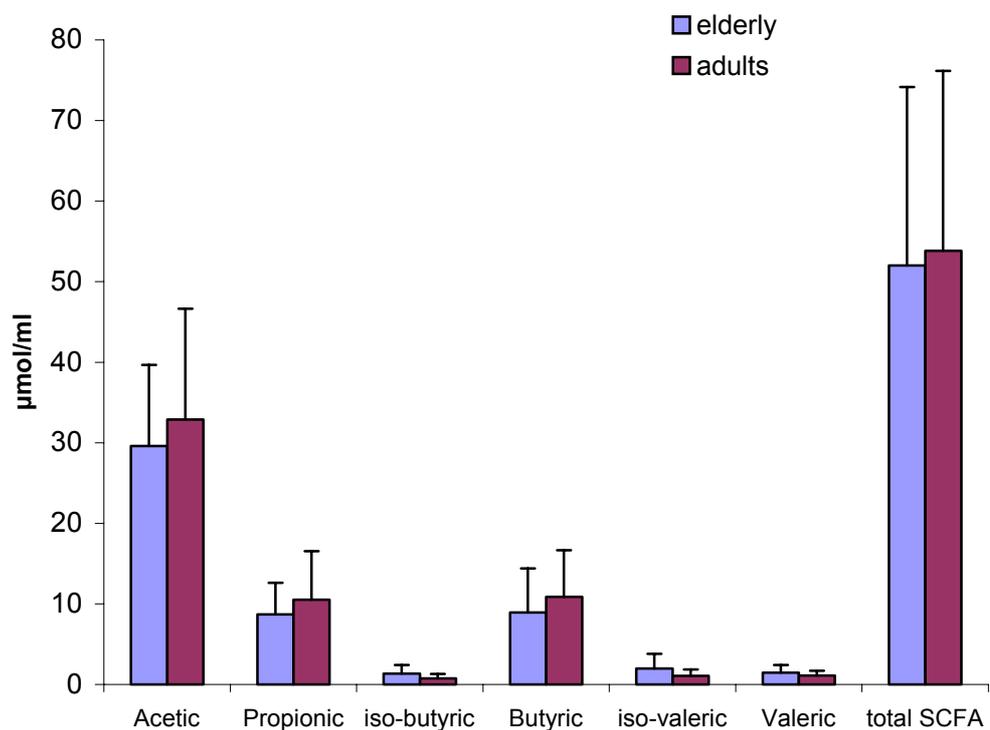


Fig. 1. Mean values of concentration of single acids and of total SCFA in elderly and adult Italian subjects.

The concentrations of faecal bile acids in elderly and adults subjects are reported in Fig. 2. Primary FBA, secondary FBA and total FBA are always higher in adults than in elderly and DCA and total secondary FBA concentrations are significantly different ($p < 0.05$) between the two groups.

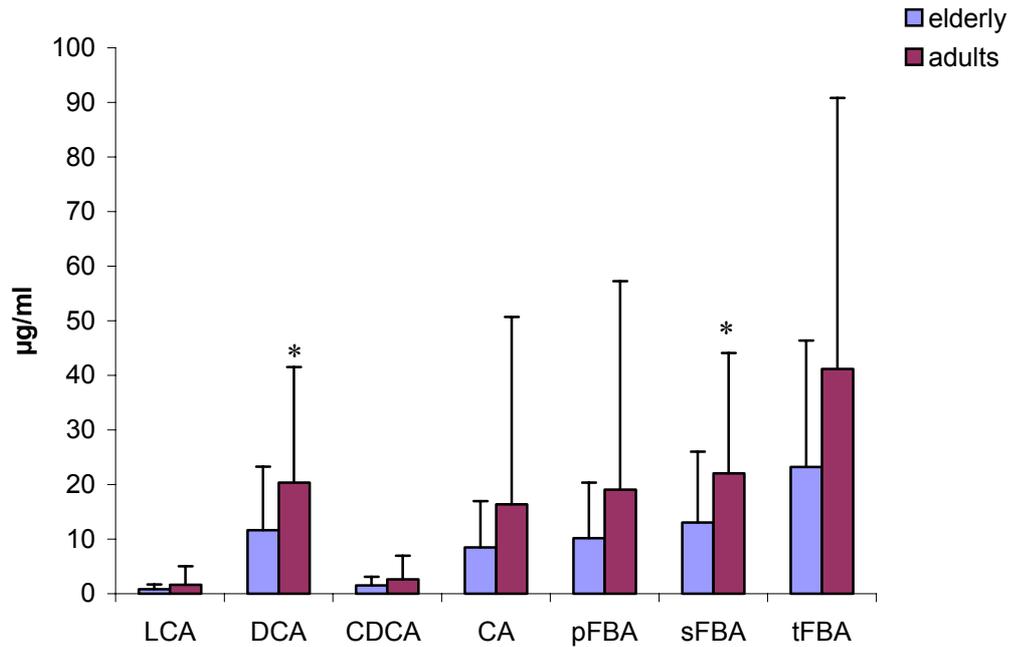


Fig. 2. Mean values of concentration of primary FBA – cholic acid (CA) and chenodeoxycholic acid (CDCA)-, secondary FBA – deoxycholic acid (DCA) and lithocolic acid (LCA)-, total primary FBA (pFBA), total secondary FBA (sFBA) and total FBA (tFBA) in elderly and adults group.
 * Significantly different from elderly group ($p < 0.05$)

Examining the mean value of pFBA/sFBA ratio (Table 1), we found a lower ratio in elderly than adults and it could indicate that in adults, even if we have a higher concentrations of pFBA, there is a lower dehydroxylation and conversion of pFBA into sFBA.

Table 1. Mean value of pFBA/sFBA ratio in elderly and adults group.

Group	pFBA/sFBA
Elderly	0.91
Adults	3.50

4. Discussion

In a world of rapidly changing food habits and stressful life styles it is more and more recognised that a healthy digestive system is essential for overall quality of life. One of the factors that is being recognised to be of major importance for the maintenance of a healthy digestive system is the colonic flora, especially its bacterial composition and the “nutrients” that it metabolises (25).

Short chain fatty acids are the main end products of anaerobic microbial metabolism in the human colon. Studies on the effect of butyrate, propionate and acetate on gut metabolism of patients and experimental animal models suffering from gut inflammation have shown that a sufficient and sustained level of SCFA may be essential for the maintenance of a healthy gut (26, 27). We observed that the SCFA concentration in elderly people is lower than in adults and these data could reflect a microflora composition change which could be associated to a higher incidence of intestinal disorders in elderly. The increase of colonic levels of SCFA might be beneficial for colon health since they determine a reduction in the colonic pH which inhibits bacterial 7- α -hydroxylase activity reducing the concentration of secondary bile acids. This action is supposed to be important because secondary FBA have demonstrated co-carcinogenic and co-mutagenic activity. Our data pointed out that in elderly there is a higher pFBA/sFBA conversion than in adults and this could be due to a modulation of intestinal microflora towards the anaerobic organisms capable of deconjugating the primary FBA to form secondary FBA, and to a higher intestinal pH.

Metabolic activities of the intestinal microflora can give rise, under specific conditions, to the formation of potential carcinogens. It is essential therefore, to have a through knowledge of dietary factors with a favourable effect on the composition and activity of intestinal microflora (28, 29).

The use of probiotics and prebiotics has become firmly established due to their beneficial effects at the nutritional and therapeutic level (30). Many authors have suggested that colon cancer could be influenced directly by reducing intestinal pH, thereby preventing growth by putrefactive bacteria. Feeding lactic acid bacteria will reduce the pH as they produce lactic and acidic acids. The lower pH will affect the metabolic activity of the intestinal flora, the action of bile acids and causing quantitative and/or qualitative alterations in the bile acid degrading bacteria. This will cause an environment unsuitable for many pathogenic bacteria which are linked in increasing the incidence of cancer. Certain strains of *Lactobacillus* are able to bind to the secondary bile acids, thereby reducing their bioavailability (31).

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SPROUTS FROM CEREAL SEEDS CONTAIN VERY POWERFUL ANTIOXIDANT MOLECULES

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The presence of biologically active substances in wheat sprouts has been previously investigated (Mancinelli et al, *Gastroenterology International*, 11, 50-51, 1998). Following this study we reported that sprouted wheat contains very high levels of two classes of molecules potentially helpful for nutritional use: a) protein kinases and phosphorylated compounds; b) redox enzymes and low molecular weight reducing substances.

In this report we show the research progress concerning the biochemical and functional characterization of the antioxidant compounds present in wheat and other cereal sprouts. Total antioxidant activity of wheat and spelt sprout extracts (prepared according to Falcioni et al, *JFS: Food Chemistry and Toxicology*, 67, 2918-2922, 2002) was measured by means of reaction with phosphomolibdic acid or potassium ferricyanide. The presence of -SH groups was also analyzed with DTNB (5,5'-Dithio-bis (2-nitrobenzoic acid)). The level of antioxidant compounds remarkably increases following the germination process. Wheat and spelt sprout extracts show a very potent radical scavenger activity against the superoxide anion generated from the xanthine-xanthine oxidase system. It is noteworthy that this scavenging activity appears quite thermo stable . A main component of the antioxidant compounds isolated from vegetal sprouts is represented by reducing glycosides. In particular the mass spectrometry analysis shows the predominant presence of an aglycon aromatic structure (MW 203) that can bind up to three hexose residues (MW $203+162=365$, $365+162=527$, $527+162=689$). The structure of the aromatic moiety is now studied by nuclear magnetic resonance (NMR). The research is also directed to carry out a methodology suitable to perform a quick isolation of large amount of sprouts antioxidant glycosides. In this perspective we perform the absorption of the substances containing aromatic structure by charcoal. Preliminary experiments show that the molecules with radical scavenging activity may be selectively eluted by ethanol at 70 °C. In conclusion a fraction with potent antioxidant activity, potentially employable in dietetic, cosmetic and pharmacological fields, may be isolated from cereals sprout extracts.

***Lactobacillus plantarum* LP01(PROBIAL) and *Bifidobacterium breve* BR03(PROBIAL) in the treatment of IBS: preliminary data.**

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According to Rome II criteria Irritable Bowel Syndrome (IBS) may be diagnosed on the presence of symptoms (Table 1) (1).

Table 1. Diagnostic criteria for IBS*

Twelve weeks**r more in the past 12 months of abdominal discomfort or pain that has two of three features:

- a. Relieved with defecation
- b. Onset associated with a change in frequency of stool
- c. Onset associated with a change in form (appearance) of stool

The following symptoms are not essential for the diagnosis, but when/if present they increase the confidence in the diagnosis and may be used to identify subgroups of IBS:

- a. Abnormal stool frequency (>3 /day or <3 day)
- b. Abnormal stool form (lump/hard or loose/watery stool) $>1/4$ of defecations
- c. Abnormal stool passage (straining, urgency or incomplete evacuation) $>1/4$ of defecations
- d. Passage of mucus $>1/4$ of defecations
- e. Bloating or feeling of abdominal distension $>1/4$ of days

*In absence of structural or metabolic abnormalities to explain symptoms

**The 12 weeks need not to be consecutive.

Some studies have shown that abnormal colonic fermentation may be an important factor in the development of symptoms in some patients with IBS (2). In man, the large gut receives material from the ileum which has already been digested and the contents are then mixed and retained for 6-12 hours in the caecum and right colon (3). Thus, the large intestine is an open system, with nutrients flowing in the caecum, and bacteria, their metabolic products, and undigested foodstuffs being excreted as faeces. The anaerobic breakdown of carbohydrate and protein by bacteria is known conventionally as fermentation. In man the major end products are the short chain fatty acids (SCFA) acetate, propionate, butyrate, the gases H₂ and CO₂, ammonia, amines, phenols and energy, which the bacteria use for growth and the maintenance of cellular function.

Fermentation is active in right colon with high bacterial growth rates and a total SCFA production of 127 mmol/l at pH= 5.4-5.9; in transvers colon there is a reduction in bacterial activity due to depletion of substrates and a total SCFA production = 117 mmol/l at pH= 6.2; in the left colon the carbohydrate fermentation is little, with a high fermentation of protein with production of phenols, indoles, ammonia(4).

The fermentations of substrates by the intestinal flora may play a key role in the use of probiotics in the treatment of IBS. Probiotics are live bacteria food supplements that benefit the host animal by improving the intestinal microbial balance. Recent studies emphasize the role of probiotics in IBS. In an open study *Lactobacillus plantarum DSM9843* has been received by patients suffering from IBS during 4 weeks with reduction of flatulence and abdominal pain. The probiotic has been recovered in faeces and rectal biopsies respectively in 84% and 34% of treated subjects (5).

In another study, to assess the efficacy of *Lactobacillus plantarum 299V* in IBS (6) patients were randomized to receive either the probiotic in liquid suspension (20 patients) or placebo (20 patients) over a period of 4 weeks. Clinical examination was performed at baseline and at the end of the study. Additionally, patients assessed their symptoms by applying a scoring system. All patients treated with the probiotic reported resolution of their abdominal pain as compared to 11 patients from the placebo group. There was also a trend towards normalization of stools frequency in constipated patients in 6 out of 10 patients treated with probiotic vs 2 out of 11 in the placebo group. With regards to all IBS symptoms an improvement was noted in 95% of patients in probiotic group vs 15% in the placebo group.

VSL#3, a composite product containing multiple strains of 3 viable lyophilized bacteria species (*Lactobacilli, Bifidobacteria, Streptococcus*), has been tested in 42 patients with IBS with advantage on pain in 81% of cases and a decrease of stool frequency from 7.2 ± 2 to 1.1 ± 1.1 ($p < 0.002$) (7); in another trial recently published VSL#3 appears to be promising in the relief of abdominal bloating in patients with diarrhoea predominant IBS (8).

A recent Editorial suggests the importance to plan randomized trials with an adequate number of patients randomly allocated to probiotics or placebo to define the possibility to use probiotics in the treatment of IBS (9).

We undertook a placebo-controlled study to define the efficacy of a composite product containing one strain of *Lactobacillus plantarum LP01 (PROBIAL)* and one strain of *Bifidobacterium breve BR03 (PROBIAL)* in the treatment of IBS.

Patients, material and methods.

Fifty patients (24 males, 26 females), mean age 40 years (range = 26-64 y) with IBS were enrolled in to the study after informed consent. All patients had a previous history of IBS according to Rome II criteria and with an exclusion of organic diseases on the basis of abdominal ultrasound and colonoscopy, treated with different drugs, without success.

Patients were randomly assigned to receive either the active preparation containing *Lactobacillus Plantarum LP01 (PROBIAL)* and *Bifidobacterium Breve BR03 (Probial)* both at a concentration of 5×10^9 CFU/ml in a powder form soluble in water twice a day, or placebo powder containing starch identical to the study product, for 4 weeks. A complete clinical examination was performed at baseline and at the end of the study.

Efficacy criteria

To evaluate treatment efficacy 2 different scores have been considered:

1. Severity of abdominal pain at different locations : Right lower quadrant (RLQ), Left Lower Quadrant (LLQ), epigastrium, back, other locations

Score: 0=no pain; severity 1,2,3= low/medium/high);

2. Severity of characteristic IBD symptoms:

constipation, diarrhea, bloating, flatulence, cephalaea, nausea, dyspepsia

Score: 0=no symptom; severity 1,2,3= low/medium/high.

RESULTS

46 patients have been considered valuable on the basis of "intention to treat": 24 in probiotics group, 22 in placebo group.

The pain score is reported in Table 1.

Pain locations	Day 0		Day 14		Day 28	
	Plac	Prob	Plac (-%)	Prob (-%)	Plac(-%)	Prob(-%)
RLQ	42	41	37 (-12) 40)	25(-	35 (-17) 54)	19(-
LLQ	61	66	47 (-23) 54)	36(-	44 (-28) 54)	31(-
Epigastrium	37	41	34 (-9) 40)	25(-	33(-11) 57)	18(-
Back	32	32	29 (-10) 16	21	28(-12.5) 31)	22(-
Other sites	20	21	18 (-10) 38)	13(-	20 (0) 21)	17(-
OVERALL	202	201	165(-18) 126(-38)		160 (-11) 97(-	52)

Pain's score in different locations after treatment decreased in probiotics group of 38% vs 18%(p<0.05) of placebo group after 14 days and of 52% vs 11% (p<0.001) after 28 days The best results were achieved for pain in RLQ and LLQ (Tab1).

The severity score of characteristic IBD symptoms significantly decrease in probiotic group vs placebo group after 14 days 49.6% vs 9.9% (p<0.001) e the data was confirmed after 28 days (44.4% vs 8.5 % ,p< 0.001).

Symptoms	Day 0 Plac Prob (n°=22 24)	Day 14 Plac(-%) Prob(-%) (n°=22 24)	Day 28 Plac(-%) Prob(-%) (n°=22 24)
constipation	23 17	20(-13) 15(-12.5)	19(-17.4) 14(-18)
Diarrhea	58 54	54(-7) 25(-44)	51(-11.1) 18(-67)
bloating	44 47	44(0) 21(-54.5)	41(-7) 15(-68)
flatulence	45 51	39(-14) 19(-63)	36(-20) 16(-68.3)
nausea	21 19	19 (-9.6) 16(-16)	18(-14.3) 12(-37)
cephalea	26 27	21(-19.3) 14(-49.2)	18(-31) 14(-49.2)
dyspepsia	33 38	30 (-9.1) 21 (-48)	31(-6.1) 22(-41.2)
OVERALL	249 243	227(-9.9) 125(-49.6)	228(-8.5) 111(-44.4)

In conclusion the preparation containing *Lactobacillus Plantarum*LP01(*PROBIAL*) and *Bifidocterium Breve* BR03 (*Probial*) both at a concentration of 5×10^7 CFU/ml may be considered an interesting tool in the therapy of IBS. Further studies are needed to confirm these preliminary data.

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Survival capacity of a Camembert microbiota in human flora associated rats, influence on intestinal metabolism and antipathogenic effect

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Little is known on the incidence of consumption of cheese on the composition and functions of the intestinal microbiota. The objective of the present study was to evaluate the survival capacity of cheese microorganisms in the intestinal ecosystem, to study their influence on intestinal microflora metabolism and their anti-pathogenic capacity.

Two experimental protocols were used.

In the first one, germ-free rats and human flora associated rats were fed with a diet containing 25% of Camembert for 6 weeks. The level of microorganisms from cheese was measured in faeces using both cultures on specific media and PCR-TTGE analyses using primers specific for the group Lactobacilli. The evolution of the predominant faecal microbiota during dairy products consumption was estimated using non-specific primers. The metabolic characteristics of the caecal microbiota were studied at the end of the experiment.

The results obtained showed that several microorganisms from cheese were found in the faecal samples of rats initially germ-free and in human flora associated rats. But, in human flora associated rats, the dominant microflora remained stable and only minor evolutions were observed. Similarly, the major metabolic activities such as short chain fatty acid production and glycolytic activities were unchanged, but azo-reductase activity, ammonia concentration were lowered whereas neuraminidase and fucosidase activities and transformation of chenodesoxycholic acid into ursodeoxycholic increased.

In the second protocole, germ-free rats were first inoculated either with a pathogen *E. coli* or with *Salmonella typhimorium* before being associated with the dominant human flora without *E. coli*. They were then fed with the diet containing 25% of Camembert or maintained on the control diet. The results obtained showed that the inoculation of the human microflora reduced the diarrhoea symptoms observed in rats infected with the pathogenic strains. Camembert consumption reinforced this effect and significantly lowered the *Salmonella* faecal levels.

These results suggest that Camembert provided probiotic candidates, able to survive during transit and to exert a potentially beneficial influence on the intestinal composition and metabolism.

BACTERIOCIN PRODUCTION BY *LACTOBACILLUS SALIVARIUS* CRL1384 WITH ANTI-LISTERIA AND ANTI-SALMONELLA EFFECTS

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Lactobacilli are important part of healthy intestinal microflora of different hosts because through many mechanisms they could inhibit pathogenic or spoilage bacteria. In particular, *Lactobacillus salivarius* strains have been employed in probiotic supplements or competitive exclusion cultures not only for human but also for chickens principally because of their high lactic acid production. However, little is known about their bacteriocin synthesis ability. Few articles report bacteriocin production by this micro-organism and no one of them have avian origin. *Lactobacillus salivarius* CRL1384 was isolated from the crop of a free-range chicken and screened for the production of antimicrobial activity, using a target panel of spoilage organisms and pathogens. This strain produces a broad spectrum bacteriocin which inhibits the growth of *Listeria monocytogenes*, *Enterococcus hirae* and some gram-negative pathogens such as *Salmonella*. This new bacteriocin is sensitive to trypsin, proteinase K, pronase E, alpha-chymotrypsin, papain and pepsin; it is not sensitive to lipase and alpha-amylase partially reduces its activity. Besides, it is heat-stable (121 degrees C for 15 min) and through an SDS-PAGE assay, the partly purified activity migrates as a peptide of 6 kDa. This bacteriocin can be synthesised by *Lact. salivarius* not only in the presence of glucose but also in the presence of sucrose or maltose, both sugars together or more complex carbohydrate molecules such as fructooligosaccharides (FOS). Genetic analyses revealed not plasmid, so we infer that the information for this bacteriocin production is encoded at chromosomal level. This result is important because that means an stable property.

STUDY AND SELECTION OF LACTIC ACID BACTERIA, ISOLATED FROM POULTRY, THAT CAN USE PREBIOTIC SUBSTANCES AS THE ONLY CARBOHYDRATE SOURCE

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Natural prebiotic substances, such as products and subproducts of sugar cane, and lactic acid bacteria, isolated from healthy chickens, were studied in order to analyze the possibility to devise an eubiotic supplement for poultry. *Lactobacillus crispatus*, *Lactobacillus johnsonii* and *Lactobacillus reuterii* were characterized using physiological and biochemical tests and analyzing the sequence of the 16S RNA fragment. All lactic acid bacteria were able to grow with brown sugar (mainly sucrose, according to HPLC analysis) as the only carbohydrate source. There were no significant differences with those that were grown in glucose medium. The amount of lactic acid produced was important and quite similar in both media.

The inhibition activity of lactic acid bacteria were studied against *S. Gallinarum*, *S. Pullorum*, *S. Thypimurium*, *S. Enteriditis*, *L. monocytogenes*, *E. hirae* and *Staph. aureus* as indicators strains. They were able to inhibit the growth of *L. monocytogenes*, *E. hirae* and *Salmonella* strains but they failed to inhibit *Staph. aureus*. The antagonistic effect was attributed to lactic acid production because inhibitory activity disappeared when the supernatants were neutralized with NaOH. The maximum activity was reached at 24 h of incubation. Below 12 h of incubation no activity was detected.

The lactic acid bacteria analyzed showed that they grow well in the presence of brown sugar and that they can inhibit the normal development of avian and human pathogens because of their ability to produce lactic acid. These results are promising because the use of prebiotics by potential probiotic strains would allow to devise eubiotic products.

Influence of iron and lactoferrin on biofilm development by *Pseudomonas aeruginosa* and *Burkholderia cepacia* isolated from cystic fibrosis patients.

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Lactoferrin (Lf) is an iron-binding glycoprotein found in high concentrations in mammalian milk, in secretions and in granules of macrophages. It is thought to be responsible for primary defence against microbial infection mainly as a result of lactoferrin sequestration of iron required for microbial growth. Many other functions have been attributed to lactoferrin including immunomodulation and cell growth regulation. Some of these functions appear to be independent of the iron-binding activity of lactoferrin. Recently, among the functions dependent on iron-binding activity, it has been demonstrated that Lf is capable of inhibiting *Pseudomonas aeruginosa* biofilm development by sequestering free iron.

Pseudomonas aeruginosa is an environmental mobile Gram-negative microorganism, opportunistic pathogen, capable of causing severe chronic infections in cystic fibrosis (CF) patients. During these infections *P.aeruginosa* may exist as a biofilm in the CF lung becoming highly resistant to antibiotic treatment. It is well demonstrated that in CF patients the serum iron level is lower than in healthy population while the sputum iron concentration exceeds plasma level reaching concentrations up to 134 μM . In addition to *P. aeruginosa*, *Burkholderia cepacia* has emerged as an important opportunistic human pathogen particularly in CF patients. The mechanisms of *B. cepacia*, motile bacterium, involved in facilitating host colonization and invasion are poorly known.

We report the effect of lactoferrin on aggregation and biofilm formation by *P.aeruginosa* and *B. cepacia*, grown under different iron availability. In the presence of low iron concentration (1 μM), both *P.aeruginosa* and *B. cepacia* are free in fluid phase and biofilm formation is inhibited, while a relevant aggregates and a noticeable biofilm formation, already in fluid phase, have been observed at high iron concentration (10 or 100 μM).

These data suggest that Lf *in vivo* could exert a noticeable function preventing both bacterial aggregation and biofilm formation of *P. aeruginosa* and *B. cepacia*.

PROBIOTIC EFFECTS ON THE INTESTINAL MICROFLORA OF AUTISTIC CHILDREN

Elisa Bertazzoni Minelli

Several reports show that children with autism may suffer from intestinal dysfunctions presenting various different features, such as small intestinal enteropathy, defective sulphation, excessive paracellular permeability, and sometimes pancreatic insufficiency and altered microflora composition. These children may present diarrhoea, constipation, abdominal pain, food intolerance, etc. AIM: the aim of our study was to evaluate the faecal flora composition of autistic children with GI symptoms and the effects of high doses of a mixture of lactobacilli and bifidobacteria (Yovis, Sigma-Tau; Italy) on the intestinal ecosystem. METHODS: stool specimens from 15 autistic children (age range: 4-10 years) were collected before and after 1 month of probiotic administration. Qualitative and quantitative composition of faecal microflora was determined by standard microbiological methods. RESULTS: faecal flora composition of autistic children presented certain differences when compared to healthy children, namely high clostridia and bifidobacteria counts and low numbers of lactobacilli. A prevalence of aerobic and anaerobic Gram-positive bacteria (staphylococci, eubacteria and cocci) was also recorded. Mean numbers of enterococci, lactobacilli, bifidobacteria and anaerobic cocci significantly increased ($P < 0.05$) following probiotic administration. In treated children we identified *Lactobacillus rhamnosus*, *L. brevis*, *L. paracasei* spp. *paracasei* and *L. plantarum* not present in faeces before probiotic administration. pH values decreased from 7.0 to 6.4. Treatment induced a few modifications in faecal enzymatic activities such as a decrease in alpha- and beta-glucosidase and beta-glucosaminidase.

CONCLUSIONS: the administration of probiotics to autistic children seems to improve the GI symptoms and intestinal microbial imbalance.

Dynamics of *Lactobacillus plantarum* 44a in the faeces of the fish tilapia (*Oreochromis niloticus*) after a single dosage in the feed

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The aim of this study was to investigate the population dynamics of *Lactobacillus plantarum* 44a in the faeces of the fish tilapia (*Oreochromis niloticus*) cultured in a recirculation system. 8 groups of fish, with 26 fish per group with an average of 70g per fish were randomly placed in 8 aquaria and fed in duplo with one of the following single dosages: 1012 CFU; 109 CFU; 106 CFU; 0 CFU of *L. plantarum* 44a contained in the feed. The recirculation system had controlled water quality parameters and both water and fish were initially devoid of lactobacilli. After feeding with lactobacilli, faeces were collected from a sedimentation tank in cumulative periods of 4 h for up to 72 and thereafter every 24 h up to 196h. Microbial analysis of the samples was carried out using selective media. A good approximation of the data of *L. plantarum* 44a population in faeces of fish is given by a two compartmental ideal mixer model. In this model, the initial population is 0, and after feeding with *Lactobacillus*, there is a short mean retention time, followed by the highest peak dependent on the dosage of *Lactobacillus* ingested. At the highest dose, the peak reaches the levels of the total anaerobic flora in the feces. For the consecutive hours and days, the *Lactobacillus* population followed an exponential decay pattern with detectable levels of organisms retained beyond a week. 10/10 randomly picked isolates from the highest dilution plates gave a fermentation pattern typical of *L. plantarum* 44a.

Probiotics and Immune response in preterm infants

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Introduction: at birth the gut is early colonized by Enterobacterias and Stafilococcus, but in preterm new borns (especially VLBW 800-1350 g.) these bacterias delaye bifido bacteria colonization and together delayed breast-feeding, deficiency of intestinal factors and multiple antibiotic treatment, support colonization of Gram negatives microrganisms very resistant to antibiotics as Klebsiella and Pseudomonas. Recently effectiveness though controversial of probiotics was observed in treatment of bowel diseases, reestablishment of normal flora in the gastrointestinal tract and stimulation of the immune system. Objectives: to evaluate and explore on immune response in preterm infants at risk of sepsis to reduce high morbidity and mortality in neonatal period.

Materials and methods: to investigate the role of cytokines in interactions between probiotic bacteria and the immune system, we measured production of $TNF\alpha$, IL 6 and IL 1β from peripheral blood and we enrolled into the study 19 newborns with EG ≤ 38 weeks (EG 33 ± 4.5) and median weight at birth $1985 \text{ g} \pm 975$ that received oral supplementation probiotic bacteria. Cytokines IL 1β , IL6, $TNF\alpha$ were measured by ELISA The results are expressed as means \pm DS. Statistical analysis by Student *t* test.

Results:

Time	IL 1β		IL 6 °		TNF α *		T0 (start) T1 (days 7) ° p<0.5 * p<0.05
	M	DS	M	DS	M	DS	
T0	0.2	± 0.7	33.60	± 32.86	1.19	± 2.05	
T1	0.9	± 7.5	22.6	± 53.5	3.33	± 15.1	

In the study 3/19 newborns have developed sepsis (15.79%). No exitus.

Release of proinflammatory cytokines (IL 6 - $TNF\alpha$) could mean that probiotics stimulate nonspecific immune response.

Conclusions: Our results suggested that probiotic bacterias could develop immuno-protective responses in preterm.

Further controlled study is needed with wider cohort to clear up these mechanisms.

Screening procedure for the selection of probiotic LAB with survival and immunomodulation potential : the combination of in vitro and in vivo techniques

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Methods

Probiotic lactic acid bacteria (LAB) should have proven health benefits. The in depth study which supports the claimed functionality can therefore be performed on a limited amount of strains only. Beside criteria of safety the strains should be screened for their survival potential as well as for their functional characteristics.

We have studied a variety of LAB for parameters related to survival and immunomodulation potential in an attempt to select strains with an elevated anti-inflammatory potential. Strains naturally present in foods have been screened for their resistance towards pepsin, pancreatin, acid and bile salts as well as for their adhesion and hydrophobicity characteristics.

Their immunomodulation potential has been assayed by measuring cytokine expression profiles of human peripheral blood mononuclear cells (PBMC 1) after incubation for 48 hours with the respective bacteria (at a ratio of 1/10). The in vitro test results were confirmed by in vivo animal trials using a TNBS induced colitis model in mice. The most performing strains have been selected for inclusion in an ongoing human clinical trial (blind, placebo controlled).

We have investigated the correlations between the different characteristics measured by the use of dedicated software.

Results

Strains studied displayed a considerable variety in resistance profiles. No clear-cut relation could be identified between resistance and taxonomic affiliation. Also, no significant correlation was found between the different characteristics measured.

The strains also showed a very diverse immunomodulation profile. Almost all strains were able to induce the anti-inflammatory cytokine IL-10, as well as variable levels of the pro-inflammatory cytokines IL-12, IFN-gamma and TNF-alfa. However, when evaluated on a quantitative basis, a limited number of strains displayed a predominant anti-inflammatory profile (high induction of IL-10) while a majority of strains showed a more neutral (moderate induction of all cytokines) or a pro-inflammatory profile (elevated levels of IL-12 and/or IFN-gamma and/or TNF-alfa; low levels of IL-10).

Selected strains with an anti-inflammatory immunomodulation profile were very effective in reducing the intestinal damage (colitis) in mice after intra-rectal administration of 100 mg/kg of TNBS. The strains with a pro-inflammatory profile were not able to reduce the colitis symptoms. Using this model we compared a variety of LAB, including bifidobacteria, as well as mixtures of varying complexity and composition.

Conclusions

Strain-based in vitro screening for resistant LAB strains will be the only reliable approach for the selection of strains which are suitable to pass the upper part of the digestive tract successfully.

For all characteristics investigated, we found a considerable degree of variation among the LAB strains; no correlation with taxonomic parameters could be established.

In general we found a good correlation between the results of immunomodulation tests on PBMC and the performance of the strains in an in vivo animal model of colitis.

The applicability of this mice model to select the most performing anti-inflammatory strains for applications in human disease situation is currently being evaluated.

LA VIA TRANS-RETTALE PER IL RIPRISTINO DELLA FLORA BATTERICA EUBIOTICA

Autore: Dott.ssa Rosanna Giuberti

Sono state selezionate 20 pazienti di sesso femminile di età compresa tra 22 e 38 anni con i sintomi di colon irritabile e che presentavano durante la prima seduta di idrocolon un livello di soglia del dolore particolarmente alto, simil colica al quale non seguiva una soddisfacente defecazione in corso di trattamento.

Alla palpazione addominale veniva avvertita in questi soggetti un colon contratto con borborigmi e meteorismo.

Al termine della seduta è stato infuso tramite canula a ingresso anale, una soluzione di probiotico (Markalat) sciolto in acqua calda nella proporzione di 50% di polvere e 50% di acqua.

In tutti i pazienti è stata eseguita ICT al dosaggio di infusione anale di un litro di acqua al minuto alla pressione massima di 70 mmbar per un tempo continuativo di 30 minuti.

Markalat polvere, contenendo estratto di camomilla, agisce efficacemente come lenitivo in caso di infiammazione delle mucose del tratto gastro-duodenale. Riduce i gas intestinali eliminando gli spasmi della muscolatura intestinale. L'estratto di camomilla svolge anche una funzione di protezione delle mucose e ne facilita i processi di guarigione da abrasioni. Nel caso di gastriti viene ripristinato l'equilibrio tra fattori aggressivi e protettivi della mucosa intestinale. Il lattosio sviluppa una ulteriore attività terapeutica nel trattamento dei disturbi disbiotici del canale gastroduodenale; stabilizza il PH nell'intestino tenue e crasso creando l'ambiente ottimale sia per la rigenerazione della flora intestinale eubiotica, sia per la direzione di simbiosi favorendo il ripristino della eubiosi acido-lattica.

È stato chiesto al paziente di non evacuare la sospensione per almeno 5 minuti all'interno del colon. I pazienti sono stati collaboranti e mediamente la sospensione è stata mantenuta per 8 minuti complessivi.

Questo trattamento ha permesso di svolgere le successive sedute di ICT con risultati più soddisfacenti rispetto al discomfort intestinale dal 30 al 50 % dei valori basali.

Inoltre i pazienti hanno avvertito una minore dolorabilità nella settimana di intervallo tra la 1^a e l'altra seduta, la presenza di feci più formate e una riduzione del meteorismo.

I risultati di questo ulteriore approccio terapeutico, aggiuntivo rispetto a quello già dimostrato efficace della ICT, sono risultati interessanti e aprono a considerazioni ponendo qualche quesito.

Innanzitutto, nei pazienti che non presentano controindicazioni alla ICT, è possibile utilizzare la via anale come via d'introduzione di sostanze con attività ritenuta ad oggi probiotica (voci bibliografiche). I risultati preliminari di questo studio devono necessariamente essere confortati da altre evidenze sperimentali.

Pur tuttavia sotto l'aspetto speculativo, si potrebbe auspicare, la possibilità di utilizzare la via transrettale per raggiungere, con adeguata e riproducibile metodologia, concentrazioni ottimali di sostanze probiotiche in situ con l'obiettivo di riportare alla eubiosi la flora batterica.

È possibile formulare in funzione di questa via terapeutica un prodotto con le caratteristiche di impatto biologico più adeguato al distretto fisiologico utilizzato per esaltare al meglio la capacità di assorbimento e diffusione del prodotto terapeutico infuso.

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INHIBITORY EFFECT OF PIG PROBIOTIC STRAINS AGAINST HOST-SPECIFIC PATHOGENS

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Infectious diarrhea of neonatal animals is one of the most common and economically devastating conditions encountered in the animal agriculture industry. Probiotic foods can be administered to human or animals in order to prevent infectious diseases, to strengthen the barrier function of the gut microflora and/ or for a non-specific enhancement of the immune system. This study was designed to evaluate the inhibitory activity in mixed cultures of lactic acid bacteria (LAB) on pathogenic strains (*Salmonella* sp. and *Yersinia enterocolitica*).

LAB were isolated from pigs faeces and selected by their potentially probiotic properties (adhesion, inhibitory activity, etc.). Competition assays were carried out using different mixed cultures. LAPTg broth were inoculated with 1×10^7 CFU/ml of individual LAB and 10^6 CFU/ml of pathogen. Cultures were incubated for 24 h at 37°C and followed by measuring the OD560 and pH. Viable microorganisms were determined from selective medium agar, after incubation at 37°C for 48 h. >From different mixed cultures after incubation lactobacillus counts did not present significant differences ($P > 0.05$) with respect to control cultures. On the contrary, in the same mixed cultures, partial inhibition of pathogens was observed. The antipathogenic effect observed in mixed cultures could be explained as a nutritional competition and acidity effect. These strains fulfill the conditions of probiotic bacteria and could be selected for elaborating pig probiotic aliments, in order to prevent infectious diseases. At the present, these potentially probiotic strains are studied about effect of oral administration to pigs.

Oral rehydration solution with a mixture of non-digestible carbohydrates in the treatment of acute diarrhoea. A multicentre randomised placebo controlled study by the ESPGHAN Working Group on Intestinal Infections

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Background

Oral rehydration therapy is an accepted treatment of acute diarrhoea. However, it does not reduce stool loss or length of illness. The objective of this multicentre randomised, double blind placebo controlled study was to evaluate the effect of a mixture of non-digestible carbohydrates (NDC) as an adjunct to oral rehydration therapy in the treatment of acute infectious non-cholera diarrhoea in children with mild to moderate dehydration.

Methods: 144 boys aged 1 to 36 months with diarrhoea defined as the passage of three or more watery stools per day for >1 but <5 days with mild or moderate dehydration (according to WHO criteria) were randomly assigned to receive hypotonic oral rehydration solution (Na 60 mmol/L, glucose 111 mmol/L) with or without a mixture of NDC (soy polysaccharide 25%, α -cellulose 9%, gum arabic 19%, fructo-oligosaccharides 18.5%, inulin 21.5%, resistant starch 7%).

Results: Intention-to-treat analysis did not show significant differences in mean 48 hour stool volume (140 ± 124 g/kg vs. 143 ± 114 g/kg; $p= 0.41$). Total duration of diarrhoea and total duration of diarrhoea in hospital were similar in both groups (130 ± 48 hours vs. 150 ± 79 hours, $p= 0.11$; and 82 ± 39 hours vs. 97 ± 76 hours, $p= 0.24$, respectively). There were no significant differences in the duration of hospital stay (111 ± 44 hours vs. 126 ± 78 hours; $p= 0.3$). Unscheduled intravenous rehydration was similar in both groups (21.4% vs. 16.2%, $p= 0.42$).

Conclusion: In boys with acute non-cholera diarrhoea with mild to moderate dehydration a mixture of non-digestible carbohydrates was ineffective as an adjunct to oral rehydration therapy.

**GENETIC IDENTIFICATION OF POTENTIALLY PROBIOTIC VAGINAL *Lactobacillus*
STRAINS ISOLATED FROM ARGENTINA**

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Objective. To identify genetically *Lactobacillus* strains isolated from human vagina, by the Amplified 16s rDNA Restriction Analysis (ARDRA). These strains, with good surface properties and antagonistic substances producers, were selected by their potential application in a probiotic product for vaginal application.

Methods. ARDRA with four restriction enzymes (*Sau* 3AI, *Hinf* I, *Dra* I and *Hinc* II) was applied to twenty-three vaginal *Lactobacillus* isolates from Tucumán, Argentina. The results of genetic identification were compared with those previously obtained by phenotypic methods (API CH 50).

Results. The genetic identification confirmed the phenotypic results at metabolic groups level (homofermentative and heterofermentative). However, at species level, the genetic and phenotypic identifications only coincided in six strains (26%), which belongs to the species: *L. salivarius*, *L. reuteri*, *L. rhamnosus*, and *L. paracasei*. Most of the microorganisms phenotypically classified as *L. acidophilus* were *L. gasseri*. The restriction profiles of two strains, previously identified as *L. crispatus*, corresponded to *L. acidophilus*. The ARDRA from three *L. delbrueckii* strains allowed the identification of a strain as *L. johnsonii*, while the digestion patterns from the remaining strains were different from the available theoretical profiles.

Conclusions. The application of ARDRA allowed the rapid identification of twenty vaginal *Lactobacillus* strains, belonging to seven different species. The genetic identification of three remaining strains is even in course. The *L. gasseri* and *L. rhamnosus* were the predominant species from the twenty-three vaginal *Lactobacillus* strains evaluated in this work. The correct genetic identification is a fundamental requirement for those microorganisms with potential probiotic use.

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A combination of galacto-oligosaccharides and *Lactobacillus* GG increases bifidobacteria to a greater extent than *Lactobacillus* GG on its own

Aims

To compare a combination of *Lactobacillus* GG (LGG) and galacto-oligosaccharides (GOS) with *Lactobacillus* GG on its own and their effects on the intestinal microbiota.

Methods

Forty-one healthy adults were randomised for a double-blind two-period cross-over study. There were two 3-week study periods with a 4-week wash-out period in between. The subjects ingested daily 65-ml of milk-based fruit juice containing either LGG (6.5×10^9 cfu) alone or LGG plus 2 grams of GOS. The faecal samples were collected at the beginning and end of both study periods. During the study periods the subjects filled in a symptom diary.

Results

The mean faecal baseline values (\log^{10} cfu/g) were 9.25 for bifidobacteria, 5.18 for LGG and 8.08 for lactobacilli. At the end of the study periods the amount of bifidobacteria was significantly greater after the ingestion of LGG plus GOS compared with LGG alone (9.54 vs. 9.31 \log^{10} cfu/g, $p=0.023$). No significant differences were seen in the amount of LGG, lactobacilli or pH. Nor did defecation frequency, consistency of stools or ease of defecation differ between the two study periods. However, though the prevalence of gastrointestinal symptoms was the same in both periods, the intensity of flatulence was significantly greater after ingestion of both LGG and GOS.

Conclusions

Ingestion of both LGG and GOS increases the bifidobacteria more than LGG on its own and thus GOS seems to have a prebiotic effect.

Probiotic *L. fermentum* ME-3 decreases atopic dermatitis clinical indices

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Over the last decade the prevalence of allergic diseases is increased in industrial countries, including atopic dermatitis (AD), known as genetic autosomal dominant chronic skin disease. The reasons and expression of AD are mainly determined by environmental factors as physical, psychogenetical stress, allergic reactions, skin infections etc.

Some lactobacilli have been successfully applied in treatment of AD (Isolauri, 2001). Our previous trial with allergic patients showed the impaired oxidative stress parameters in skin and blood (Kaur et al. 2001).

We have developed fermented goat milk with probiotic *L. fermentum* ME-3 (Kullisaar et al. 2003). The aim of our study was to determine oxidative stress parameters in patients with AD and the change of lactobacilli species in gut of patients with AD before and after consumption of goat milk fermented with probiotic lactobacilli *L. fermentum* ME-3.

The effect of probiotic goat milk on oxidative stress level was evaluated in three groups of total 18 patients; 25-35 years old): placebo group, goat milk fermented with antioxidative lactobacilli and goat milk fermented with nonantioxidative lactobacilli.

Consumption of probiotic goat milk by patients with AD significantly improved blood antioxidative indices: total antioxidative capacity and reduced glutathione levels increased and the level of oxidized low density lipoprotein (oxLDL) in blood and the content of iron in skin decreased compared to placebo and nonantioxidative lactobacilli group. Oxidative stress markers of skin and the score values of patients with AD significantly decreased only in the AD patients group, who consumed goat milk fermented with antioxidative probiotic *L. fermentum* ME-3.

The consumption of goat milk fermented with antioxidative probiotic *L. fermentum* ME-3 diminishes severe oxidative stress both in blood and in skin. This may be one of the reasons for the decrease of atopic dermatitis clinical indices in patients of AD.

Probiotic potential of Lactobacilli isolated from food and animal sources

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A total of 52 *Lactobacillus* species from the ACA-DC collection of the Agricultural University of Athens, isolated from dairy foods and animals, were screened for their potential use as probiotics. Specifically, they were examined on the basis of properties relevant to their survival in human gastrointestinal tract such as resistance to low pH, bile salts and proteolytic enzymes. A number of important probiotic properties were also studied for these strains, such as the ability to hydrolyse bile salts, the potential antimicrobial activity against gram ⁺ pathogens, such as *H. pylori*, *E. coli* and *S. typhimurium*, the adhesion to intestinal epithelial cells in vitro as well as the immunogenic potential in vitro by stimulation of peripheral mononuclear blood cells (PBMCs). Finally, the safety of these strains was evaluated, examining their haemolytic activity and resistance to selected antibiotics. Several strains were found to have such a combination of desirable attributes that allows their use as potential probiotics. Specifically, *L. plantarum* ACA-DC 146 was found to have good survival ability in conditions similar to the GI tract, as well as being highly adhesive to intestinal epithelial cells and able to induce a strong pro-inflammatory reaction. On the other hand, *L. casei* Shirota, a well-known probiotic, was found to induce a strong anti-inflammatory reaction, which was utilized in an animal TNBS colitis model, in which results indicate a protective effect against TNBS colitis in mice treated with the Shirota strain.

ENDOTOXINEMIA AND BENZODIAZEPINE-LIKE SUBSTANCES IN COMPENSATED CIRRHOTIC PATIENTS: A RANDOMIZED STUDY COMPARING THE EFFECT OF RIFAXIMINE ALONE AND IN ASSOCIATION WITH A SYMBIOTIC PREPARATION

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The aim of the present investigation was to test study BZDs profile in patients with viral cirrhosis under different combinations of rifaximine and of a novel symbiotic.. Our study groups consisted of 24 patients with a confirmed diagnosis of HCV-related Child B liver cirrhosis. Patients were randomly allocated into three groups: A) Rifaximine 400mg t.i.d. for 2 weeks; B) SCM-III (L. acidophilus, L. helveticus and Bifidobacteria in a ion- and vitamin-enriched medium, Named srl, Italy) 10ml t.i.d for 2 weeks; C) Rifaximine 400mg t.i.d. for 1 week followed by SCM-III 10ml t.i.d fo 5 weeks. At weekly interval, blood samples were withdrawn to test BDZ-like substances, ammonia and endotoxin. Rifaximine treatment brought about a significant early drop of BDZs ($p < 0.01$ vs pre-treatment and vs control) till 4th week of observation when a gradual increase took place with return to pre-treatment values at the 6th week. Symbiotic treatment was comparably effective while given to patients but significantly elevated BDZs level were noted starting from the 3th week. Similar phenomena were noted for endotoxin and ammonia although symbiotic seemed more effective against endotoxin and rifaximine against ammonia increase. However, the sequential treatment rifaximine-symbiotic brought about a sustained normalization of BDZs, ammonia and endotoxin throughout the 6-week study. The present pilot study suggests that a rifaximine-symbiotic regimen could be an effective tool in compensated liver cirrhosis to limit some triggering factors of hepatic encephalopathy while being amenable to long-term use and devoid of significant side effects.

SPONTANEOUS BACTERIAL PERITONITIS ASSOCIATED TO EXPERIMENTAL CIRRHOSIS: COMPARATIVE EFFECT OF DIFFERENT THERAPEUTIC OPTIONS ON ENDOTOXINEMIA AND HAEMODYNAMICS DERANGEMENT

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The aim of this investigation was to assess the role of different therapeutic options to modify gut microecology in experimental cirrhosis and cytokine cascade and splanchnic and systemic haemodynamics. After the 6th week of CCL4 administration, rats were allocated into 5 treatments A) saline b.i.d; B) lactulose 0.5g b.i.d.; C) rifaximine 1mg b.i.d; D) 2ml b.i.d of a probiotic mixture, E) one week of rifaximine followed by five weeks of probiotic. Rats with cirrhosis and ascites showed a significantly high level of either portal, splanchnic and systemic endotoxin and TNF-alpha concentration ($p < 0.05$). Either C-, D- and E-treatment significantly decreased plasma endotoxin and TNF-alpha level in each of three tested sites ($p < 0.01$). Total fecal Gram-negative aerobic bacteria markedly decreased together with an increase of enterococci in the E-group and less in other groups. Treated rats showed a significant decrease of bacterial peritonitis and E-treatment was the most effective regimen. All treatments significantly reduced the percentage of culture-positivity of mesenteric lymph node and portal vein samples, E-treatment being the most effective. As compared to control, rats with cirrhosis showed a significantly lower mean arterial pressure and systemic vascular resistance but higher cardiac index and portal pressure. SBP further worsened the systemic vascular resistance but this was partly improved by E-treatment. These data suggest that the association of non-absorbable antibiotics with a probiotic, is able to ameliorate the abnormal systemic vasodilatory response in the course of severe liver cirrhosis, probably through the beneficial effect on endotoxin and indirect inhibition of TNF-alpha release.

Tolerance of probiotics and prebiotics

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There is a growing interest in the field of both probiotics and prebiotics as randomised controlled trials have shown that they may have clinical benefits in various physiological or pathological situations. They are used in drugs, food supplements or functional foods. The tolerance of the available products is usually excellent, however, they may have side effects which will be reviewed in my talk.

Potential side effects are more acceptable for probiotic or prebiotic drugs (for which a risk to benefit ratio can be estimated and which can be more easily monitored using the classical pharmacovigilance methods) than for food products which have to be "very safe" as they are consumed by the general population without restriction. My main messages are listed below:

Prebiotics :

1. The tolerance at low doses is excellent
2. Prebiotics at high doses may induce gaseousness, bloating and eventually diarrhoea
3. These effects are dose-dependent but the threshold varies between individuals and is lower in subjects with irritable bowel syndrome
4. There seem to be a metabolic and sometimes clinical adaptation to chronic consumption in some subjects which leads to a better tolerance

Probiotics :

1. The tolerance of commercial probiotics is excellent.
- 2- The rare cases of infections observed with *Saccharomyces boulardii* occurred only in hospitalised patients who had an indwelling catheter and who were treated with the yeast for severe medical conditions
- 3- There is no evidence that ingested probiotic lactobacilli or bifidobacteria pose any greater risk of infection than commensal strains
- 4- There is insufficient knowledge on the risks or benefits (or risk to benefit ratio) of probiotics in immunodeficiency.

Tracking the fate of *Bifidobacterium longum* W11 strain during a human trial.

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Orally consumed viable bacteria with proposed beneficial health effects, the so-called probiotics, are increasingly used to treat disorders like viral, bacterial and radiotherapy-induced diarrhoea, constipation, inflammatory bowel disease and food allergy. An assessment of the survival and persistence of the ingested probiotic bacterium is required in order to correlate the observed effects to its action. The real in vivo persistence of some strains has to be assessed by means of genetic tools, based on DNA amplification and analysis, in order to identify the administered strain.

Objective.

To evaluate the potential of a novel probiotic strain, *Bifidobacterium longum* W11, the active ingredient of Zirfos[®], a synbiotic dietary supplement, to survive and persist into the gut of human subjects.

Material and Methods.

The experimental group consisted of 10 healthy adults, without no immediately (1 week) past history of consuming probiotic containing products. No antibiotics therapy was applied either during the entire trial nor during the week immediately before the trial started. The study group was randomly divided into a placebo (4 subjects) and treated group (6 subjects). Microbiological and genetic analysis were performed in a blind conditions. codes were broken after genetic identification of the studied strain has been achieved. The study period was 15 days divided into a treatment period of 12 days and a follow up period of 3 days.

Faecal samples were collected from all volunteers after the control period, the administration period, and the post-test period. The samples were plate counted to assess the presence of bifidobacteria. Randomly selected CFU of bifidobacteria (about 10% of colonies counted on readable plates), were isolated and cultivated in liquid medium. The genetic identification with RAPD-PCR analysis was then used to identify, among the isolated bifidobacteria, those belonging to the *B.longum* W11 strain.

Results.

The presence of the *B.longum* W11 was detected in all treated subject. The genetic identification method performed well to identify the strain in the treated group and was able to discriminate between treated and placebo group during this blind trial. All treated subjects showed an increase of bifidobacteria counts during the treatment period. No persistence of this strain was detected after discontinuation of the treatment, at least at the highest plate dilutions.

EFFECT OF TEMPERATURE, pH AND CULTURE MEDIA ON THE GROWTH AND LACTIC ACID PRODUCTION OF *Lactobacillus acidophilus* CRL 1259.

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Objective. To study the effect of temperature (30, 37 and 45°C) pH (5, 6.5 and 8) and culture media (MRS or LAPTg broths) on the growth and lactic acid production by *Lactobacillus acidophilus* CRL 1259 isolated from human vagina. This microorganism was previously selected by its capability to inhibit the growth of different uropathogenic microorganisms.

Methods. The growth of *L. acidophilus* was determined spectrophotometrically (540nm) and by viable counts. The production of lactic acid was analyzed by the pH decrease and by the Lactic dehydrogenase test. The results were evaluated by the Gompertz and nonlinear mixed-effects models. The statistical programs SAS 8.2, SPSS 10 and S-Plus 2000 were used.

Results. The optimal conditions both for the growth and lactic acid production by *L. acidophilus* were: pH of 6.5 or 8.0, and temperature of 37°C. The growth was higher in LAPTg than in MRS. However, the lactic acid production was more efficient in MRS. The initial pH of the culture medium and the temperature of incubation exerted significant effects on all the growth parameters (increase of biomass, growth rate and lag phase). However, the culture medium used only affected significantly the final biomass.

Conclusions. The application of the Gompertz model and the statistical software help to determine in a very fast way the effect of different factors on the growth and lactic acid production by *L. acidophilus* CRL 1259. The results obtained in lab experiments allow considering the inclusion of *L. acidophilus* CRL 1259 in a probiotic product for vaginal application.

The strains were licensed to ANIDRAL, a Molfin-Alce group, from Italy.

BOVINE VAGINAL *Lactobacillus acidophilus* PRODUCES H₂O₂ AND INHIBITS *Staphylococcus aureus*.

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The hydrogen peroxide-positive *Lactobacillus* participate in the stability and equilibrium of the vaginal microbiota. Our research is directed to the selection of beneficial *Lactobacillus* for probiotic use in the bovine vaginal tract, to prevent the uncontrolled bacterial colonization causing metritis in the postpartum. *L. acidophilus* CRL 1421, H₂O₂ producer, was assayed with *Staphylococcus aureus* in LAPTg broth at 37°C under static and agitated conditions. The number of microorganisms was determined in MSA (*S. aureus*) and MRS agar (*Lactobacillus*), measuring pH and H₂O₂ during the growth. The damage of *S. aureus* cells was evaluated by transmission electron microscopy.

In the *L. acidophilus* CRL 1421 cultures, aeration increased the growth rate and H₂O₂ production, but the final biomass decreased. The final pH of aerated cultures was 0,46 pH units higher than in static cultures. The Minimal Inhibitory Concentration of H₂O₂ on *S. aureus* was 1.65 mmol l⁻¹. In the mixed cultures, the growth of *S. aureus* was inhibited after 3 h. of incubation, but after 24 h. 10² cells ml⁻¹ remained viable. Under aerated conditions, the inhibition was observed from the beginning of the growth, and a complete inhibition was obtained before 24 h. The inhibitory effect was partially reverted when catalase was added, being evident from 6h. of incubation. The *Lactobacilli* supernatant produced a strong damage in the *S. aureus* cell structure, observing disintegration and loss on the cell wall, which agrees with the decreased viability of the staphylococci cells in *Lactobacilli* supernatants.

Conclusion: The *L. acidophilus* 2014 inhibits a bovine potentially pathogen by the production of antagonistic substances, suggesting the application for probiotic purposes. This strain could be combined with other probiotic strains to be used in the restoration of the vaginal microflora of cows during postpartum .

SURVIVAL OF PROBIOTIC VAGINAL LACTOBACILLI IN GELATIN CAPSULES UNDER REFRIGERATED STORAGE

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Objective: Our research group works in the development of a novel probiotic vaginal formulation for therapy and prevention of urogenital tract infections. The aim of the present work was to evaluate the survival rates of three human vaginal probiotic lactobacilli included as freeze-dried powders with different excipients into gelatin capsules during a storage period of 6 months.

Methods: The strains used were *L. acidophilus* CRL 1259 (high lactic acid-producer), *L. paracasei* CRL 1289 (H₂O₂ producer) and *Lactobacillus salivarius subsp. salivarius* CRL 1328 (bacteriocin-like substance producer). The microorganisms were harvested at stationary phase, washed with sterile distilled water, concentrated ten-fold and resuspended into the following individually and combined pharmaceutical excipients: 8% lactose, 6% skim milk and 2.5% ascorbic acid. The suspensions obtained, with a cell density of about 5×10^9 UFC/mL, were freeze-dried and incorporated aseptically into gelatin capsules. The capsules were stored at 4°C and viability and antimicrobial substances production were determined at defined time intervals.

Results: *L. acidophilus* CRL 1259 was highly resistant to all the storage conditions tested and after 6 months a significant loss of viable cells (82%) was observed only in capsules containing lactose+milk as protectors. *L. paracasei* CRL 1289 dramatically decreased in capsules containing lactose or milk and survival was improved in those containing ascorbic acid individually and combined. *L. salivarius subsp. salivarius* CRL 1328 was the most sensitive strain with a decrease of viable cells ranging between 35-95%. Cells survived better with ascorbic acid and mixtures of protective agents. All the strains tested retained their ability to produce antimicrobial substances.

Conclusions: Survival rates varied with the strain tested and the excipient used. Ascorbic acid significantly improved survival of the three strains tested and could be considered as a good excipient for the preparation of vaginal products.

The strains were licensed to ANIDRAL, a Molfin-Alce group company.

Prevention of antibiotic associated abdominal disorders with probiotics

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Diarrhoea and abdominal disorders are common adverse effects of antibiotic therapy. Probiotics are known to prevent antibiotic associated diarrhoea in children. The aim of the study was to investigate if probiotic bacteria reduce or prevent abdominal disorders during antibiotic therapy in adults.

A total of 94 outpatient adults with a 20-day oral antibiotic therapy (macrolide 36%, penicillin or kefalosporin 35% or other) for acute infectious disease (mainly respiratory infections) were included. Subjects were randomised to receive capsules containing either probiotic mixture of 4 strains (2 Lactobacillus, Propionibacterium and Bifidobacterium) or placebo for 21 days in a double blind randomized trial. Ingestion of study capsules and antibiotics began at the same day. Subjects filled in a daily diary about abdominal complaints during the 3 weeks of capsule ingestion.

There was no difference between the study groups in abdominal disorders, consistency of stools or defecation frequency. Abdominal complaints were minor: the mean weekly sum of all symptoms were 8.6 (\pm SEM 1.1) in the probiotic group and 9.2 (\pm 1.2) in the placebo group ($p=0.68$), while the theoretical maximum score was 84. Three patients in both groups had diarrhoea.

The amount and incidence of abdominal disorders were lower than expected in both study groups. Therefore, probiotic mixture neither reduced nor prevented antibiotic associated abdominal disorders. Another reason for a negative result may be too heterogeneous antibiotic treatments. More studies are needed with higher dosages of antibiotics and probiotics.

Intestinal microbial populations in children with Autistic Spectrum Disorders

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Autism is a developmental disorder characterized by a spectrum of symptoms. Children with Autistic Spectrum Disorders (ASDs) tend to suffer from severe dietary or gastrointestinal (GI) problems. Such symptoms may be due to a disruption of indigenous gut flora promoting the overgrowth of potentially pathogenic microorganisms, such as toxin-producing bacteria. The faecal flora of patients with ASDs was studied and compared to a healthy control group. Faecal bacterial populations were assessed through the use of culture independent fluorescent *in situ* hybridisation (FISH), using oligonucleotide probes targeting predominant gut flora components. Clostridia counts from the faecal flora of ASDs patients were much higher ($>1.5 \log_{10}$ cells/g faeces) than in healthy persons. This may indicate an overgrowth of clostridia in the gut, contributing towards gut dysfunction. Strategies to reduce clostridial levels harboured by ASDs patients or to improve their gut microflora, while stimulating numbers and activity of more beneficial gut bacteria e.g. the bifidobacteria and lactobacilli, may be of benefit in gut health. The future role of probiotics/prebiotics may help to alleviate gut disorders common in such patients. Further gastrointestinal studies may drive theories forward in the development of more effective treatments in ASDs individuals

Clinical volunteer trial using probiotic capsules

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ABSTRACT

Background: Probiotic strains affect beneficially the host's health, expressing antagonistic activity against different pathogenic bacteria and decreasing the risk of infection. They can also influence some health parameters (e. g antioxidative parameters, blood cholesterol level, microbial balance in gastrointestinal tract)

Objective: The aim was to determine the health-promoting effect of a probiotic antimicrobial and antioxidative *Lactobacillus fermentum* ME-3 strain in healthy volunteers.

Material and methods:

The study group consisted of 22 healthy adults of both males and females (from age 24 to 64). The volunteers were randomly divided into two subgroups: study and placebo group. The duration of a double blind clinical trial was 10 days.

Participants filled daily the questionnaires about general welfare, gut health and stool frequency. The study group members took 3 capsules per day containing probiotic strain *L. fermentum* ME-3 (per capsule 10⁹ cfu); the placebo group received the capsules containing only saccharose and microcellulose. Fecal samples were collected to assess changes in gastrointestinal lactoflora and persistence of the ingested probiotic strain. Urine samples were collected to evaluate changes of antioxidative parameters by isoprostane test (ng/ml) during the clinical trial.

Results:

In capsules the viability of *L. fermentum* ME-3 after trial was the same as prescribed (10⁹ per capsule). There were no significant differences in the general welfare, intestinal gas production and frequency of stool between the study and placebo groups. In the study group the probiotic strain was detected in low counts (10⁵ cfu per g faeces). There was found a decrease in isoprostane values in the study group ($p < 0.05$).

Discussion and conclusions:

The colonisation of probiotic strain ME-3 was relatively low though the viability of capsulated product was good. This may be due to the relatively low daily dose for a person or too short duration of the clinical trial. However, the trend for lowering the amount of isoprostane as an antioxidative index in urine samples seems remarkable.

Thus, the ingestion of *L. fermentum* ME-3 containing capsules improves the healthy volunteers antioxidative parameters.

Evaluation of the survival of *Bifidobacterium animalis* DN-173 010 in faecal samples from healthy adults

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Twelve adults ingested 10¹¹ *B. animalis* DN-173 010 per day during 7 days in fermented milk or in lyophilised form. Faecal samples were collected prior to- (D0), at the end (D7) and 10 days after the end of the supplementation (D17). *B. animalis* DN-173 010 was enumerated by immunodetection on colonies. The faecal microbiota was analysed by FISH coupled with flow cytometry using rRNA targeted probes. A species-specific probe for *B. animalis* was developed and applied to quantify the probiotic strain. Faecal DNA was subjected to PCR-TTGE to assess species dynamics of bifids.

At D7, by immunodetection *B. animalis* DN-173 010 was detected at levels >10⁷ ufc/g faeces in all the donors but 2. At D7, By FISH, in the yoghurt group, a transient increase of the dominant phylogenetic groups tested was observed. *B. animalis* DN-173 010 was consistently detected in 8 subjects at D7 and still detectable in 4 subjects at D17. At D7, by PCR-TTGE, all volunteers but one harboured a double band co-migrating with the double band obtained with DNA from *B. animalis* DN-173 010.

This study demonstrates the transient modulation of predominant groups of the human faecal microbiota during the intake of *B. animalis* DN-173 010 in a fermented milk. At D7, the species-specific probe for the probiotic allowed to detect it at dominant levels in 8 of the 12 volunteers. The combination of culture, FISH and PCR-TTGE allowed the specific detection of the probiotic *B. animalis* DN-173 010 in 8 out of the 12 donors.

**CHARACTERIZATION AND PRODUCTION OF PROBIOTIC MICROORGANISMS:
LACTOBACILLUS CRISPATUS A FUNDAMENTAL COMPONENT OF VAGINAL MICROFLORA.**

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Introduzione

In the last few years special scientific attention has been focused on the application of probiotics. There are many commercial products based on lactic acid bacteria that were demonstrated to have a beneficial effect on the gut microflora balance thus improving the wellness of the host. Even if many lactic acid bacteria have been demonstrated to deliver benefits for the digestive apparatus, it is also very interesting to evaluate the possibility of using certain strains of LAB in pharmaceutical preparations for prevention and treatment of vaginal infections (1). Because the role of these microorganisms has changed over time, from simple starters in the food industry to important ingredients in pharmaceuticals, it is particularly important to achieve high biomass yield in their cultivation. We recently proposed an innovative fermentation strategy for high cell density production of *L. delbruekii* ssp. *bulgaricus* (2), in this research work we studied the physiology and growth behaviour of a human isolate, *Lactobacillus crispatus*, in order to apply the MF strategy to improve biomass yield. This strain is part of the vaginal microbiota of healthy women, together with *L. acidophilus*, *L. casei*, *L. brevis*, *L. fermentum*, *L. jensenii*, *L. plantarum*, the pool is often referred to as "Döderlein bacillus"(3). These gram positive, generally microaerophilic, microorganisms are able to ferment a wide range of carbohydrates to lactic acid. Because of this characteristic they are responsible for the acidification of the vaginal environment, furthermore many strains are able to produce H₂O₂, the coupling of these two actions is believed to prevent pathogen attacks.

In the framework of these research we analysed the metabolic response to variation of the growth conditions, with peculiar attention to the production of hydrogen peroxide, lactic acid and exopolysaccharide, that could be responsible for bacterial adhesion to the host tissues.

Materials and Methods

Lactobacillus spp strains have been isolated from vaginal tampons of healthy women of age comprised between 20-45 years, in Man Rogosa Sharp MRS agar (OXOID), incubating for 48h at 37°C under microaerophilic conditions. The species has been identified analyzing the carbohydrate fermentation profile using the API System 50CH (BioMérieux). The isolated samples were maintained as stock in MRS medium added with glycerol (15% v/v) at -80°C. The production of H₂O₂ was determined using the PeroxiDetect Kit (Sigma Aldrich).

Shake flasks cultivations were carried on in a rotative shaker at 37°C pH 6.5 160 rpm, using different carbon and nitrogen sources. Growth was followed by absorbance measurements at 600 nm, cell counting at the microscope and wet and dry weight measurements. In addition, lactic acid production was quantified by HPLC (Supelco C610P column, and glucose consumption was analysed using a Dionex Chromatographer.

Batch experiments were completed on a Biostat CT fermenter equipped with a digital control unit, and remote control via MFCS-wisn software.

Results

The experiments demonstrated the ability of the strain to grow on a wide range of semi-defined media, also producing a high amount of lactic acid. The complex component tested were yeast extract, bactocasitone, soya peptone and casein hydrolysate. The highest biomass yield was achieved using yeast extract. Concerning the carbohydrate sources, growth experiments in shake flasks were completed using glucose, lactose, trehalose maltodextrins and saccharose. All of them were shown to be metabolized to lactic acid, a better yield was achieved when using saccharose or trehalose. In particular, a biomass yield of 6 g/L wet weight was achieved when using glucose, with a production of lactic acid of 12 g/L, while on saccharose 7,4 g/L of wet biomass and 11 g/L lactic acid were produced, finally when using trehalose the biomass yield was 7 g/L and lactic acid concentration was 13 g/L. It was also evidenced that *L. crispatus* was able to grow on starch (0.2% w/v) even if to a minor extent, delivering an acidification from pH 6.8 to pH4. We are currently exploring the possibility of using whey as substrate in batch experiments, so that the medium cost will be drastically reduced.

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Novel fermented probiotic dairy products as functional food

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Different probiotic lactobacilli have been applied in functional food aimed for improvement the health indices. *Lactobacillus fermentum* ME-3 (DSM 14241), a strain of healthy human intestinal origin, expressing high antimicrobial activity against *Escherichia coli*, *Salmonella Typhimurium*, *S. enteritidis*, *Shigella sonnei*, *Staphylococcus aureus* and possessing high antioxidative activity (TAA) has been used as a probiotic supplement in a semi-soft smear-ripened cheese.

Purpose of the study. The aim of present study was to design various fermented milk products as novel functional foods with probiotic strain ME-3 and to check the viability of a probiotic strain ME-3 and stability of its probiotic properties in different dairy products.

Methods. Four different dairy products were analysed as carriers of the probiotic ME-3 strain: yoghurt with four different flavours, three different flavoured creams, sour cream and kefir. All products were industrially manufactured by adding the freeze-dried probiotic ME-3 (5×10^8 CFU mg⁻¹) culture directly in vat in the dose of 150 mg l⁻¹. The reisolates of ME-3 from the products were tested for TAA and their antimicrobial activity against abovementioned pathogens.

Results. The viable counts of the strain were found to be high in all yoghurt variants (average 3×10^8 CFU ml⁻¹). In the other dairy products *L. fermentum* ME3 survived in counts of 10^6 CFU ml⁻¹. The flavour additives did not affect the viability of the probiotic strain ME-3. All novel products had excellent mouth feel, organoleptic and texture quality. The antimicrobial activity ME-3 reisolates from all tested products remained high against all tested pathogens. The strongest inhibition of pathogens was seen in ME-3 reisolates from yoghurts. Average width of inhibition zone of all tested pathogens in millimetres $20,4 \pm 1,9$. The reisolates from other products showed approximately similar antimicrobial activity ($19,3 \pm 1,7$). Mean TAA value of the original ME-3 was 26%. The total antioxidative activity expressed by the strain reisolates varied in different products, being highest in sour cream (46%).

Conclusions. The probiotic properties of ME-3 sustained in fermented milk products though in different products their expression was not similar. Thus, fermented dairy products are suitable vectors for delivering the strain ME-3 into gastrointestinal milieu of consumers.

Involvement of bacteria with special regards to *Lactobacillus* in pathogenesis of inflammatory bowel disease (IBD)

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Purpose of the study. Animal models of inflammatory bowel disease (IBD) are useful tool in studies on mucosal immune response uniquely involved in IBD pathogenesis and in defining a role of bacteria this process. It is known that normal bacterial flora participates in experimental colitis since animals mice housed under germ-free conditions never develop colitis. However, it seems that not all bacteria have the same capacity to develop colitis. Our studies were arranged to follow changes in microbial flora related to chronic bowel inflammation in mice models.

Methods used. We have used in this study two animal models of IBD: SCID mice receiving CD4+, CD45RBhi T cells and showing symptoms of chronic bowel inflammation in about 8 weeks after transfer and Gai2 mutant mice spontaneously developing colitis. The whole gastrointestinal tract of the animals and appropriate controls was aseptically removed during necropsy, ligated on both ends, immersed in a special transport medium with glycerol and transported in dry ice for microbiological analysis. After thawing, the tract was opened, content removed and 5 different samples of mucosa taken from stomach, proximal and distal ileum, colon and caecum dissected and homogenised. Weighed samples of both content and mucosa were serially diluted in pre-reduced medium and plated on a variety of solid pre-reduced media used for cultivation of a wide range of aerobic and anaerobic bacteria and yeasts, and incubated at appropriate conditions. After counting, representative colonies were subcultured and isolates identified at species level using both standard phenotypic methods and PCR technique with species-specific primers. Localisation of both cultivable and non-cultivable members of the colon flora in relation to mucosa was studied using different immunochemical techniques as well FISH method with appropriate probes. The in vitro interactions of selected bacteria isolated from mice with antigen presenting cells were also tested using different cytokines assays.

Summary of the results. No organisms reported previously as specifically related to experimental IBD in mice such as *Bacteroides vulgatus*, *Helicobacter hepaticus* or other *Helicobacter* species were found. Moreover, no growth of *Campylobacter* and sulphur-reducing bacteria was recorded. The cultivable strict anaerobic species were found in small numbers or only occasionally. The bacterial flora of the colon content and samples taken from mucosa of stomach, proximal ileum, colon and caecum of both colitis and control animals was composed of Gram (-) rods, mostly *Escherichia coli*, *Enterobacter agglomerans*, *Citrobacter* sp. and *Klebsiella* sp., Gram (+) cocci: *Enterococcus faecalis*, different species of *Staphylococcus* and *Aerococcus viridans*, and lactic acid bacteria, mostly different *Lactobacillus* species, and more rarely *Lactococcus* sp., and *Leuconostoc* sp.

Colon contents of colitic Gai2 mice but also T cell transfer SCID mice showed significantly higher numbers of Gram (-) rods than those of control animals. These differences were not found in stomach and proximal ileum samples. The numbers of Gram (+) cocci in samples of the IBD mice were, with the exception of colon, and proximal ileum, lower than in controls. On the other hand, populations of lactobacilli adherent to colon mucosa were significantly higher in mice of both models with IBD.

Microbial population in control animals were localised almost exclusively on mucosal layer surfaces while these in colitic mice were displaced to reach a close proximity to colon mucosa.

Conclusions. Our data indicate that although no defined bacterial species seem to be related to these experimental models of IBD, evident quantitative changes in indigenous bacterial colon flora in both SCID mice after T cell transfer and Gai2 mutant mice reflect development of chronic colitis.

Since in animals with colon inflammation, bacterial populations, and especially Lactobacillus are more closely related to colon than in health mice, bacterial products, including those of Lactobacillus species may be involved in the process by either perpetuating or modifying the course of the inflammation by interactions with mucosal immune cells.

Effects of probiotic bacteria on atopic dermatitis symptoms in infants

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Background: Probiotic bacteria are suggested to reduce symptoms of atopic dermatitis in food-allergic infants. We aimed to investigate whether probiotic bacteria have any beneficial effect on atopic dermatitis.

Methods: Follow up of severity of atopic dermatitis by the SCORAD system in 230 infants with suspected cow's milk allergy receiving, in a randomized double-blinded manner, concomitant with elimination diet and topical skin treatment, Lactobacillus GG, a mixture of four probiotic strains, or placebo for 4 weeks. Four weeks thereafter, cow's milk allergy was diagnosed with a double-blind placebo-controlled milk challenge in 120 infants.

Results: In the whole group, severity of atopic dermatitis (SCORAD 32.5) decreased by 65%, but with no differences between intervention groups in infants with atopic dermatitis or cow's milk allergy immediately after the intervention or 4 weeks later. In IgE-sensitized infants, however, the Lactobacillus GG group (n=46) showed a greater reduction in SCORAD scores than did the placebo group (n=46), -26.1 versus +19.8 (p=0.036), from baseline to 4 weeks after the intervention. Exclusion of infants who had received antibiotics during the study reinforced the findings in the IgE-sensitized subgroup: +28.5 in the Lactobacillus GG group (n=36) versus +19.7 in the placebo group (n=33)(p=0.016).

Conclusion: Treatment with Lactobacillus GG may alleviate atopic dermatitis symptoms in IgE-sensitized infants.

Molecular marker of probiotic effects

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Previously we have demonstrated that the quantity of faecal sIgA is significantly higher after stress-situations (surgical operations, infections, unadequate nutrition, overloads) when the level of protective groups of microorganisms (bifidobacterium, lactobacilli, bacteroids) becomes lower and the level of pathogenic microorganism increases. The increase of faecal sIgA indicates an immune response reflecting a short-term immune activation in order to protect the mucose from injury by pathogenic microorganisms. The increase of sIgA level indicates immune response in the direction to form mechanisms of adaptation to new environment by synthesis of mucose sIgA.

Now in the clinical investigation we have used the measurement of the sIgA level for monitoring of therapy with antibiotics. The sIgA levels increased in the early stages of disbacteriosis. In clinical trial we used the measurement of faecal sIgA for estimation of the effect of bacteriotherapy with probiotics. The first group is 96 persons with dysbacteriosis 1-4 degree manifestation. The sIgA level was ($M \pm m$) 875 ± 119 mcg/g feces before bacteriotherapy. After bacteriotherapy (with bifidobacterin and lactobacterin) the sIgA level decreased to 288 ± 101 mcg/g feces.

The second group is 17 healthy children 1-6 old. They have the sIgA level $28,7 \pm 15,5$ mcg/g feces. The third group is 67 children 1-8 old with recurrent respiratory diseases and disbacteriosis 1-2 degree manifestation: children up to 1 old have the sIgA level $99,8 \pm 45,5$ mcg/g feces; children 1-5 old have the sIgA level $93,0 \pm 42,2$ mcg/g feces; children 5-8 old have the sIgA level $139,9 \pm 101,9$ mcg/g feces. In clinical trial we used the measurement of faecal sIgA for estimation of the effect of bacteriotherapy with probiotics eufhorins B and L. We have shown that the decrease of faecal sIgA was followed by increase of bifido- and laktobacteria. Bacteriotherapy with probiotics eufhorins B and L was effective in 51,6%. This effect was in accordance with the levels of bifido- and laktobacteria and clinical symptoms. In the cases when bacteriotherapy was not effective (level of bifido- and laktobacteria did not increase), faecal sIgA level was the same, or increased.

Conclusions. sIgA is molecular marker of adaptation defence of organisms. We suggest that this marker may be diagnostic indicator of dysbacteriosis, may be useful for the evaluation of the effects of bacteriotherapy and selection individual cure.

Probiotic potential of *Streptococcus macedonicus* strains

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A total of 38 *Streptococcus macedonicus* strains were screened for their potential use as probiotics. They were examined for properties relevant to their survival in the human gastrointestinal tract such as resistance to low pH, gastric proteolytic enzymes and bile salts. Their ability to hydrolyze bile salts and their antimicrobial activity against Gram-negative pathogens, such as *H. pylori*, *E. coli* and *S. typhimurium*, were also investigated. Finally, the safety of these strains was evaluated, with respect to their haemolytic activity and resistance to selected antibiotics. According to our results, none of the strains showed haemolytic activity. Low MIC values (< 8 µg/ml) were obtained for vancomycin, teichoplanin, tetracycline, and penicillin with all strains. This was also the case with the majority of the strains (> 84%) for cloxacillin, tetracycline, and bacitracin, while all strains were highly resistant to ampicillin, neomycin, paromomycin, streptomycin and sulfathiazole (MIC > 64 µg/ml). Concerning their ability to survive under GI tract conditions, the viability of the strains was not affected by pancreatin (3 mg/ml at pH 2) or bile salts (2% at pH 8), while 13 strains were able to hydrolyze bile salts. At pH 2 or in the presence of pepsin (3 mg/ml at pH 2) low survival of 10⁻³ % was observed for all strains tested. At pH 1, 22 strains were not able to survive at all, 14 of them were more resistant with survival of 10⁻³ %, while two strains, namely *S. macedonicus* ACA-DC 198 and 205 showed a survival of 10⁻² %. Finally, *S. macedonicus* ACA-DC 198 was the only strain exhibiting clear inhibition against two *H. pylori* strains, which was due to the bacteriocin (macedocin) produced by this strain.

EFFETTO DELLA SOMMINISTRAZIONE DI LATTOFERRINA SULLO STATO IMMUNO-VIROLOGICO DEI BAMBINI HIV-INFETTI : UNO STUDIO PILOTA.

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Obiettivo: Valutare l'effetto della somministrazione di lattoferrina bovina sullo stato immunologico e virologico dei bambini HIV-infetti.

Metodi: A cinque bambini (3 femmine e 2 maschi; età 2-13 anni) con infezione HIV acquisita verticalmente, in terapia antiretrovirale (4/5 con due farmaci, 1/5 con tre farmaci) invariata da almeno 18 mesi, e monitorati dalla nascita, è stata somministrata oralmente per tre mesi una dose di 3 g/die di lattoferrina bovina. Lo stato immunologico e virologico dei bambini è stato valutato trimestralmente da 9 mesi prima (-9) dell'inizio (0) della supplementazione a tre mesi dopo (+3).

Risultati: Nei 4/5 (80%) dei bambini la carica virale, dopo tre mesi di somministrazione, era minore dei valori rilevati nei 9 mesi precedenti (Tabella). La riduzione percentuale della carica virale dopo tre mesi di somministrazione, rispetto alla carica virale media dei 9 mesi precedenti, è risultata non negativa in tutti i bambini (minimo 0%; massimo 78%), con una media (intervallo di confidenza al 95%) del 48% (6%;92%).

Età ^a (mesi)	Sesso	Carica virale (copie x 1000/ml)				
		Mese di supplementazione				
		-9	-6	-3	0	+3
24	M	206	63	86	181	45
30	F	69	36	14	92	86
84	F	148	460	399	119	63
108	F	120	66	75	72	62
156	M	82	23	27	27	10

^aall'inizio della supplementazione.

In ciascun bambino, i valori di CD4 dopo tre mesi di somministrazione di lattoferrina sono risultati comparabili a quelli rilevati nei 9 mesi precedenti. La differenza tra i valori percentuali di CD4 dopo la somministrazione di lattoferrina e i valori medi pre-somministrazione era compresa tra -4% e 0%. Nessun evento avverso è stato osservato nei bambini durante i tre mesi di somministrazione.

Conclusioni: La somministrazione di lattoferrina bovina potrebbe migliorare lo stato virologico dei bambini HIV-infetti. Studi più ampi e con adeguata potenza sono necessari per confermare i risultati dello studio presente, e valutare l'effettiva efficacia della somministrazione di lattoferrina per migliorare lo stato immunologico e virologico dei bambini HIV-infetti.