September 4/6, 2005

ATTI
ABSTRACTS

3rd PROBIOTICS & PREBIOTICS NEW FOODS

ROME
Università Urbaniana

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Società Italiana di Medicina del Benessere

Oltre la nutrizione
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UNDER THE PATRONAGE OF

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SUNDAY, SEPTEMBER 4

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FACULTY

H. Aarts

A. Anadòn

G. Barbara

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E. Bruzzese

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H. Szajewska

T.A. Tompkins

M.C. Urdaci

P. Valenti

A. Wilcks

Antibiotic Resistance : pros and cons of antibiotics

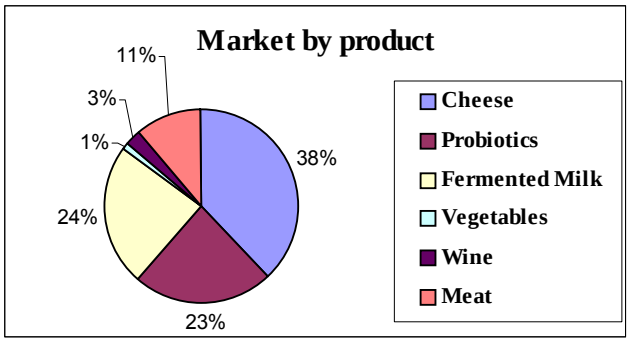
Patrice Courvalin
Unité des Agents Antibactériens, Institut Pasteur, Paris, France

Classically, resistance to antibiotics is divided in insensitivity (also designated intrinsic or natural resistance) inherent to a genus or a species that defines the spectrum of activity of a molecule, and acquired resistance present only in some members of a taxonomic group. Advantage of resistance in a probiotic is that it can be co-administrated with antibiotics but the drawbacks are the potential of horizontal transfer of resistance determinants to bacteria pathogenic for mammals and, in case of infection by the probiotic (alteration of digestive epithelium, immunocompromised host), availability of only a limited number of drugs. Insensitivity and acquired resistance can be due to the same biochemical mechanism (mainly, enzymic detoxification of the drug, modification of the target, and decreased intracellular accumulation of the antibiotic). The risk of horizontal transfer of resistance determinants to human or animal pathogens depends on the genetic basis of resistance. Insensitivity and acquired resistance by mutation are presumed to present a minimal potential for spread because of the chromosomal location of the responsible loci. By contrast, acquired resistance mediated by mobile genetic elements (conjugative or mobilizable plasmids, transposons conjugative or not) is considered as presenting the highest degree of danger for dissemination. However, any bacterial gene can become mobile as long as the right selective pressure is exerted and, thus, the distinction between fixed chromosomal genes and mobile determinants is dépassé. Since microorganisms, mainly Gram-positive, used as feed additives should not contribute to the genetic pollution by resistance determinants we will consider the strategies for elucidation of the genetic basis of multiresistance à propos *Bacillus clausii*.

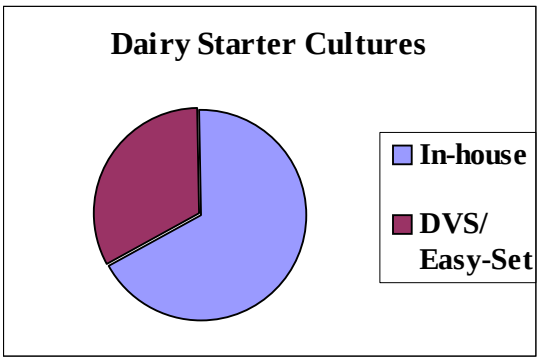
Assessing drug resistance in lactic acid bacteria: How industry and academia can collaborate

Svend Laulund, Chr. Hansen A/S, Boge Alle 10-12, DK-2970 Horsholm, Denmark, on behalf of European Food & Feed Cultures Association (EFFCA). www.effc.org www.aceart.net svend.laulund@dk.chr-hansen.com

The use of microorganisms in the production of food goes back thousands of years. Looked at it in this context, the awareness of the beneficial effect was established a long time ago, but the knowledge about the effect of the individual microorganisms is quit recent. It is estimated that 25% of the food and feed consumed in the world is undergoing a fermentation process with microorganisms. In this presentation, the use of lactic acid bacteria is the subject only and not yeast that is used in beer, wine and bread production.
The majority of lactic acid bacteria (also know as starter cultures) are used in dairy for acidification of milk for cheese making, ripening of cheeses and fermented milk. Probiotics with beneficial health effects are used in dairy products as well as in food supplements and in agriculture as feed additives. To a smaller degree, lactic acid bacteria is used in meat production of sausages but also as protective cultures on sliced meat. In preservation of vegetables, lactic acid bacteria is used for olives, cucumber, cabbage (sauerkraut), and for silages making in feed production. In the last 10 to 15 years the understanding of malolactic fermentation in the production and maturation of wine has led to the industrial use of lactic acid bacteria in this area as well.

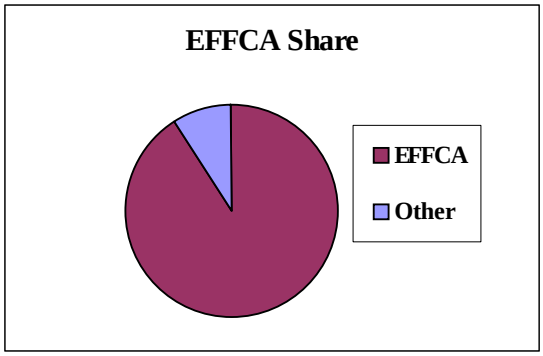


It is estimated that approximately 2/3 of all dairies produce their own in-house starter cultures. This process takes place in two to three fermentation steps, starting with a small amount of milk inoculated with a relatively low concentration of lactic acid bacteria. To exert the desired reaction the cultures are propagating to the required concentration by up-scaling in steps with 1% to 0.1% inoculate (culture) to the fermentation media (milk). The media can be inoculated with an in-house defined culture, undefined culture(s) or with an industrial-made bulk culture. In some cases the inoculation is made with the remains of the previous production, this is also known as “back slopping”. In minor productions of so called traditional cheese, parts of the microorganisms comes from the agricultural environment, as un-pasteurized milk is used for this kind of cheeses, which gives rise to a spontaneously fermentation as well.



The remaining 1/3 of dairies use industrial starter cultures in their production, in a manner that the milk can be inoculated directly with 0.01 to 0.005 % of a very highly concentrated and well defined strain a so called Easy-Set, Direct Vat Set or Direct Vat Culture (DVS or DVC) with no intermediate growth step. In this way the numbers of cell multiplications and the total fermentation time at the dairy is highly reduced. The contamination risk is also minimized to a great extent. The result is a uniform and certified quality of the final product. The industrial starter cultures can be provided in freeze-dried as well as in frozen form. The frozen cultures gives a quick start whereas the freeze-dried cultures facilitate an easier transportation and storage.

It is estimated that the member companies of European Food & Feed Cultures Association (EFFCA) are delivering more the 90% of the industrial made starter cultures.



A natural property of microorganisms is resistant to antibiotics. The resistance to antibiotics in therapeutic use is a growing concern within public health. Resistance is proportional with the increased use of antibiotics. In an investigation published in Lancet February 2005 by Herman Goossens, it is proved that there is a clear correlation between the use of penicillin and the prevalence of penicillin non-susceptible *Streptococcus pneumonia*.

The intake of food and feed with millions to billons of lactic acid bacteria per milliliter are not suspected to be a major concern, but from a theoretical point of view, horizontal transfer of antibiotic resistant genes can take place in the food or feed during production or in the human - or in the animal intestinal system. Despite concern that the use of microorganisms in the food and feed chain contributes to the development of resistant bacteria, research was, before the start of the ACE-ART project, yet to provide the data and methods necessary for the development of an effective risk management strategy.

From 1999 to 2001, the EU SSC (European Unions Scientific Steering Committee) published opinions on antibiotic resistance in microbial (probiotics) feed additives. In 2001 EU SCAN (European Unions Scientific Committee on Animal Nutrition) published an opinion with guidelines on demands for documentation on MIC (minimum inhibitory concentration) to a range of clinically relevant antibiotics in probiotic feed additives. At that time no validated and international recognized methods existed for testing this in lactic acid bacteria including bifidobacteria. For industry to provide the needed data required by SCAN was a huge challenge. Especially as available reference data on normal resistance was and still is very limited. An investigation in EFFCA was made on how to cooperate on this mutual problem. But as EFFCA members are also competitors, the sharing of experience and sharing of data was not straightforward. One or more independent partners were needed if confidential information should be validated and shared in a coded manner. Five "old" partners from authorities, universities, the industry and an industrial association met in May 2002 and discussed the possibility for collaboration and investigation in a solution to this problem. In the European Union's 6th Framework Programme a priority on assessment of antibiotic resistance in food related bacteria was identified and an Expression of Interest was made as the ACE-ART project. The project was approved with 14 partners and with the industrial association EFFCA as a platform for providing bacterial strains, associated data and scientific input.

The outcome of the project will hopefully provide regulatory bodies, EFSA, FAO/WHO, CRL (Central Research Laboratory) and others with scientific information and validated test methods with an adequate background for implementing proportional guidelines and regulation, which can support, and not prevent, the development of finding new and better microbial products to improve the level of food and feed safety. ACE-ART's input to the European Food Safety Authorities (EFSA) FEEDAP Panel has already contributed to a revision of the former SCAN opinion. An updated version of "Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance" was adopted on 25 May 2005. Another example illustrating the value of the program is the collaboration between ACE-ART and a working group under IDF (International Dairy Federation) and ISO (International Organization for Standardization). The aim is to harmonize the analytical methods applied for assessing resistance in probiotics. The protocols and procedures developed by ACE-ART are under evaluation and implementation by the working group.
The starter culture industry (EFFCA) will use the microbiological breakpoint as reference data in the quality control and the genetic tools to investigate for intrinsic or acquired resistance in the research and development for new and improved cultures.
The previously mentioned use of undefined starters, back slopping and spontaneous fermentation will be very difficult, not to say impossible, to analyze for antibiotic resistance on a continuous schedule. The question is - as is the case with the QPS (Qualified Presumed Safety) approach - can we accept that this area of fermented product will still be outside the scope and control, even after the results from the ACE-ART project has been disseminated? The defined starter cultures will be even safer. Will microorganisms used in undefined back slopping and spontaneous fermentation still persist as a potential threat to the safety of fermented food products?

Phenotypic assessment of drug resistance in lactic acid bacteria and bifidobacteria

Jaana Mättö
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Introduction

Lactic acid bacteria (LAB) are used in a large variety of fermented food and feed applications. Due to their reported health benefits lactobacilli and bifidobacteria are increasingly used as probiotics. LAB and bifidobacteria have a long history of safe use and they do not usually create any safety concern. However, extensive use of antibiotics for treatment and prophylaxis of microbial infections in humans, animals and even in plants have led to increase of antibiotic resistance in bacteria. Therefore, monitoring of antibiotic susceptibility profiles of non-pathogenic bacteria linked to the food chain has also become relevant. Contrarily to clinically important bacterial species, there are no established standard methods and breakpoints available for susceptibility testing of non-pathogenic food-associated bacteria. Therefore, various culture media and test procedures have been used in susceptibility testing of these bacteria. However, even minor changes in the test protocol may cause difference in the susceptibility test results and the microbiological breakpoints need to be linked with the test procedure.

Aim

The WP1 of the ACE-ART project focuses at phenotypic assessment of antibiotic susceptibility of non-pathogenic food associated bacteria representing selected *Lactobacillus*., *Lactococcus*, *Streptococcus thermophilus* and *Bifidobacterium* species. The overall aim is the differentiation of strains with atypical (potentially acquired) antibiotic resistance(s) from the normal susceptible population. In order to achieve this goal microbiological breakpoints for selected antibiotics are defined by using the required large number of strains of each target species. Strains with atypical resistances are further characterised to reveal the impact of the antibiotic resistance determinant on the strain properties. One specific aim of the project is to provide evaluation of the role of antibiotic use in animal and plant production and in the prophylaxis and treatment of disease in humans on the level of antibiotic resistance in bacteria. This will be targeted by comparing strains isolated from various habitats and time era and by studying the impact of antibiotic challenge on the prevalence of antibiotic resistance.

Description of work and results achieved

Nine institutes from eight European countries are involved in the WP1 of the ACE-ART project (Table 1). The activities of the project consortium related to the phenotypic assessment of antibiotic susceptibility are outlined in the Fig. 1.

Strain collection

More than 1300 strains representing 20 species of LAB and bifidobacteria are included in the project strain panel (733 lactobacilli, 116 *Lc. lactis*, 90 *S. thermophilus* and 383 bifidobacteria). Strains originate from various ecological habitats (human, animal, dairy and plant), geographic locations (mainly different regions in Europe) and time era (from before 1950 to recent isolates). The members of EFFCA (the European Food and Feed Culture Association) have collaborated in the establishment of the strain collection by providing their production strains for characterisation in the project (7 % of the strains). A database was created for handling the strain data.

For each bacterial species or group a minimum of 50 strains per species is included in defining the microbial breakpoints for the selected antibiotics. For most of the species the minimum number of strains has been achieved during the first project year. Since isolation and species- and strain-level identifications are still continued, the number of strains included in the database will slightly fluctuate throughout the project.

Table 1. Scientists and institutes involved in the phenotypic assessment of antibiotic susceptibility.

Institute	**Target bacteria****
Project coordinator L. Morelli, L. Tosi Istituto Microbiologia UCSC-, Piacenza, Italy	*Streptococcus thermophilus,* lactobacilli
M. Danielsen, A. Wind, S. Laulund Chr. Hansen A/S, Copenhagen, Denmark	lactobacilli
B. Mayo, A. Margolles Instituto de Productos Lácteos de Asturias, Villaviciosa, Asturias, Spain	lactococci, lactobacilli and bifidobacteria
Workpackage 1 leader J. Mättö, M. Saarela VTT Biotechnology, Espoo, Finland	bifidobacteria
L. Axelsson Norwegian Food Research Institute, AAS, Norway	lactobacilli
A. von Wright, J. Korhonen University of Kuopio, Kuopio, Finland	lactobacilli, lactococci
W. Kneifel, K. Domig, S. Mayrhofer University of Natural Resources & Applied Sciences, Vienna. Austria	lactobacilli, bifidobacteria
G. Huys Ghent University, Lab of Microbiology, Ghent, Belgium	lactobacilli
S. Lindgren, M. Egervarn National Food Administration, Stockholm Sweden	lactobacilli

** Each institute is responsible for selected species within the target genus/genera.

Assessment of antibiotic susceptibility

The focus on the phenotypic assessment of antibiotic susceptibility is in selected clinically relevant antibiotics namely tetracycline, erythromycin, streptomycin and for bifidobacteria and *Lb. acidophilus* group additionally vancomycin. In addition, clindamycin, gentamicin and ampicillin susceptibilities are assessed for a large number of strains. Moreover, an extended antibiotic panel (i.e. antibiotics described in the SCAN document) is used for screening of multi-drug resistances in strains showing atypical susceptibility profiles for the primarily tested antibiotics. Susceptibility assessment will mainly be performed by using the Etest or a broth microdilution technique, but agar dilution and disk diffusion techniques are additionally used for a subset of strains.

Antibiotic susceptibility assessment has been initiated by studying the impact of test parameters on the susceptibility test results. The most critical factor influencing on the susceptibility test results was the choice of the test medium, which had an impact on the MIC-values of several antibiotics and in all target bacterial groups. In addition, inoculum size and incubation time had a large influence on the MIC-values, which increased upon prolonged incubation and by increasing the inoculum size, especially in lactobacilli. Although changes in test procedures had an influence on the actual MIC-values, it was still possible to differentiate resistant and susceptibility populations from each other by using variable test procedures. However, to be able to compare the susceptibility data between different laboratories and to determine susceptibility of a single isolate harmonised procedures and defined microbiological breakpoints are needed.

The method harmonisation was initiated from choosing a suitable test media. Growth performance of more than 300 strains representing 11 *Lactobacillus* species, 28 *Lc. lactis* strains, 31 *S. thermophilus* strains and more than 100 strains representing eight *Bifidobacterium* species were studied on LSM agar or broth (supplemented with cysteine for bifidobacteria). All except the *S. thermophilus* and some of the *B. bifidum* strains showed good growth on the LSM (+cys).

Based on the method comparisons and growth performance studies, test protocols were defined for each target bacterial group with some species-specific modifications. By using the standardised procedures a method harmonisation study involving all partners of the WP1 (Table 1)

was performed by using the the Etest and the borth microdilution (tailor-made VetMIC^{TM} panels) techniques. In the harmonisation study susceptibility of representatives of 25 species to the seven antibiotics listed above was assessed in five repeats in three different laboratories.

Characterisation of strains with atypical resistances

During preliminary screening the majority of the strains have not showed atypical resistances to the assessed antibiotics. However, occasional strains have been resistant to tetracycline and to lesser extent to erythromycin. A wide distribution of MIC-values for streptomycin has been observed among LAB and bifidobacteria, and for some species streptomycin resistance may be intrinsic. Strains with atypical antibiotic resistances have been prevalent among isolates recovered from antibiotic-associated environments e.g. tetracycline resistance among *Lactococcus* isolates from fish originating from a farm with a history of tetracycline use. All strains with atypical (possibly acquired) resistances will be subjected to a more detailed characterisation including assessment of multi-drug resistances and the effect of antibiotic resistance on other strain properties. Moreover, they are further unravelled in the WP3 for molecular characterisation of the antibiotic resistance determinants.

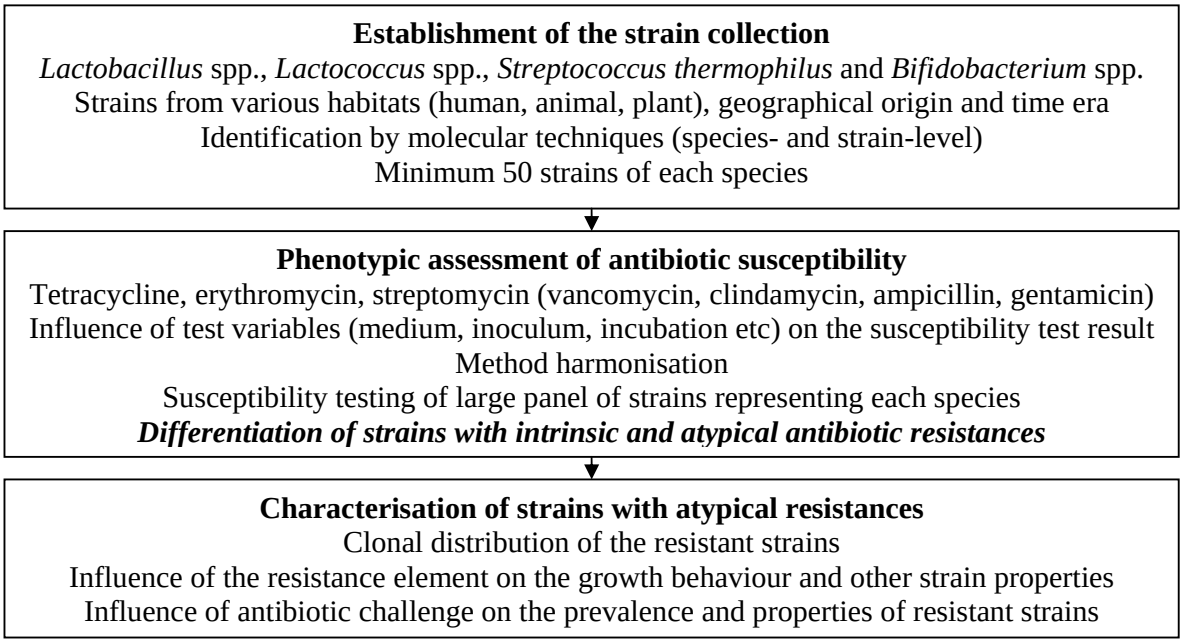

Fig. 1. Outline of the WP1 of the ACE-ART project.

References

SCAN. Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human and veterinary importance. European commission (EC) 2001.

NCCLS. Methods for antibiotic susceptibility testing of anaerobic bacteria: approved standard-sixth edition. M11-A6, Vol. 24 No. 2.

List of relevant publications related to the topic are available at the www pages of the ACE-ART project (www.aceart.net).

Transferability of drug resistance genes harboured by lactic acid bacteria

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In Work-package 2 of ACE-ART, gene transfer studies are conducted in order to generate qualitative and quantitative data on transferability of resistance genes from antibiotic resistant Lactic Acid Bacteria (LAB) to other LAB and to more distantly related bacterial genera. Horizontal gene transfer is investigated both in vitro and in vivo. Although gene transfer has been studied throughout the last decades there are only few examples of standard protocols available, that gives detailed description of the experimental conditions. Therefore one of our objectives is to establish a set of in vitro mating conditions that will produce comparable results in any laboratory.
Additionally, model systems of the animal and human gut are developed for investigation of horizontal gene transfer in vivo. Transfer of tetracycline and erythromycin resistance from wild-type LAB was demonstrated in a worst-case model.
This model consisted of germ-free Sprague-Dawley rats that were given a single dose of the recipient Enterococcus faecalis (rifR, fusR) that colonized the gut. After a week wild-type Lactobacillus plantarum (tetM and/or ermB) was given daily. Additionally, the streptomycin treated mouse intestinal model has been used in a pilot study to test the ability of E. coli to receive plasmids in vivo. Currently we are investigating transfer in a bovine rumen model that consists of fresh rumen fluid extracted from fistulated animals. These animals are on a variety of diets producing different fluids that are transferred to test tubes. These studies will provide data to a critical evaluation of the risks associated with genetic transfer of antibiotic resistance from LAB in the food chain.

Genetic Mechanisms of Resistance in Lactic Acid Bacteria.

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Within the framework of the ongoing EU-funded project ACE-ART (www.aceart.net), one of the work packages (i.e. WP3) is dedicated to the study of the genetic basis of atypical antibiotic resistances in *Lactobacillus, Lactococcus*, *Streptococcus thermophilus* and *Bifidobacterium* strains mainly from food origin. For this purpose, various molecular methods are being developed. Besides single PCR assays, a multiplex MQDA- PCR method integrating the principles of SNaPshot fluorescent labelling and capillary electrophoresis (CE) has been developed for the simultaneous identification of multiple antibiotic resistance genes. For instance, tetracycline genes *tet*(L), *tet*(O), *tet*(S), *tet*(M), *tet*(T) and *tet*(K) can be amplified simultaneously and subsequently analysed by CE. In addition, a thematic microarray containing gene specific oligonucleotide probes for several classes of antibiotic resistance genes was developed and used to screen strains of the above mentioned lactic acid bacteria (LAB). The screening efforts ((multiplex-) PCR and microarray) resulted in the detection of a number of atypical genes. For instance, *erm*B was found in *Streptococcus thermophilus*, *tet*(M) in *Lactococcus lactis* and *tet*(O) in *Bifidobacterium*. Plasmid isolation methods, anchor PCR and sequencing are used for the precise determination of the location of the genes. Another aspect of resistance, multidrug resistance, is specifically studied in *B. breve*. It was proven that a gene with similarity to the secondary transporters, *bbmR*, encodes a membrane protein conferring resistance to the macrolides erythromycin, azythromycin, dirithromycin and clarithromycin in *Lc. lactis*. It was also found that BbmR probably contributes to intrinsic antibiotic resistance in *B. breve* to, at least, macrolides. The preliminary characterization of BbmA1 and A2 suggested that they could build up an active heterodimeric MDR transporter.

Introduction:

The spread of antibiotic resistance among bacteria is recognised as a serious problem that eventually can complicate medical treatment of bacterial infections. The EU-project "Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain" (ACE-ART), will provide a critical evaluation of the role of antibiotic use in agriculture and in the prophylaxis and treatment of diseases of humans. ACE-ART is focussed on non-pathogenic bacteria. As such, lactic acid bacteria (LAB) are chosen as model organisms, representing commensals distributed in plants, animals and humans and furthermore commercially applied in food and feed. Within WP3 mechanisms of resistance are studied at the molecular level. Relevant gene(s), genetic element(s) or mutation(s) involved are characterised and their possible association with extra chromosomal DNA, such as conjugative plasmids, or other mobile elements like integrons or transposons are determined. Based on the phenotypic data provided by WP1 bacterial strains belonging to the ACE-ART collection are screened for the presence of antibiotic resistance genes/mutations by (multiplex-) PCR assays, small-scale microarray hybridisation experiments and sequence analysis. Subsequently, a subset of strains are studied in more detail, which includes copy number and their location within the genome. Genes of interest are those responsible for

resistance to Erythromycin (ERY, MLS group;5 genes), Tetracycline (TET; 5 genes), Streptomycin (STR; 3 genes) and Vancomycin (in bifidobacteria VAN; 2 genes). Nevertheless, microarray analysis with the ability to screen for a larger set of genes is included. WP3 also deals with the characterisation of mechanisms involved in the intrinsic resistance to several antibiotics since this feature is becoming more common in LAB. Known and unknown M(ulti) D(rug) R(esistance) transporter mechanisms are studied. The study of unknown MDR transporter mechanisms is feasible by searching for MDR homologous sequences in the huge and growing amount of information available in public DNA and protein databases. Especially a lot of information is available about LAB, which makes them to a large extent suitable for these kind of studies.

Obtained results:

Strains under investigation. More than 100 strains assigned to the species *B. longum, B pseudocatenulatum, B. breve, Lb. plantarum, Lb. helveticus, Lb. rhamnosus, Lb. acidophilus, Lb. johnsonii, Lb. reuteri, Lb. amylovorus, Lb. fermentum, Lc. lactis* subsp. *lactis* and subsp. *cremoris* and *S. thermophilus* were selected based on their phenotypic resistance profile for further investigation. In addition, a list of control strains (among which also non-LAB strains) containing described antibiotic resistance genes was made available for the partners.

Molecular tools. For the purpose of molecular characterisation, various tools were developed. Single PCR tests for genes belonging to the antibiotic resistance classes MLS, streptomycin, tetracycline and vancomycin and species-specific tests were developed. In addition, PCR primers already published in literature were implemented. Besides these single PCR tests, a multiplex PCR system was developed for the detection of different antibiotic resistance genes simultaneously. This system employs a patented method with specific primers harbouring a common 5' head sequence. The amplified products were visualised using the ABI PRISM SNaPshot multiplex kit (Applied Biosystems) in combination with CE. Both primers and SnaPshot probes were designed for the following genes known to be present in lactobacilli and other LAB: *tet*(M), *tet*(S), *tet*(O), *tet*(W), *tet*(K), *tet*(L), *tet*(T), *erm*B, *erm*GT, *erm*C, *erm*A, *erm*TR, *msrA, mefA, aadE, mecA, vanA, vanB and aac-aphD*. Furthermore, a thematic microarray was developed containing approximately 300 oligonucleotide probes representing genes belonging to the following antibiotic resistance classes; Aminoglycosides, ESBL, Chloramphenicol, MLS, Sulfonamides, Tetracyclines, Trimetoprim and Vancomycin.

Observed resistance and genes involved. The observed erythromycin resistance phenotype in *S. thermophilus* strains isolated from Italian raw milk and cheese samples was due to the presence of the rRNA methylase *erm*B gene. To our knowledge, this is the first reporting of *erm*B in *S. thermophilus.* Atypical resistance patterns were also studied in *Lc. lactis* isolated from dairy environments (71 strains), lactobacilli strains from dairy and human origin (101 strains) and bifidobacteria isolated from faeces and mucosa of healthy people (76 strains). Atypical resistance patterns were found in 41 strains. For some of these strains the genes involved were analysed by PCR. Two *Lc. lactis* strains that were resistant to tetracycline contained *tet*(M), and one of them also had an internal fragment of *tet*(K). All bifidobacteria resistant to tetracycline gave amplification products with universal tet primers and specific primers for *tet*(W). However, no resistance genes were found in a set of tetracycline-resistant lactobacilli. In contrast, lactobacilli isolates resistant to erythromycin gave an amplification product with specific primers to detect *erm*B. Amplicons have all been sequenced, and the comparisons in databases proved the sequences to be homologous in high extend to their corresponding antibiotic resistant gene. For the analysis of a large number of lactococcal strains isolated from milk samples in Poland PCR tests specific for various tetracycline and

vancomycin genes were used. Among those strains *tet*(M) and *tet*(S) giving high tetracycline resistance levels are found (96 µg/ml, at least). *Lb. casei/paracasei* strains (n= 110) displaying very low MIC breakpoints for tetracycline and erythromycin and as such not displaying atypical resistances for these two antibiotics. The MIC range of susceptibility to streptomycin within these strains was much broader, which may be indicative for the fact that some of the strains tested contain atypical resistance for this agent. One remarkable finding in the microarray screening experiments was the detection of *tet*(O) in *Bifidobacterium*.

*Location of antibiotic resistance genes.*Work is in progress to confirm the possible linkage of the *erm*B and *tet*(S) in the genome of *S. thermophilus*. Furthermore, southern blot analysis showed that both *tet*(M) and *tet*(K) were plasmid encoded in *Lc. lactis*.The *tet*(M) gene was located on a rather big plasmid (estimated size range of 30-40 kb), whereas *tet*(K) was located on a small plasmid (estimated size of 3.5 kb). In these two *tet*(M)resistant strains, both ends of the tetracycline-resistant Tn916 transposon first isolated from *Enterococcus faecalis* were present, which suggested that the gene in *Lc. lactis* was similarly arranged as in *E. faecalis*. Within the 6 Tet^R strains from the Polish collection, the presence of one or multiple plasmids was confirmed.

Multidrug resistance. Intrinsic resistance was specifically studied in bifidobacteria. Four protein sequences with a high homology to MDR transporters, two ABC transporters (ATP dependent), and two secondary transporters (proton motive force dependent) were selected from the genome of *B. breve*. The genes coding for this proteins were cloned in *Lc. lactis* and the proteins were expressed using a nisin inducible plasmid. Susceptibility tests using E-test strips were performed with 22 different antibiotics from which was observed that the cells containing one of the secondary transporters, named BbmR, become significantly resistant to all the macrolides used for this study, azythromycin, erythromycin, dirithromycin and clarithromycin. Western blot analysis with a “his” tag variant of BbmR showed that it concerns a membrane protein. Growth experiments in liquid medium containing the four macrolides indicated that BbmR-expressing cells grow better in the presence of macrolides than control cells. Similar experiments were carried out for the transporters BbmA1 and A2. The preliminary characterization of BbmA1 and A2 suggested that they could build up an active MDR transporter.

Kefir: a border line probiotic between innovation and tradition

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Introduction: Research on fermented milks (FM) has grown dramatically in the past 20 years. FM have probiotic effects since their consumption leads to the ingestion of large numbers of live bacteria which exert health benefits beyond basic nutrition. Kefir is a refreshing fermented-milk with a slightly acidic taste originated many centuries ago, in the Northern Caucasus Mountains. Its use is currently being expanded because of its unique organoleptic properties and its long tradition of health benefits. It has a uniform creamy consistency, a slightly sour refreshing taste, with a mild aroma resembling fresh yeast (or beer like). Kefir has a slightest hint of natural effervescent zesty tang. There are an assortment of approx. 40 aromatic compounds, which contribute to the unique flavour and distinctive pleasant aroma of kefir. To round this all off, kefir may contain between 0.08% to 2% alcohol. Two types of kefir exist: sugary, a fermented sweetened water; and milk, a fermented milk beverage.

Kefir grains: Kefir distinguishes itself from the more known fermented milk yogurt in that it is traditionally made only from a natural-starter known as “kefir grains”. They resemble small cauliflower florets, and each grain is 3 to 20 mm in diameter. The grain's bio-structure, is created through the efforts of a symbiotic relationship, shared between a vast mixture of specific friendly Lactic acid bacteria (LAB) and yeasts. They are a soft, gelatinous white biological mass (biomass), comprised of protein, lipids (fats) and a soluble-polysaccharide Kefiran complex. The microbes and yeasts not only create the bio-matrix structure, they are harboured by the very structure that they create; abiding either on the surface (interior and exterior), or encapsulated within the bio-matrix itself. The grains are formed in the process of making kefir and only from pre-existing grains. The grains include primarily lactic acid bacteria (lactobacilli, lactococci, leuconostocs) and yeasts, and include acetic acid bacteria and possibly other microorganisms. The overall organization of microorganisms of grains is not completely elucidated. Some observations suggest surface areas consisting of vast irregularity or roughness, contain higher yeast activity. While smoother areas are mainly where bacteria predominate. Yeasts and bacteria cells, particularly yeasts, seem to form large surface concentration (micro-colonies) along the protrusions over the surface; streptococci seem to intertwine with other bacteria, without forming colonies. Research suggests internal structure of the grains show a predominance of *Lactobacilli* with few yeasts; cells are not bound to one another but encapsulated within a muco-polysaccharide believed to be produced by the encapsulated microorganisms. Other research suggests stained sections of grains studied under a microscope, showed that yeasts were mainly located on the edge of the internal cavities, and occasionally along the peripheral channels of the matrix. While the exterior was mainly occupied by bacteria. Short and long rod-shaped bacteria and yeast, formed separate colonies both on the outside and inside of the grain. Internally, filaments of encapsulated cells, extending outwardly from a population of long rod-shaped bacteria. One microorganism in particular, *Lb. kefiranofaciens* is found to be responsible for the formation of the soluble polysaccharide, Kefiran. This research suggests that the encapsulated bacteria may be responsible for the propagation of kefir grains. The type of medium, temperature and the amount of time that the grains are left in the same milk, all these factors influence growth-structure activity of kefir grains. A vast variety of different species of microbes have been isolated and identified in kefir grains. Such species are among four genus groups; *Lactobacilli, Streptococci - Lactococci, Acetobacter* and *Yeasts*. Bacteriocin may also be present, especially if the appropriate strains of lactic acid bacteria are present in the grains. Our

findings demonstrated that kefir grains cultured with buffalo's whey and cow's whey at room temperatures in aerobic condition increase weight to 120 % in 20 days.

Figure 1: kefir grains

Kefiran: A soluble gel polysaccharide [PS] discovered in kefir grains, was unique enough to be given its own name, kefiran [KGF-C]. Dry kefir grains consist of a matrix of which approx. 45% is kefiran. The PS is composed of two mono-saccharides: Glucose and Galactose in almost equal proportions. Kefiran is produced at the centre of the grain, synthesized by homofermentative Lactobacilli species including *Lb. kefiranofaciens* and *Lb. kefiri*. These particular Lactobacilli are encapsulated within the centre of the grain, where anaerobic conditions are favourable for kefiran synthesis in the presence of ethanol. Experiments performed with mice have revealed kefiran exhibited anti-cancer properties.

Kefir Process: There exist several methods of producing kefir (see Figure 2). Food scientists are currently studying modern techniques to produce a kefir with the same characteristics as those found in traditional kefir, but without some of its drawbacks. Kefir can be made from any type of milk: cow, goat, sheep, skimmed milk, coconut, rice or soy. There are many choices for milk: pasteurized, unpasteurized, whole fat, low fat, skim and no fat.

Traditional process: The traditional, method of making kefir is currently achieved by directly adding kefir grains (2-10%) to milk that has been pasteurized and cooled to 20-25°C. After a period of fermentation lasting around 24 hours, the grains are removed by filtration. The beverage, itself containing live microflora from the grain, is then ready for consumption. The grains grow in the process of kefir production, and are reused for subsequent fermentations. Grains can then be dried at room temperature and kept at cold temperature (4°C). For a longer conservation, they can be lyophilized (freeze-dried) or frozen. Kefir is stored at 4°C for a time then is ready for consumption. A second method, known as the "Russian method", permits production of kefir on a larger scale, and uses a series of two fermentations. The first step is to prepare the cultures by incubating milk with grains (2-3%), as just described. The grains are then removed by filtration and the resulting mother culture is added to milk (1-3%) which is fermented for 12 to 18 hours. Several problems associated with traditional kefir have led to a more modern method of production. The traditional method produces only small volumes of kefir, and requires several steps, each additional step increases the risk of contamination. In addition, the grains themselves are not well understood, and are not well controlled. Strong pressure from the CO_2 gas content can lead to the explosion of the recipient unless appropriate containers which resist the escaping of gas are used. Finally, the shelf-life of traditional kefir is very short, less than three days.

Recent process: To resolve the above difficulties, some producers in Eastern Europe have begun using concentrated lyophilized cultures made from grains. These mother cultures are then used as bulk starters for direct inoculation of the milk. More control over the process and fewer steps provide a more consistent quality.

Current areas of research: Attention is now being turned toward producing kefir from pure, defined cultures.This method will allow for a better control of the microorganisms involved, an ease of production, and a more consistent quality. The product will also have a longer shelf-life of 10 to 15 days at 4°C. It will also permit various modifications of the product to achieve certain health or nutritional benefits.

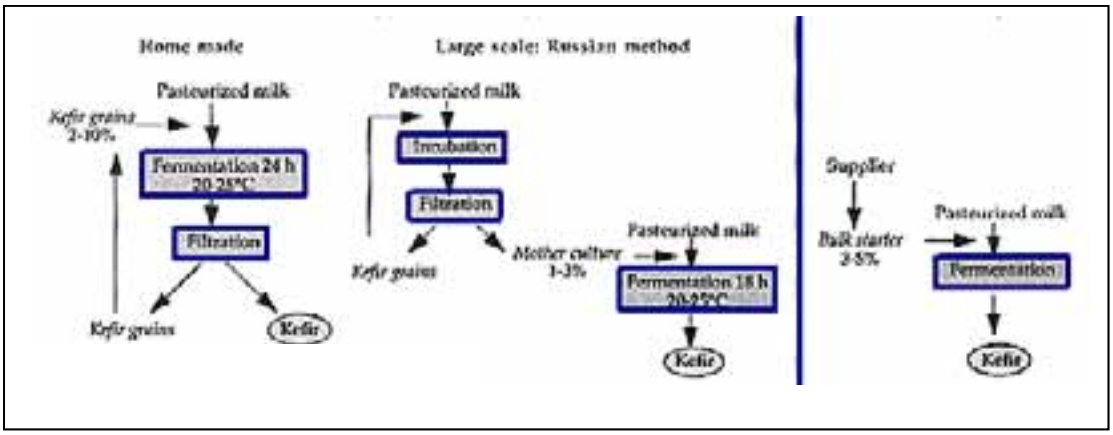

Figure 2: different methods of fabrication of kefir

Many health benefits related to the consumption of kefir have been observed, but rigorous research using modern scientific methods is in its early stages.

Kefir as a probiotic: Kefir contains live active cultures of normal flora which is made of vary strong strains of microorganisms that help to over take pathogenic organisms, repopulate the digestive tract and aid in digestion. The microorganisms predigest the protein that enchanting protein digest and absorption and also use the lactose thus many people whom have lactose intolerance problem can consume kefir.

The microbiological, chemical, and nutritional composition of kefir: The major products formed during fermentation are lactic acid, CO2, and alcohol. Many aromatic compounds, including diacetyl and acetaldehyde are present in kefir. Diacetyl is produced by Str. lactis subsp. diacetylactis and Leuconostoc sp.. The pH of kefir is 4.2 to 4.6. As in yogurt, the lactose content is reduced in kefir and the b-galactosidase level is increased as a result of fermentation. In addition to beneficial bacteria and yeast, kefir contains vitamins, minerals and essential amino acids that help the body with healing and maintenance functions. Kefir is rich in Vitamin B1, B12 , calcium, amino acids, folic acid and Vitamin K. It is a good source of biotin, a B vitamin that aids the body's assimilation of other B vitamins, such as folic acid, pantothenic acid and B .

Health benefits of kefir: Regularl kefir consumption can help relieve all intestinal disorders, promote bowel movement, reduce flatulence and create a healthier digestive system. The antibacterial, immunological, antitumoral and hypocholesterolemic effects of kefir have been investigated in recent studies. Kefir possesses antibacterial activity in vitro against a wide variety of gram-positive and gram-negative bacteria and against some fungi. Several studies have investigated the antitumor activity of kefir and polysaccharides from kefir grain. Kefir plays an important role of controlling high cholesterol levels in this way protecting from cardio vascular damage.

Conclusion: Kefir is a traditional product that provides variety to the range of fermented milks already available. It offers unique organoleptic properties and may in addition prove to possess certain health benefits that remain for now still shrouded in mystery. The current challenge in the dairy industry is to find a way to benefit from kefir's traditional roots, and at the same time to find a less complex and more practical way to produce a high-quality kefir with the same characteristic taste and texture. In the meantime, researchers should continue their efforts to demonstrate specific health benefits and to uncover the mechanisms involved.

HIGH CELL DENSITIES AND METABOLITES PRODUCTION IN *LACTOBACILLUS PLANTARUM* CULTIVATION

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Introduction

This research work is focused on the physiological characterization of *Lactobacillus plantarum* DSMZ 12028 specifically focusing on its probiotic potentialities as assessed exploiting *in vitro* experiments. This microorganism is known for its lipolytic ability [1], due to a glycerol ester hydrolase, EC 3.1.1.3, and has interesting applicative potential being a good L(+) lactic acid and exopolysaccharides (EPSs) producer. Furthermore related strains proved to secrete bacteriocins. We aim to achieve high density fermentations in order to promote *L. plantarum* use as a starter in food industry, and eventually as a probiotic strain in food supplements. In addition the selection of optima conditions for metabolites production may broaden the applicatication of these molecules (e.g. EPSs and bacteriocins).

Lactic acid bacteria are considered to be weakly lipolytic compared with the activities of psychrotrophs, micrococci or brevibacteria and little is known about their contribution to cheese lipolysis and flavour development [2]. In food fermentations the ability of produce organic acids and EPSs is technologically very relevant. The EPSs produced *in situ* result in a natural product with no need of additives to improve the structure of yogurth and cheese. Some studies indicate that EPSs from lactobacilli may be beneficial for human health because they mediate the probiotic effect of different strains. The strain selected of *L. plantarum* was able to survive a simulation of the digestive process *in vitro* [3], a first requisite for a definition as probiotic microrganism. However, studies on its metabolites and eventual interactions of EPS with living intestinal cells is very important to assess its ability to beneficially influence human and/or animal health.

From a technological point of view the best way to improve biomass yield in the fermentation of LAB is to limit the accumulation of lactic acid that is responsible for growth inhibition. However, it is of key importance to evaluate the parameters of inhibition kinetics to design custom tailored processes that achieve the target also minimizing process costs. Various strategies have been lately reported in literature, but frequently the developed processes were focused on the production of a “single product” (either biomass or lactic acid or EPS). Here we present a particularly efficient strategy based on a membrane bioreactor that with the exchange of exhaust medium through microfiltration permit to prolong growth also recovering exocellular products in continuous [4].

Materials and Methods

A semidefined medium was formulated similarly to the one previously reported [4], as complex components we generally used bactocasitone. Few experiments were performed using whey, and also adding oils to simplified media in order to characterize the lipolytic activity of the strain on vegetable fats.

Fermenter experiments

The fermenter used was a Biostat CT, Braun Biotech International (Melsungen,Germany), 2 L working volume, equipped with a DCU and connected to a PC for remote control via MFCS-win software.

The vessel was modified: two polypropylene microfiltration (MF) modules, assembled as previously described (Schiraldi et al., 2000), were inserted into specific stainless steal holders

which were fixed to two baffles, and they were connected to a peristaltic pump (model 313U, Watson Marlow, England) which supplied the driving force for transmembrane flux. Prior to fermentation, the MF modules were sterilised *in situ* together with the medium. During cultivation backflushings were operated by simply inverting flux for 1-2 min every 30/60 min. Successively a solution containing the salts of the medium recipe was pumped reversing the flux to increase the cleaning efficiency.
Lactobacillus plantarum DSMZ 12028 was grown at T = 30°C, pH = 6.5, the stirring velocity was initially set at 100-200 rpm and only with air in the head space without aeration.When truly anaerobiosis experiments had to be performed the medium was sparged with nitrogen after sterilization prior to inoculation for at least 30 min.
Experiments in batch mode were carried out also using a Biostat Q (Braun Biotech Int.), that permitted to compare different medium at once with its four independent vessel.
The fed-batch experiments started in batch mode, using semi-defined, then a concentrated nutrient solution containing 400 $g \cdot L^{-1}$ glucose, 100 $g \cdot L^{-1}$ bactocasitone and 25 $g \cdot L^{-1}$ yeast nitrogen base was added in the late exponential phase (6-8 h) following either a step or a linear profile (implemented through the digital control unit, DCU), in order to increase the rate of glucose addition. At about 22 h experiments a second feed stock solution was prepared containing less complex components (i.e. 25 % of the nominal concentration).
The MF experiments started in batch mode, switched to fed-batch and h later to MF mode. Each phase duration was settled upon evaluation of lactate formation, glucose consumption rate and their influence on specific growth rate. Throughout the MF experiments few samples, opportunely diluted, were plated on MRS agar to prove the cell viability.
In addition to cell mass, organic acids produced, consumption of substrates (measured by HPLC) and EPS production, it was also evaluated the ability of secreted bacteriocins to inhibit growth of few pathogenic strains. Using plate tests it was also possible to asses the kinetic of production of this molecule.

Results and Discussion

MF experiments improved 4-fold the biomass and lactic acid production in comparison with batch cultures: about 30 OD_{600} were obtained in 44 hours, with a productivity in lactic acid of 2,64 g/l·h and a yield of 0,80, for the total amount of 126,43 g, while a batch culture in the same conditions produces only 35 g.
Bovine whey was compared to a mixed bovine-ovine whey in batch cultures; both solutions were supplemented with glucose or malt extract and a complex nitrogen source, such as bactocasitone or soy peptone. The best results were obtained with the mixed origin whey, probably due to the richer lipidic fraction still present after cheese making: 11 OD_{600} were obtained with malt extract as carbon source and 10 OD_{600} on glucose, in comparison with the 9,7 OD_{600} obtained on a semidefined medium. Milk whey also stimulated a heterolactic fermentation of *L. plantarum*, and in particular the synthesis of isobutyrric acid in amount 36-48% of total organic acids produced. In the experiments performed with a supplement of vegetable oils 1% p/v in a SDM medium, a prolonged lag phase was observed in comparison with standard SDM. In the first 24h of culture olive oil supplement and peanuts oil supplement reached 5,5 OD_{600} and 6,3OD_{600} respectively, while standard SDM reached 8 OD_{600}, but after 55h olive oil was metabolized reaching 10 OD_{600}, and on peanuts oil we achieved 13,2 OD_{600}, while decrease in absorbance was registered on standard SDM. As for the lactic acid production, when olive oil and peanuts oil were added to the medium, yields on sole glucose reached respectively 0,85 and 1,43, both superior to the ones previously obtained.

Preliminary experiments on membrane separated fractions showed that also this strain is secreting a bacteriocin during growth and that the inhibition activity is deputed to a molecule of molecular weight comprised between 3 and 10 KDa.
Further investigations of *in vitro* adherence to human cells are in progress in order to assess the possible use of *L. plantarum* as probiotic strain.

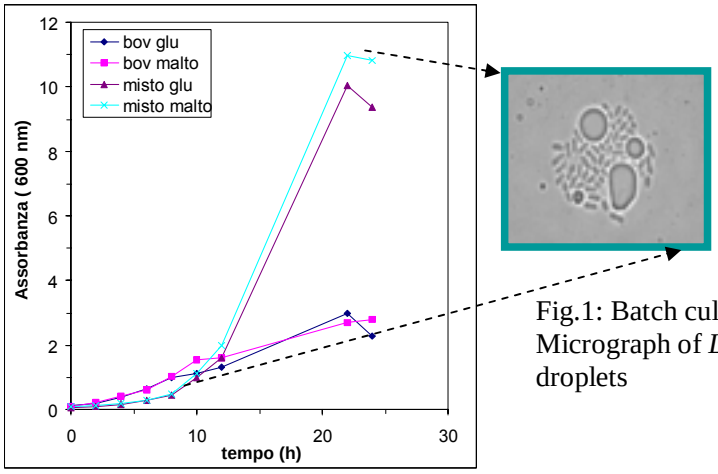

Fig.1: Batch cultivation on different exhaust whey. Micrograph of *L.plantarum* cells covering the fat droplets

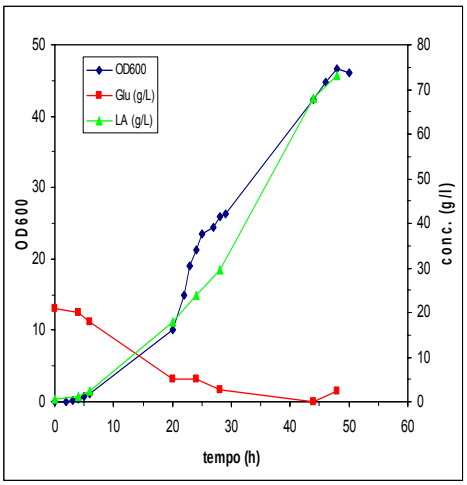

Fig.2: MF fermentation of *L.plantarum :* glucose consumption and LA production during the experiment.

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Dietary modulation of blood pressure

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Background

Hypertension is a world-wide risk factor for cardiovascular diseases, including coronary heart disease, peripheral arterial disease and stroke. Nutritional factors have a considerable impact on the treatment and prevention of hypertension. The life-style factors, such as nutrition, moderate alcohol use, exercise and abstinence from smoking are important part of the treatment of hypertension. Several studies have shown that a low intake of sodium and a sufficient intake of potassium, calcium and magnesium help lower blood pressure and prevent the development of hypertension. High intake of fibre and unsaturated fatty acids has been associated with a reduction in blood pressure in several studies. Epidemiological and intervention studies have shown that consumption of milk and milk products are inversely related to the risk for hypertension. Current evidence on the connections between milk peptides and blood pressure also suggests that milk peptides may have antihypertensive activity.

Milk peptides and blood pressure

Ile-Pro-Pro and Val-Pro-Pro have been shown to reduce blood pressure in spontaneously hypertensive rats (SHR) after a single oral administration (Nakamura et al., 1995). They also prevent the development of hypertension in SHR after long-term, twelve and thirteen weeks, oral feeding (Sipola et al., 2002, Sipola et al., 2001). At the end of the twelve-week treatment period systolic blood pressure was 17 mmHg lower in the group receiving *L. helveticus* LBK-16H fermented milk containing Ile-Pro-Pro and Val-Pro-Pro than in the control group receiving water ($p<0.001$) and 12 mmHg lower in the group receiving the tripeptides in water than in the control group (Sipola et al., 2001). In a thirteen-week study, *L. helveticus* LBK-16H fermented milk-containing Ile-Pro-Pro and Val-Pro-Pro tripeptides attenuated the development of hypertension more effectively than water ($p<0.001$) or the *L. helveticus* and *S. cerevisiae* fermented milk containing half as much the same peptides than *L. helveticus* LBK-16H fermented milk ($p<0.001$) (Sipola et al., 2002). It has been shown that α-Lactorphin (Tyr-Gly-Leu-Phe) lowers blood pressure dose-dependently in SHR and in normotensive Wistar-Kyoto (WKY) rats. The blood pressure was measured with continuous radiotelemetric monitoring and the maximal reductions in systolic and diastolic blood pressure were 23 ± 4 and 17 ± 4 mmHg, respectively (Nurminen et al., 2000).

Lactobacillus helveticus fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptide has also been shown to decrease systolic and diastolic blood pressure in hypertensive subjects (Hata et al., 1996, Seppo et al., 2003, Seppo et al., 2002, Tuomilehto et al., 2004). In a placebo-controlled study on hypertensive subjects, *L. helveticus* and *S. cerevisiae* fermented milk reduced systolic and diastolic blood pressure ($p<0.05$) during the eight-week intervention more than placebo-fermented milk (Hata et al., 1996). In an eight-week placebo-controlled study on 17 hypertensive subjects systolic and diastolic blood pressures were lowered more in the group receiving *L. helveticus* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides than in the control group receiving normal fermented milk fermented with *Lactococcus sp.* mixed culture ($p=0.05$ and $p<0.05$) (Seppo et al., 2002) and also in the long-term clinical study (21 weeks) systolic and diastolic blood pressure were decreased more in *L. helveticus* fermented milk group ($n=22$) than in the control group receiving fermented milk ($n=17$) (SBP 6.7 ± 3.0, $p=0.030$ and DBP 3.6 ± 1.9, $p=0.059$) (Seppo et al., 2003) (Figure 1). Tablets, containing Ile-Pro-Pro and Val-Pro-Pro tripeptides, have been shown to decrease blood pressure in mild or moderately hypertensive

subjects (Kajimoto et al., 2001). Milk that has been fermented using *Lb. casei* and *Lc. lactis* and that contains γ-aminobutyric acid (GABA) reduced blood pressure during a 12-week treatment period. Systolic blood pressure lowered more in fermented milk group than in the control group ($p<0.05$), but diastolic blood pressure of the fermented milk group did not differ from the control group (Inoue et al., 2003). In an other eight-week-long study systolic blood pressure was significantly lower in the group receiving yoghurt fermented with two strains of *Streptococcus thermophilus* and two strains of *Lactobacillus acidophilus* and in the group receiving yoghurt fermented with one strain of *Enterococcus faecium* and two strains of *Streptococcus thermophilus* compared to the group receiving yoghurt fermented with two strains of *Streptococcus thermophilus* and one strain of *Lactobacillus rhamnosus* (Agerholm-Larsen et al., 2000).

Mechanisms of the antihypertensive effects of milk peptides

Angiotensin-converting enzyme inhibition

One mechanism by which milk-derived peptides can reduce blood pressure is inhibition of ACE (Maeno et al., 1996, Mullally et al., 1996, Nakamura et al., 1995, Nakamura et al., 1995, Nurminen et al., 2000, Sipola et al., 2002, Takano T, 1998, Yamamoto et al., 1999). This is the mechanism that has been studied most in relation to the antihypertensive effects of milk peptides. ACE is an enzyme that plays a crucial role in the function of the renin-angiotensin system (RAS). The RAS is an important regulator of blood pressure and fluid and electrolyte balance (Brown and Vaughan, 1998). In the RAS angiotensin I is converted to angiotensin II by ACE. Angiotensin II is a strong vasoconstrictor that induces release of aldosterone and therefore increases sodium concentration and furthers the blood pressure. ACE inhibitors have two effects on the renin-angiotensin system. They reduce production of angiotensin II and inhibit the degradation of the vasodilator bradykinin by Kininase II, the same enzyme as ACE.

Several antihypertensive peptides that inhibit ACE have been isolated from milk products and the ACE inhibition activity of these peptides has been determined. The IC_{50} values are the concentrations at which ACE activity *in vitro* is inhibited by 50%. The relationship between ACE inhibitory peptides and the chemical structure has not been confirmed, but it has been suggested that peptides, with hydrophobic amino acids at the C terminal position could be the most likely ACE inhibitors (Ondetti and Cushman, 1984).

Peptides derived from casein by *L. helveticus* proteases have been shown to have ACE inhibitory activities (Yamamoto et al., 1994). ACE inhibitory activity of casein-derived tripeptides Ile-Pro-Pro and Val-Pro-Pro has been shown *in vitro* (Nakamura et al., 1995). *L. helveticus* fermented milk-containing Ile-Pro-Pro and Val-Pro-Pro raised plasma renin activity in SHR during long-term treatment (Sipola et al., 2002). Also ACE activity in aorta of SHR was reduced after single oral administration and after long-term treatment with *L. helveticus* and *S. cerevisiae* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides (Masuda et al., 1996, Nakamura et al., 1996). Antihypertensive peptides with insignificant ACE-inhibitory activity have also been isolated from milk products (Maeno et al., 1996, Nurminen et al., 2000, Yamamoto et al., 1999). Therefore milk-derived peptides could also affect blood pressure by mechanisms other than ACE inhibition.

Conclusion

The future challenge is to understand the mechanisms and absorption of antihypertensive ingredients and thus to find more possibilities to use these ingredients and products as a dietary treatment of hypertension.

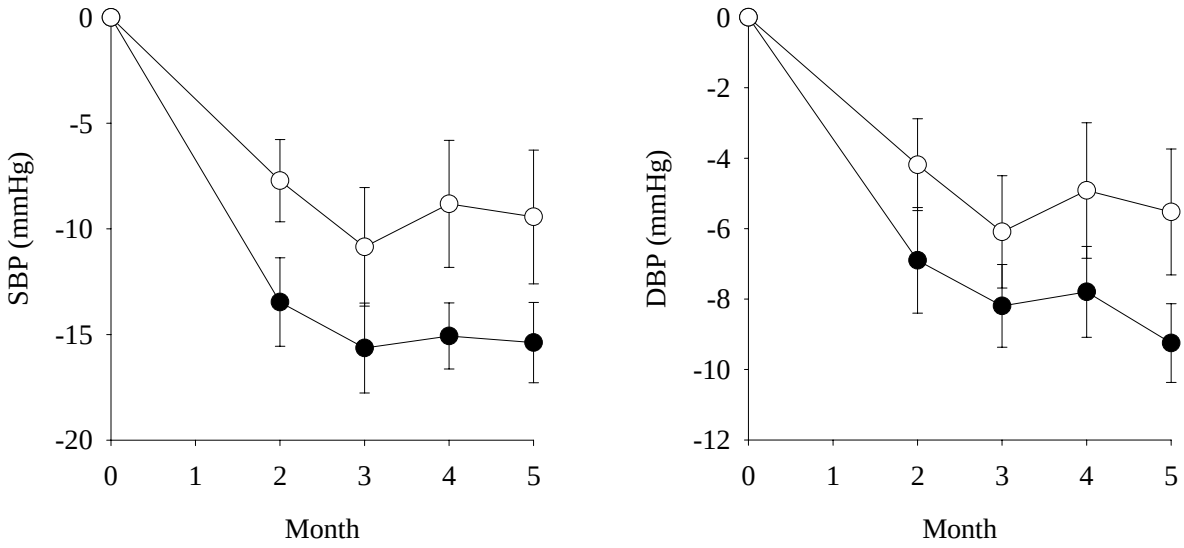

Figure 1. Blood pressure lowering effect of fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides (Seppo et al. 2003). ○ = control product ●= fermented milk containing IPP and VPP

DIETARY MODULATORS OF GUT MICROBIOTA IN INFANT FORMULAS

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Introduction

During early infancy diet is thought to be a dominant factor regulating the sequence of gut microbial colonisation and composition. In addition to promote normal gastrointestinal functions and protect against pathogenic bacteria, gut microbiota plays a key role in maintaining a disease-free state of the host, as specific strains provide the immune system with stimuli resulting in tolerance and maturation to nonallergic mode. During the first months of life the two primary sources of nutrition for infants are human milk and infant formula. Human milk is considered to be a complex biological system, making exclusive breast feeding the golden standard for infant nourishment. Whenever breast-feeding is not available in adequate amounts, cow's milk based infant formulas (starting formulas) provide a safe and healthy food for growth and development, although they cannot replicate the bioactive properties of human milk.

Gut microbiota biomodulators

It has been suggested that protective commensal microflora be regarded as part of the human body, as one of the body's most metabolically and immunologically active organ ("the microbial organ") (1).

It is generally accepted that breast and bottle infants have different flora compositions: lactobacilli and bifidobacteria dominate the colonic flora of the breast-fed infant whereas the formula-fed infant has a more diverse flora. The establishment of gut microbiota begins at birth and the composition of commensal bacteria colonizing the intestine is in part dependent on mode of delivery (natural vaginal delivery *vs* caesarean section), neonatal nutrition (breast milk *vs* formula feeding) and antibiotic administration. The type of postnatal colonisation and the intensity and the timing of the microbial stimulus represent key protective modulators of the naïve immune system against atopy (2). As the critical time period during which immune-modulation with long-lasting effects is considered crucial is early infancy, it could be of clinical interest to selectively manipulate colonic flora in formula-fed infants, by using prebiotics (non-digestible oligosaccharides), These *gut microbiota biomodulators* beneficially affect the intestinal ecosystem and correct the imbalanced composition. Herein, the opportunity for gut flora dietary manipulation arises in infants fed formulas supplemented with (3-4).

The term **prebiotic**, coined by Gibson and Roberfroid in 1995 (5), indicated *a non-digestible food ingredient that affects the host by selectively targeting the growth and/or activity of one or a limited number of bacteria in the colon*. More recently, Van Loo defined a prebiotic effect as *a food-induced increase in numbers and/or activity predominantly of bifidobacteria and lactobacilli in the human intestine* (6). In order for a food ingredient to be classified as a prebiotic, it must be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract, be able to alter the colonic flora in favour of a healthier composition and induce luminal or systemic effects that are beneficial to the host health. Although any foodstuff that reaches the colon (resistant starch, dietary fibre, some peptides and proteins) is a candidate prebiotic, current prebiotics seem to be confined to non digestible oligosaccharides (NDOs). Of all the NDOs, inulin, fructo-oligosaccharides (FOS), galacto-

oligosaccharides (GOS) and lactulose have been the most thoroughly investigated and a prebiotic effect has been convincingly proved (6-7-8). Inulin and FOS are compounds with a vegetable origin. Inulin is a group of fructose polymers (or fructans) linked by ß(2-1) bonds that limit their digestion by upper intestinal enzymes; chain lengths of these fructans range from 2 to 60 units. Oligofructose is a mixture of oligosaccharides composed of fructose units linked together by ß-1,2-glycosidic linkages and part of them are terminated by a glucose unit. The total number of fructose or glucose units in an oligofructose molecule generally ranges between 2 and 8 (9). Industry obtains FOS from beetroot via transfructosilation, using fructosilfuranosidase synthesised by *Aspergillus niger* (10). Galacto-oligosaccharides are animal compounds provided by cow's milk. GOS, consisting of chains of galactose with a terminal glucose molecule, vary in chain lenght from 2 to 7 monomers which are linked mainly by ß (1-4) linkage (11). They are obtained from lactose via transgalactosilation, using ß-D-galactosidase synthesised by *Bacillus circulans*.

The predominance of bifidobacteria and lactobacilli in the gut microbiota of breast-fed infants is essentially due to the prebiotic effect of human milk oligosaccharides (HMOs), representing its third largest solute (12). Furthermore, HMOs are potent inhibitors of bacterial adhesion to intestinal epithelial surfaces, which represents the initial stage of the infective process (13). The highest concentrations of HMOs can be found in colostrum (20 g/L), but even mature milk contains oligosaccharides in concentrations up to 13 g/L. Recently, 130 different oligosaccharides have been characterized and many more are expected to be identified (14). They are formed by sialic acid, *N*-acetylglucosamine, L-fucose, D-glucose, and D-galactose. Resistance to hydrolysis by amylases and glycosidases in the small intestine is a prerequisite for the HMOs to reach the large intestine, where they could serve as a preferred substrate for bifidobacteria and lactobacilli (15).

Prebiotics in infant formulas: closer to the reference

The composition of starting formulas, that are based on bovine milk, has been adjusted to that of human milk with respect to most of the constituents. The latest phase of formula development has been to add non-nutritional "bioactive" components and protective factors, but the constituents that are currently receiving most attention are oligosaccharides. Normal starting formulas do not contain oligosaccharides as the complexity and variety of HMOs make it impossible for the food industry to reproduce them. However, if the composition and structure cannot be imitated, the effect and function can be mimicked. The addition of non-digestible carbohydrates (that have a comparable prebiotic effect to HMOs) to starting formulas brings this alternative, second choice infant feeding one step closer to the golden standard (human milk).

Boehm (16) demonstrated in formula fed preterm infants that an experimental prebiotic oligosaccharide mixture of 90% low-molecular-weight galacto-oligosaccharides (GOS) and 10% high-molecular-weight fructo-oligosaccharides (FOS), at a concentration of 1 g/dl, stimulated the growth of intestinal *Bifidobacteria*, compared with a control group fed on the same formula but supplemented with maltodextrin as placebo ($p = 0.0008$). More recently, the same author found that at the end of a 28-day feeding period in preterm infants fed with the prebiotic formula, the sum of the pathogens was lower, compared to the group fed on the placebo formula ($p = 0.039$) . Moro (17) analyzed in 90 term infants the bifidogenic effect of two test starting formulas supplemented with the same oligosaccharide mixture at different concentrations (0.4 g/dl and 0.8 g/dl). In the control formula, maltodextrin was used as placebo. At the end of the 28-day feeding period, the number of *Bifidobacteria* was significantly increased for both groups receiving prebiotic formulas versus the placebo group ($p < 0.001$), and the effect was dose dependent. The number of *Lactobacilli* also increased significantly in both groups fed on the supplemented formulas versus placebo ($p< 0.001$), but was not dose dependent. Recently, a starting formula with the GOS/FOS mixture and hydrolysed whey protein was compared

with a standard formula for the effect on the gut microbiota composition (18). The results obtained by using fluorescence *in situ* hybridisation (FISH), showed that in the prebiotic group *Bifidobacteria* increased whilst the proportion of *Clostridia* and *E.coli* was reduced and the ratio of *Bifidobacteria* to *Clostridia*, which has been reported to be related to atopic diseases (19), was shifted in the direction of non atopic-infants ($p<0.05$). In a recent randomized double-blind study, 154 healthy formula-fed term infants, aged younger than 2 weeks, were randomized to receive either this new prebiotic formula or a standard formula until the age of 12 weeks (20). The aim of the study was to determine the concentration of bifidobacteria and to investigate the nutritional adequacy of the formula containing GOS and FOS and partially hydrolyzed proteins, as recommended by ESPGHAN (21). When compared with the standard formula, the prebiotic formula led to higher counts of bifidobacteria in the faeces and supported satisfactory growth.

Recently, Moro performed a RCT in 41 term infants with family history of allergy, randomised to be fed with either a formula supplemented with GOS/FOS mixture or with the same quantity of maltodextrines as placebo. The aim of this study (*in press*) was to evaluate the prevention effect on the occurrence of allergic biomarkers (IgE, IgG4, IgE/ IgG4 ratio). Infants fed prebiotic formula had significant decreased IgE levels, increased IgE/ IgG4 ratio (an important predictive biomarker for the development of allergy) and IgG4

Maturation of the gastro-intestinal tract is a dynamic process that is still in progress at birth; the small intestine functions as a selective barrier, which is reflected by selective permeability to macromolecules. As the barrier function is still developing at birth, increased intestinal permeability is a normal finding in the neonatal period. It can have beneficial effects (uptake of larger nutritional molecules and development of systemic tolerance), but also disadvantages (increased uptake of infectious agents leading to infection, inflammation, and hypersensitivity). The aim of our recent study (Miniello and Francavilla, *submitted*) was to assess the timing of maturation of the gut barrier by the measure of the intestinal permeability (IP) in exclusively breast fed full term infants born by vaginal delivery, with no family history of atopy and in formula fed infants randomised to receive either a formula supplemented with GOS/FOS mixture or placebo. The trial shows that in breast fed term infants born by vaginal delivery the IP is high at birth, has a rapid decline in the first 10 days of life, reach the median normal value at day 20 and completes within the first two months of life. Interestingly, infants receiving the formula supplemented with prebiotics showed an IP pattern similar to breast fed infants that was significantly different than that of infants fed with the non supplemented formula at day 10 and 20 of life. This may have profound implications in the timing of antigen permeation and subsequent sensitisation.

Menard demonstrated that active metabolites released by probiotic bacteria (*Bifidobacterium breve* BbC50 and *Streptococcus thermophilus* St065) exert anti-inflammatory effects, by inhibiting the realise of TNF-α and by stimulating IL-10 secretion, a cytokine with anti-inflammatory properties. These two lactic acid bacteria are used to ferment formula milks.

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Effect of prebiotics in preventing intestinal and respiratory infections in infants.

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Intestinal immune system development begin soon after the birth in parallel with the microbiological colonization of the gut. There are evidence that a temporal relation exists between the appearance of bacterial in the gut and the presence of circulating immunoglobulins. Namely, gut colonization by Bifidobacteri and Lactobacilli has been showed to able to increase IgA and IgM serum levels (1). More recently it has been shown that newborns whose gut was colonized by Bacteroides fragilis during the 1st month, had a higher number of Ig-producing cells during the 2nd month (2).

It seems clear that intestinal microflora is able to actively influence the mucosal immune response of the gut and, at the same time, microbiota play a pivotal role in the host pathogens interaction and in development of food tolerance. It is now clear that the host-pathogens interaction involve in the gut, immune and non-immune mechanism. The latter are mainly represented by tight junctions, mucus and intestinal peristalsis, which are able to block the adhesion and penetration of microbes; other non immune-mechanisms consist of different substance with antimicrobial properties: lisozyma, dephensines, lactoferrin and intestinal microflora.

Intestinal microflora plays its antimicrobial effect through several mechanisms: nutrient and receptors competition, production of antimicrobial molecules and short chain fatty acid (SCFA). Although these mechanisms are able to modify the intestinal microenvironment and thus exert an antimicrobial effect, it is now of major interest the immunomodulatory ability of intestinal microflora.

In vitro and in vivo data have demonstrated that intestinal microorganisms are able to influence any aspects of immune response, by directly acting on immune cells or by amplifying the enterocyte-mediated immunity (3).

It is now possible to modify the intestinal microflora by administration of microorganisms able to colonize the gut (probiotics), or alternatively by using substances that are able to stimulate the growth of specific bacteria in the colon (prebiotics).

Several evidence indicate that Lactobacillus GG is effective in the treatment of Rotavirus gastroenteritis in children (4). The efficacy of LGG in the prevention and treatment of enteric infections is widely established (5) and it is likely to be related to a competitive effect with selected pathogenic bacteria, and/or to the release of antimicrobial substances and/or to the stimulation of the immune response (6). The mechanism seems likely to be associated to the ability of LGG to stimulate the mucosal immune response during the acute phase of Rotavirus infection (5).

In addition some probiotics have been found to be effective in the prevention of acute diarrhea both in children and adults, especially in population at high risk. LGG was found to reduce the incidence of acute diarrhea in malnourished Peruvian children (6) mainly in children not breast-fed. In addition, a mixture of Bifidobacterium bifidum and Streptococcus thermophilus (7) and LGG alone (8) were both found to reduce the incidence of diarrhea in hospitalized children.

Because of the interaction between the gut lymphoid associated tissue (GALT) and the bronchial associated lymphoid tissue (BALT) the protective effect of probiotics involves the respiratory tract. It has been showed that the prolonged administration of LLG protects children aged 1 to 6 years against respiratory infections (9). As upper respiratory infections are widely common the beneficial effect of probiotics would also results in economic advantages. These data were not confirmed using other probiotics (10).

Prebiotics are not digestible food able to stimulate the growth of selective intestinal micro-organisms such as Bifidobacteria. Bifidobacteria are the prevalent specie of bacteria in intestinal flora of breast fed infants. Maternal milk contains not digestible substances acting as a prebiotic. Several oligosaccharides show an anti-infective effect. Fucoso-oligosaccharides are able to inhibit

the binding between E. coli termostable toxin and its specific receptor on intestinal epithelial cells, guanilato-cyclase (11). Other oligosaccharides inhibits the ability of Campylobacter jejuni in colonizing the gut (12). In recent years several evidence showed that the adjunct of prebiotics to standard formula are effective in stimulating the growth of Bifidobacteria and Lactobacilli in term and (13) preterm infants (14). No evidence are available showing the effect of prebiotics on clinical parameters as incidence of infectious episodes. Our preliminary results support the hypothesis that supplementation with prebiotics not only modify the intestinal flora but also showed clinical beneficial effects. In a prospective study children assuming for 12 months a formula containing a mixture of fructooligosaccharides and galactooligosaccharides (GOS/FOS) showed a lower number of intestinal and respiratory infections than children assuming a standard formula. During the 12 months of follow up, 543 acute infections were recorded, of which 222 in the group of children assuming GOS/FOS and 319 in children assuming the standard formula. A significant reduction in the incidence of intestinal infections was observed in children assuming probiotics. In the same group a lower, but not significant, number of upper respiratory infection was recorded. The data support the hypothesis that the addition of probiotics may be effective in reduce the recurrence and the severity of the infections A significant lower number of children with more than three respiratory infection was registered in prebiotic group. The use of antibiotics for treatment of respiratory infection was also lower in children assuming prebiotics. Finally, the same enriched formula is able to determine a more pronounced increase in body weight and height in the first six months of follow up.

In conclusion intestinal microflora seems to play an important role in the development of immune system and in the prevention of intestinal and extraintestinal infections. The early use of substances able to beneficially affect the composition of intestinal microflora, has the potential to promote immediate and long term effects on the health and well-being of infants. However, randomized controlled trials are needed to confirm these effects and to demonstrate the absence of harmful consequences from their use.

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PREBIOTICS IN HUMAN MILK.

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The microbic colonization of human intestine begins at birth, when from a sterile state the newborn is exposed to an external environment rich in various bacterial species. The kind of delivery has an important influence on the composition of the intestinal microflora in the first days of life. Thereafter, irrespective of the type of delivery, the development of the intestinal flora is mainly influenced by the kind of feeding. In fact, breast-fed infants show a microbic flora characterized by the predominance (90%) of Bifidobacteria and Lactobacilli, the so called bifidogenic flora. On the contrary, the bottle-fed infant develops a mixed flora with lower number (about 50%) of Bifidobacteria.

Since the beginning of the nineteenth century, it has been shown that human milk contains substances which favour the growth of bifidogenic flora in the intestine of breast-fed infants, thus having a prebiotic effect. The first data concerning the nature of these compounds emerge from Schonfeld's studies: he proved that the prebiotic effect derives mainly from non-protein portion of human milk. Later, Gyorgy et al found that the growth-promoting bifidus factor was made up of a mixture of oligosaccharides called "Gynolactose". Thereafter, Khun et al. identified N-acetyl-glucosamine containing oligosaccharides as the prebiotic factors.

A prebiotic effect has also been proposed for other milk substances: in particular, lactoferrin and nucleotides.

Bullen et al. hypothesized that lactoferrin in human milk can exclude iron from bacteria and limit their growth. Bifidobacterium bifidum, that do not have an obligatory requirement for iron can proliferate instead. Their studies showed that human milk or purified human lactoferrin limits the growth of several strains of E. coli. Moreover, a direct bactericidal effect of human milk lactoferrin was showed against Streptococcus mutans and V. cholerae. Therefore, the effect of lactoferrin should be ascribed to an inhibitory effect on a pathogenic flora rather than a direct stimulus to the development of Bifidobacteria.

A role for nucleotides in establishing fecal flora in infants was suggested by the work of Gill et al. In their study infants fed with nucleotide supplemented formula had a fecal flora somewhere between that of the breast-fed infants and standard formula-fed infants. A subsequent study by Balmer et al, however, failed to show any positive effect of adding nucleotides to an infant formula on the proportion of Bifidobacteria in the fecal flora.

On the contrary, strong evidence exists on the prebiotic properties of human milk oligosaccharides. These substances are carbohydrates made up of 3-9 monosaccharide units and are quantitatively the third component of human milk, after lactose and lipids. They reach the highest concentration in colostrum (more then 20 gr/L) and then decrease, after about two weeks, to approximately 12 to 14 gr/L in mature milk. On the contrary, cow's milk contains less than 1 gr/L oligosaccharide (fig. 1).

Human milk oligosaccharides are synthesized in the mammary gland by specific enzymes, the glycosyltransferases, by adding sequentially monosaccharide units (galactose, fucose, N-acetyl-glucosamine, sialic acid) to the basic molecule of lactose, thus forming compounds with both linear and branched structures. A peculiar characteristic of such substances is that monosaccharides, of which they are comprised, are bound by specific bonds resistant to the enzymes present in the newborn intestinal wall (lactase, saccharase-isomaltase, maltase-glucoamylase, amylase). As a

consequence most oligosaccharides ingested with mother's milk pass through the small intestine undigested and reach the colon.

At this level, undigested oligosaccharides are used by the microflora. They cause a "biomass effect" characterized by the selective stimulation of the Bifidobacteria and Lactobacilli development.

Recent studies have shown that the pattern of carbohydrates present in breast-fed infant feces is almost the same as that of ingested milk. On the contrary, only traces of oligosaccharides are present in bottle-fed infant feces. It follows that the considerable difference in the composition of the intestinal microflora between the breast-fed newborn and the bottle-fed one is strictly related to the different milk oligosaccharide content.

In conclusion, oligosaccharides are the most important prebiotics present in human milk.

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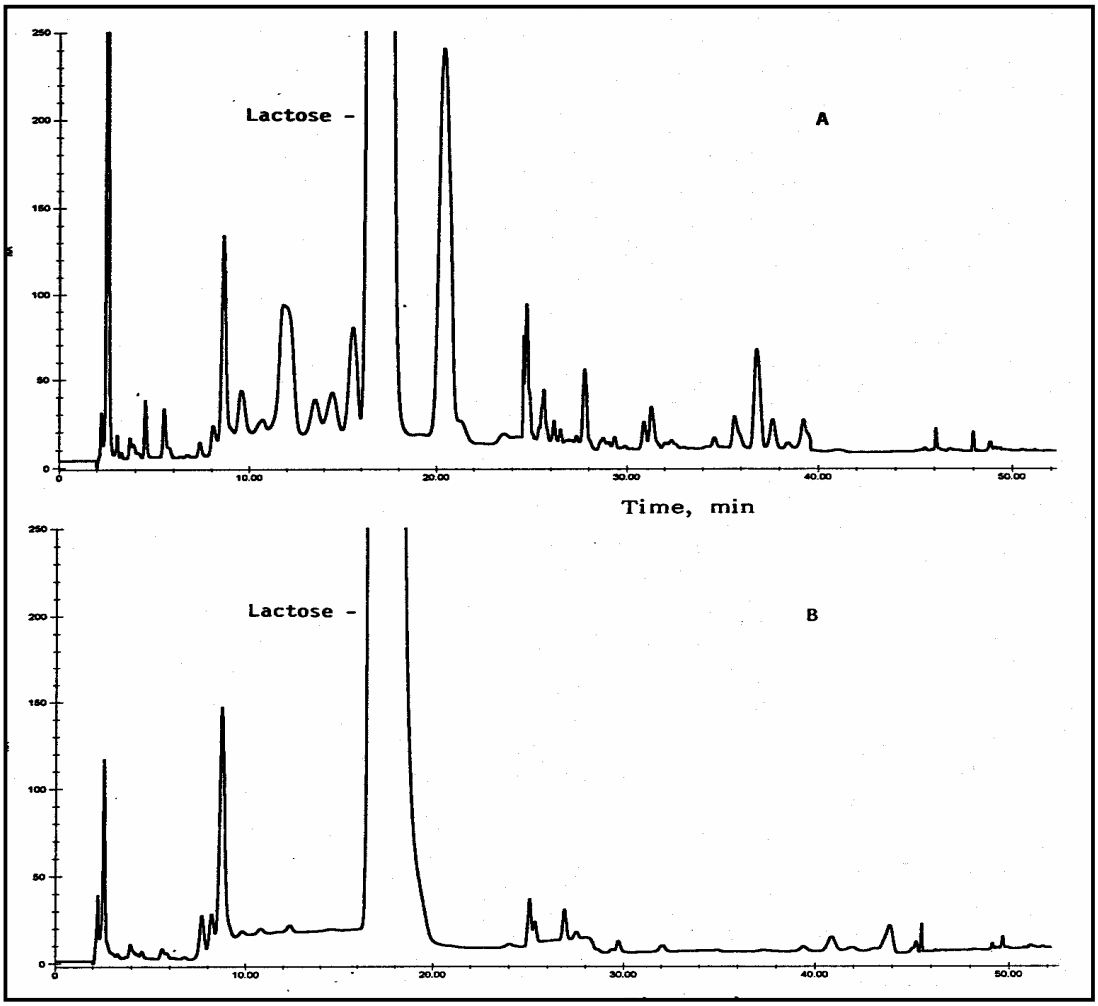
Figure 1. High performance anion exchange chromatography of milk carbohydrates. A) human milk oligosaccharides and B) cow's milk oligosaccharides.



The Effect of Probiotics on the Integrity of the Intestinal Mucosa.

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Abstract:
The effect of probiotics on the function and integrity of the intestinal barrier may be one of the prominent mechanisms whereby probiotics provide benefit to the host. While our understanding of bacteria-host interaction is increasing, those specific gut microflora that can promote maturation of the intestinal mucosa and can modulate inflammatory events are under study. There are a number of reported mechanisms by which probiotics may exert their influence on host barrier integrity at multiple levels including the intestinal epithelial cell layer, the supra cell layer and the submucosal elements. The literature discusses the role of adhesion, modulation of the immune system and translocation events. This presentation will explore pre-clinical and clinical reports from studies conducted by the Institut Rosell and with interjected supporting reports from the literature. Specifically, the presentation will examine the ability of probiotic strains of Lactobacilli and Bifidobacteria to adhere to human cells in culture and examine some of the surface molecules associated with the adhesive properties. Results will be presented on the regulation of mucin gene expression and various interleukins and cytokines both *in vitro* and *in vivo* and the impact of these microbes on the inflammatory process. This will be followed by a discussion on the application of how some of these microbes maintain membrane impermeability by reducing cytoskeletal rearrangement of the human epithelial cells. The results are viewed in light of new pharmacological studies.

What is the Intestinal Mucosa?
The intestinal mucosa is generally defined by its three distinct layers (1. a monolayer of epithelial cells; 2. lamina propria; and 3. muscularis mucosae). The intestinal epithelial layer is comprised of a dynamic, self-renewing population of epithelial cells that includes secretory cells (such as mucus-secreting goblet cells), endocrine cells found in the crypts of Leiberkuhn and the mature micro-villi presenting absorptive epithelial cells which take up nutrients from the lumen and transport them into blood, fulfilling the basic function of the digestive system. The tight junctions of the columnar epithelial monolayer can sometimes be interrupted by specialized epithelial M cells in regions called Peyer's Patches. These cells transport antigens and microbes from the apical surface through the cytoplasm to the basolateral side and the rest of the gut-associated lymphoid tissue (GALT) in the lamina propria. The lamina propria contains loose connective tissue, lymphatics, blood vessels and nerves. This rests upon the muscularis mucosae which is comprised of smooth muscle fibres and elastic connective tissue.
There is another supra epithelium layer, a polymeric mucus gel bilayer, which functions as both a semi-rigid protective barrier and a fluidized bio-film lubricant. The thicker upper mobile phase of this bilayer is represented by secreted mucins such as MUC2 and the thinner more rigid phase is represented by membrane-tethered mucins such as MUC3. This mucin bilayer is the primary contact with the intestinal microflora.
The integrity of the intestinal mucosa defines the 'Gut Barrier Function'.

What Is the Gut Barrier Function?

The gut barrier function is a complex set of mechanical, innate and adaptive impediments that act to reduce or eliminate potentially harmful components from the intestinal lumen, such as microbes and endotoxins, from traversing the epithelium and entering the aseptic organs, tissue and circulating blood. This barrier function is perceived to be three distinct barriers, namely the ecological barrier (commensal gut microbes), the mechanical barrier (mucous epithelia) and the immune barrier (secretory IgA and cells of the immune system within the lamina propria). Often, the mechanical or structural epithelia is considered to be the predominant aspect of the gut barrier.

Effect of Microbes on the Development of the Intestinal Mucosa

In mammals, the proper development of the intestinal mucosa is dependent upon the exposure and presence of the commensal microflora during post-natal maturation. An elegant study by Hooper *et al* (2001) introduced *Bacteroides thetaiotamicron*, a normal human and mouse gut microbe, into germ-free mice. This microbe normally colonizes the gut during the weaning period and correlates with the maturation of the gut development, specifically a decrease in ileal epithelial lactase activity. This activity is a marker of gut development. Hooper *et al* observed in the adult germ-free mice a similar reduction in this marker when *B. thetaiotamicron* was given suggesting similar maturation was occurring. Furthermore, they observed a 280-fold increase in the villus epithelial expression of small proline-rich protein-2 (sprr-2) mRNA. The sprr family members contribute to the barrier functions of the epithelia, especially in squamous cells where they have been observed as a component of the cornified cell envelope and as a cross-bridging component to the desmosomal protein, desmoplakin.

Effect of Probiotics on Mucin Expression

Work by Mack *et al* (2003) demonstrated that probiotic lactobacilli can adhere to HT-29 cells in culture and increase the expression of mucins (MUC2 & MUC3). Furthermore he has gone on to show that this effect requires viable lactobacilli and has observed that, in the intestines of rats fed probiotics, the increased mucin expression was both regional and time-dependent. For example, rats receiving oral administration of *Bifidobacterium bifidum* R0071 showed significantly increased levels of rMUC3 after 2 days in the jejunum (204%) and ileal segments but after 10 days only baseline levels were observed. Colonic segments did not show any increased expression (see poster of Godwin, Hyde & Mack at Rome meeting).

Adhesion of Probiotics to Intestinal Epithelial Cells of the Intestinal Mucosa

Many of the pharmacodynamic properties of probiotic microbes are purported to occur due to direct interaction of the microbe with the epithelial cells lining the intestinal mucosa. Correlation of *in vitro* adhesion to *in vivo* residence time has yet to be determined but it is generally considered that strains that show better adhesive properties will make more effective probiotics. There are many *in vitro* assay systems using various human gastric or intestinal cell lines such as KatoIII, Caco-2, T84 or HT-29 cells. The HT-29 cells tend to be the most commonly used because of their shorter doubling time. Determination of adhesion can be done by direct enumeration of the microbes, or labelling the bacteria with biotin or radioactive markers. A variety of competition assays

can be done with antibodies against the strains, or carbohydrates or glycosphingolipids in order to help elucidate the nature of the adhesion. For example, M. Kostrzynska of Ag-Canada, Guelph, ON has evaluated the adhesion of *B. infantis* R0033 in detail. Dr. Kostrzynska *et al* observed that the adhesion of this microbe to Caco-2 cells could be inhibited in part by fucosyllactose, sulfatides and fetuin (see Table 1) but asialo-fetuin and other carbohydrates and glycolipids had no effect. The fucosyllactose and sulfatide anti-adhesive effects were additive suggesting the involvement of two separate receptors whereas fucosyllactose and fetuin inhibitions were averaged suggesting the involvement of a single receptor. Sulfatides have been implicated previously in the adhesion of pathogenic *H. pylori* (Wadstrom *et al*, 1997). Thus, it would appear that there may be direct competition for binding sites on the epithelial cell surface.

Table 1: Inhibition of adherence of *Bifidobacterium infantis R0033* to Caco-2 epithelial cells by various compounds.

Compound	**Inhibition of Adherence (%)**
2'Fucosyllactose	28 +/- 5.9
3'Fucosyllactose	30 +/- 4.0
Lewis-B tetrasaccharide	24 +/- 2.7
L-Fucose	0
D-Lactose	0
Fetuin	61 +/- 3.5
Asialofetuin	0
Sulfatides	39 +/- 8.7
2'Fucosyllactose + Sulfatides	69
2'Fucosyllactose + Fetuin	47
2'Fucosyllactose + Glucosamine	23
Sulfatides + Fetuin	72
Sulfatides + Glucosamine	27

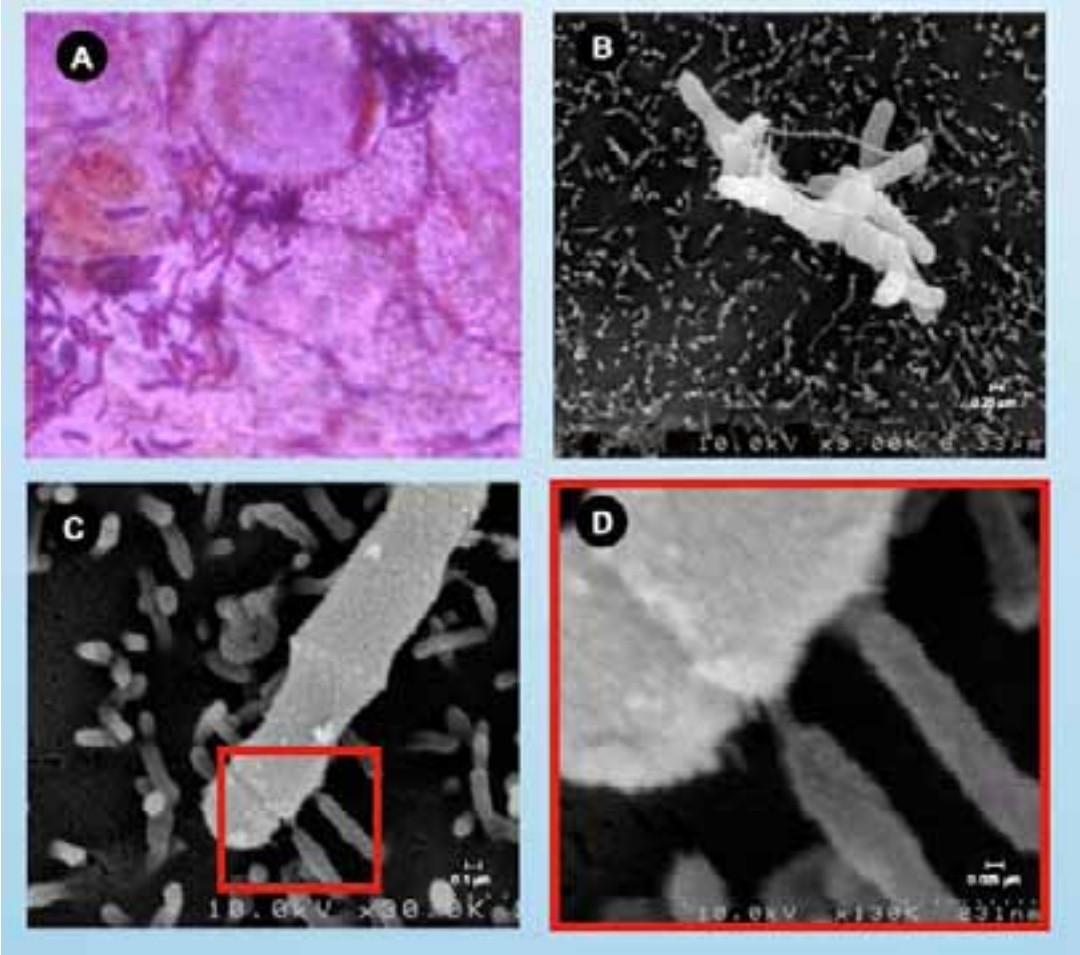

Figure 1. Scanning electron microscopy of *B. infantis* R0033 adhering to Caco-2 intestinal epithelial cells. A. Light microscope image of *B. infantis* R0033 adhering to Caco-2 cells. B-D. Scanning electron microscope (SEM) images of *B. infantis* R0033. C. Intimate interaction between R0033 and microvilli. D. inset of C.

Probiotic Impact on Tight Junctions, Permeability and Translocation.

Microbial competition for sites of adhesion can be demonstrated by running exclusion, competition and displacement experiments between probiotic strains and enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *E. coli*. Sherman *et al* (2005) demonstrated that *L. acidophilus* R0052 and *L. rhamnosus* R0011 could exclude (i.e., they need to be present first) the adhesion of EHEC and EPEC but they could not effectively compete with nor displace the pathogen adhesion to polarized T84 epithelial cells in culture. There was a corresponding reduction in the number of attaching and effacing lesions induced in response to both EHEC and EPEC infections when lactic acid bacteria were pre-incubated with host cells. Both EHEC and EPEC decreased trans-epithelial electrical resistance (TEER) to 20% of control values, whereas the lactobacilli had no effect on TEER. There was a time-dependent attenuation of the drop in resistance produced by EHEC and EPEC when T84 cells were pre-treated with probiotic microbes, showing that tight junctions were maintained. Moreover, immunofluorescence microscopy showed that viable probiotic agents reduced the number of foci of rearrangements of alpha-actinin by the *E. coli*.

It can be shown in rat models that increased chronic psychological stress (water avoidance stress, WAS) induces intestinal barrier dysfunction, increases bacterial adhesion and translocation, and initiates mucosal inflammation. Pre-treatment of these animals with the probiotic microbes *L. acidophilus* R0052 and *L. rhamnosus* R0011 completely abrogated WAS-induced bacterial adhesion and prevented the translocation of bacteria to mesenteric lymph nodes.

Studies by other groups have suggested a role for probiotics in maintaining the expression of zonula occludens 1 (ZO-1) tight junction protein (Montalto *et al*, 2004; Resta-Lenert and Barrett, 2003). The expression of ZO-1 can be downregulated by histamine and by NSAIDs such as ASA. Brzozowski *et al* (2005) induced ulcers in rats then demonstrated that treatment with ranitidine, an anti-ulcer agent, or ASA, a NSAID, increased the gastric colonization of *Candida albicans*. In this model, treatment with the probiotic Lacidofil® (*L. acidophilus* R0052 and *L. rhamnosus* R0011) reduced the colonization and allowed ulcer healing. Concurrent with this there was a significant drop in plasma TNF-α and IL-1β. Controlling inflammatory events across the intestinal mucosa may be the most important contribution of probiotics.

Impact of Probiotics in Controlling Pro-inflammatory Events in the Intestinal Mucosa.

Studies examining the *in vitro* immune response by human intestinal epithelial cell lines have shown that all probiotic microbes do not elicit the same response. Wallace *et al* (2004) observed that *L. rhamnosus* R0011 suppressed IL-8 levels in HT-29 cells that constitutively express this chemokine yet it upregulates IL-10 levels. Another chemokine, RANTES, was also downregulated by this probiotic as was the pro-inflammatory cytokine, TNF-α. RANTES acts as a chemoattractant for monocytes and T cells, and causes the release of histamine from basophils, which may contribute to its role in exacerbating such diseases as asthma, arthritis and inflammatory bowel disease.

Elevated levels of IL-8 may contribute to bacterial attachment to epithelial cells by upregulating adhesion proteins (Baggiolini *et al*, 1995). This pathway tends to feed back upon itself, for example, IL-8 induction by *Helicobacter pylori* appears to be involved in its ability to produce peptic ulcer disease (Hersh *et al*, 1998).

In animal models where IL-10 has been knocked out, nearly any irritation (ex. *E. faecalis*, fats, etc.) will produce a state of chronic inflammatory colitis. It is interesting to note that this seems to be linked to defective ability to produce MUC2 (Schwerbrock *et al* 2004)

Studies with genetically modified *Lactococcus lactis* expressing human IL-10 (described by Shanahan as "turbo probiotics", 2001) offer the best chance to treat inflammatory bowel diseases. Dr. Steidler is sure to provide more exciting details on this development.

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Live, genetically modified *Lactococcus lactis* in IBD therapy
Lothar Steidler

Introduction

The intestinal microflora is a key component of all complex metazoans metabolism and immune system. As well as adding to health, the intestinal commensals sometimes drive pathology. An example is inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC)[1;2]. Treating these kinds of disorders with selected or genetically modified (GM) microflora therefore appeals to the creative mind.

IBD represents an important issue in public healthcare. Alongside a notable increase in immune diseases driven by normally harmless antigen such as asthma and autoimmune pathologies, the incidence and prevalence of IBD has since the early 1950s either continued to increase or has stabilized at a high rate - 2.5 per 1000 population - in most western countries. The increase in the incidence of UC appears to precede the increase in the incidence of CD by about 10-15 years[3;4]. This was again demonstrated clearly in recent studies conducted in Iceland[5] and Sweden[6]. The number of people affected continues to rise in those regions where IBD had been less common, such as in western Hungary, where IBD now has become as frequent as in Western European countries[7].

CD and UC typically display a frequent recurrence after effective induction therapy. Conventional chemical therapeutics are effective to a certain extent but improved maintenance therapy remains the greatest unmet medical need in treating IBD[8]. High profile drugs such as anti-TNF monoclonal antibodies (infliximab)[9], steroids or immune suppressive chemicals are still required for remission induction. Although such medications are definitely prominent therapeutics in severe IBD, many remain hesitant to utilize such highly powerful tools for long term treatment. The explosion in our knowledge of molecular immunology and genetic engineering may however provide an alternative: the design of GM bacterial therapeutics.

Genetically modified *Lactococcus lactis*

Over centuries food-grade bacteria, such as *L. lactis;* used in the production of Gouda, Cheddar, Mozzarella and Camembert type cheeses, have extensively been consumed by humans. Impact of these organisms on health is very well documented and has on the whole been considered as supportive to the well-being. Nowadays, with the industrialization of food and feed production, this impact is monitored systematically. As pathologies associated with their consumption are extremely scarce, their nutritional use is hardly ever disputed. One intrinsic advantage of *L. lactis* therefore lies in the knowledge that they are not pathogenic, not even when given overt opportunity, as would be the case during an ongoing intestinal disease.

L. lactis can be genetically modified (GM) to secrete fully functional cytokines and various other regulatory proteins derived from eukaryotes. Simply by local application, these GM *L. lactis* can actively deliver such cytokines to the mucosa. The mechanism by which this occurs involves *in situ* synthesis of the recombinant proteins. By use of this principle we developed novel therapeutic approaches for the treatment of IBD.

Interleukin-10 (IL-10) is a cytokine that suppresses inflammation and plays a central role in T-cell driven tolerance induction and maintenance. Attempts have been made to administer recombinant IL-10 to IBD patients. The results were not completely

successful because injection of high concentrations of IL-10, required to attain the necessary level in the intestine, led to moderate side effects[10-14]. This impedes the long-term use of IL-10 at high doses. Moreover, oral administration is hampered by the extremely acid sensitivity of IL-10. We tried to circumvent all these drawbacks by constructing an IL-10 secreting *L. lactis* strain[15]. Daily oral doses of this strain could efficiently cure colitis in a DSS-induced murine model and prevent the onset of colitis in IL-10 knockout mice. When the bacteria were killed by UV-treatment the positive result was abrogated. This shows that the *in situ* production of IL-10 is essential for curing colitis. After oral administration these recombinant bacteria arrive at the inflicted area and produce the therapeutic agent. Because of this localized production it can be speculated that side effects associated with systemic administration can be reduced or even avoided.

Trefoil factors (TFF1, 2 and 3) form a class of non-mitogenic peptides that are important in the protection and repair of the intestinal epithelium[16] and accordingly are promising tools for treatment of acute colitis. Acute intestinal inflammation – acute colitis - is characterized by extensive epithelial ruptures. Treatment thereof may be a means to prevent the onset of IBD, but the number of therapeutic strategies for acute colitis is rather limited. Accordingly, the need for new methods is evident. Despite extreme resistance of TFF to acid and enzymatic degradation, no successful oral TFF formulation has been reported. The main reason for this is that luminal administered TFF stick to the mucus of the small bowel and are removed from the lumen at the caecum[17]. Active *in situ* delivery of TFFs in the colon by localized synthesis from *L. lactis* circumvents this problem.
GM *L. lactis* are able to produce and secrete biologically active murine TFF in suitable quantities to allow for oral application and subsequent adequate production *in situ*[18]. Daily intragastric administration of the TFF secreting strains, prior to or during disease induction, resulted in significant protection against DSS-colitis as observed by reduced mortality, reduced loss of body weight, substantial improvement of the colon histology and reduction of inflammatory infiltrate. The protective effect requires *de novo* TFF synthesis by live *L. lactis*. Oral administration of high amounts of purified mTFF1 did not ameliorate acute colitis, whereas rectal administration did, albeit much less effective than orally administered mTFF secreting *L. lactis*. Basolateral contact between colonocytes and the GM *L. lactis* cells now probably enable TFF to accumulate out of reach of complexing mucins and allow them to interact with the putative basolateral TFF receptors on enterocytes[19].
Ptgs2, a known TFF target gene[20; 21], is strongly induced in the intestine of mice treated with mTFF secreting *L. lactis*. This proves that recombinant TFF were biologically active *in situ* in the colon. Ptgs2 contributes to the healing and down-regulation of the inflammatory responses in the gastrointestinal tract [22; 23]. Inhibition of Ptgs2 by meloxicam substantially abrogated the prophylactic effect of mTFF producing *L. lactis*.

Prospects
Systems such as the ones described above offer fascinating possibilities for future use in medicine. The deliberate release of live GM bacteria, as would occur following medical application, however raises legitimate concerns. Before they can be applied they have to be redesigned to reconcile medical effectiveness and biological safety.
We established adequate means for inheritable growth control of engineered *L. lactis* by genetically exchanging the chromosomal thymidylate synthase gene *thyA*, the gene

encoding an mandatory enzyme in the synthesis of the DNA constituents thymidine and thymine, for the IL-10 gene[24]. The resulting GM *L. lactis*, Thy12, is strictly dependent on the presence of thymidine or thymine for its growth and survival. In contrast to deprivation of any other metabolic mutant of its complementing metabolite, thymidine starvation of Thy12 leads to induced cell death, due to increased DNA damage and subsequent induction of SOS-repair genes and fragmentation of the DNA. This phenomenon had already been reported for a long time and is known as thymineless death[25]. Readily amenable systems based on this phenomenon have however not been described up to now. This approach has received approval from the Dutch authorities for the conduct of the first clinical trial ever that utilizes a live GM bacterium as a therapeutic. It may well be that the conduct of this clinical trial leads us into the development of a completely novel pharmacology: GM bacterial therapeutics.

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Highlight on Lactobacillus reuteri

Chairpersons: M. Campieri, G. Dobrilla

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Symposium " Highlight On *Lactobacillus reuteri*"
Rome, September 5th 2005

Symposium Chairpersons Introduction

Lactobacillus reuteri the emerging probiotic

Prof M Campieri, Prof G Dobrilla

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Probiotics are nonpathogenic microorganisms of the indigenous intestinal microflora that protect their host, and in some cases they can prevent disease.
They contribute to the mucosal barrier function and stabilize intestinal permeability.
Some probiotics have been shown to modulate the mucosal immune response and reduce gastrointestinal inflammation in the treatment of many conditions including infectious diarrhea and chronic diarrhea, inflammatory bowel disease, irritable bowel syndrome, and food allergy in infants.

Lactobacillus reuteri, the research breakthrough in probiotics, has been shown to have superior efficacy compared to the many other strains that lack scientific backing and valid stability data.
Animal and human studies have shown that *L. reuteri* possesses desirable properties of a probiotic microorganism, including well documented safety and functionality combined with excellent technological characteristics.
In fact, no other strain of lactobacillus has the diverse health benefits of *L. reuteri*.
Its supporting scientific documentation should change what one expects from a probiotic.

Lactobacillus reuteri can prevent diarrhoea binding sites on the gut mucosa, preventing pathogenic bacteria from adhering to the mucosa.
Lactobacillus reuteri also produces compounds, reuterin and reutericyclin, that act as local antibiotics against more pathogenic organisms.
The wide spectrum of pathogens inhibited by *L. reuteri* includes *Helicobacter pylori.*

This biological feature of *L. reuteri* opens new and interesting prespects in the treatment and prevention of *H. pylori* infection in humans.

Moreover some recent studies in children show that re-establishment of the ecological balance of the intestinal flora with the *Lactobacillus reuteri* administration could play an important role in treatment and prevention of atopic dermatitis and in infantile colic in the new born.

The establishment and maintenance of innate immune tolerance is mediated by T helper 1 cells and linked in some way to the faecal flora.
If the Th1 response is particularly robust, the allergic response mediated by T helper 2 cells tends not to be so strong.
Lactobacillus reuteri may prevent atopy by supporting the microbiota, strengthening the Th1 response, and reducing the allergic response.

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Lactobacillus reuteri
A Unique Immunoprobiotic Agent for Improving Human Health

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Abstract

Elie Metchnikoff (1) introduced the 'probiotic concept' over 100 years proclaiming: "A general belief is that microbes are harmful. This belief is erroneous. There are many useful microbes, amongst which the lactic bacilli have an honorable place."

Only recently, however, have some of Metchnikoff's purported 'honorable lactic bacilli' been isolated and identified. A enterolactobacillus species - *Lactobacillus reuteri* - was discovered to be a rich source from which to isolate 'honorable' strains of Metchnikoff's 'lactic bacilli'. And, over twenty years of intensive research on this species have brought his 'probiotic concept' into the mainstream of biomedicine.

This species was discovered to produce and secrete a unique antimicrobial substance, termed reuterin, and it was found to be one of only few *Lactobacillus* species indigenous to the human and animal GI tract.

This information led a group of American and Swedish microbiologists on a twenty year quest, commencing in 1985, to evaluate host-specific strains of *L. reuteri* for probiotic efficacy in humans and animals.

Probiotic efficacy being defined at that time as: Live commensal microbes administered orally in adequate amounts able to confer health effects on the host by improving its intestinal balance. The quest proved successful.

Host-specific strains of *L. reuteri* strains were found to confer broad-spectrum protection from an assortment of microbial and chemical-associated diseases in humans and in various animal model systems. Human clinical trials also proved successful, and strains of *L. reuteri* were shown to meet all the requirements demanded of true probiotics.

Recent studies aimed at obtaining an understanding *L. reuteri'* s mode(s) of action revealed unique immunomodulatory activities, and it has been proposed that strains possessing such activities be henceforth referred to as immunobiotics rather than as probiotics.

However, certain strains of *L. reuteri* are known to produce numerous substances that may also enhance the health and well being of hosts through non-immunological processes. Therefore the term -immunoprobiotic - rather than the term - probiotic - has been proposed for *L. reuteri*. This report is a synopsis of *L. reuteri's* twenty year journey from probiotic to immunobiotic to immunoprobiotic status - a journey that parallels development and evolution of the 'probiotic concept' itself.

Lactobacillus reuteri - A Unique Probiotic

Commencing with studies initiated in 1985, it was discovered that when compared to their placebo-treated counterparts, oral formulations of host-specific strains of *L. reuteri* administered to their respective animal model hosts conferred significant broad-spectrum protection from an assortment of microbial (bacterial, protoazoan, viral, and fungal) infections, chemical (methotrexate) challenges, and environmental (cold stress) stressors.

Successful placebo-controlled, double-blinded human clinical trials were also conducted during this period. They revealed significant biotherapeutic effectiveness in treating rotavirus diarrhea and significant prophylactic effectiveness in preventing community-acquired diarrhea in children (2).

The *L. reuteri* ATTC 55730 strain used in human clinical trials is unique in comparison to other probiotic strains in that it was isolated from the nursing milk of a young healthy Peruvian mother.

The many successful human clinical trials carried out using this strain, and the successful animal field trials using other host-specific strains, indicated that this particular species of 'lactic bacilli' had developed a truly synergistic relationship with its respective human and animal hosts.

This unique *L. reuteri*-host relationship was subsequently confirmed when it was shown to be one of only three or four 'lactic bacilli' species indigenous to both human and animal GI tracts. Based on continued laboratory studies and numerous clinical trials it was shown that *L. reuteri* strains meet all the requirements demanded today of a *bona fide* probiotic, namely:

Requirements	***L. reuteri* Compliance**
Species/strains identified	**Yes -** Genetically & physiologically identified; genome sequenced.
Physiology, metabolism known	**Yes -** Heterofermentative metabolism & physiological properties well studied
Ecosystem determined	**Yes -** GI tract and mammary ducts.
Safety determined	**Yes -** In infants, adults, animal models.
Antimicrobial produced	**Yes -** Reuterin, reutericyclin, bacteriocins.
Survival in gut & colonization	**Yes -** Excellent colonization.
Efficacy determined	**Yes -** In humans and various animals.
Mode(s) of action determined	**Yes -** Beneficial immunomodulation
Commercial availability	**Yes -** W/ patents, insured OTC viability as capsules, tablets, straws, powders, drops, yogurts, drinks, lozenges, and gums.
Clinical studies	**Yes -** To be summarized by Dr. Eamonn Connolly
Publication in peer-reviewed journals	**Yes -** Over 120 publication to date

These and other investigations carried out during the past twenty years confirmed *L. reuteri* as a *bona fide* probiotic.

They also contributed significantly to validation of the 'probiotic concept' (2). It is noteworthy that during this same period clues were obtained as to *L. reuteri's* underlying mode(s) of action. It was shown, for example, that when compared to placebo controls, *L. reuteri* administrations resulted in (a) significant down-regulation of GI tract inflammations caused by microbial infections or chemical insults, and (b) a significant increase in $CD4^+/CD8^+$ T cell ratios in avian ileal tissues in both infected and control animals.

To the best of the author's knowledge, this was the first observation of a probiotic effect detected in a healthy host. And, as will be noted below, this effect has now also been observed in a human host.

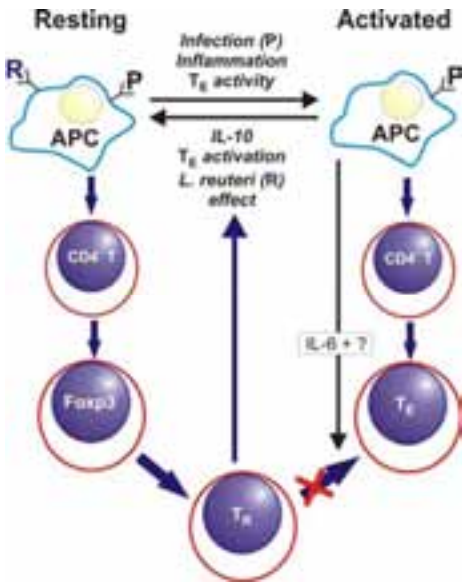
Lactobacillus reuteri - A Unique Immunobiotic

In recent years, rapid advances have been recorded concerning various molecular events underlying *L. reuteri's* mode(s) of action. For example:

- The above described increase in the avian gut $CD4^+/CD8^+$ T cell ratio attributable to *L. reuteri* administrations has now been seen in the human ileum as well (3). This is the first *in situ* study with human subjects to show that oral administrations with *L. reuteri* resulted in specific colonization of these cells throughout the GI tract, from the stomach to the large intestine. Particularly noteworthy in this study, however, were the following two findings. **First,** when compared to subjects examined prior to *L. reuteri* administration, those examined after probiotic administrations were found to have increased levels of $CD4^+$ T cells in their ileal region.

  Secondly, and to the best of the author's knowledge, this is the first demonstration of a 'probiotic effect' on a healthy human host other than merely establishing that colonization occurred. This finding of a 'probiotic effect' on healthy humans addresses an often asked generic question: Why should a healthy person consume *L. reuteri*?

***L. reuteri* and Dendritic Cell Interactions:**

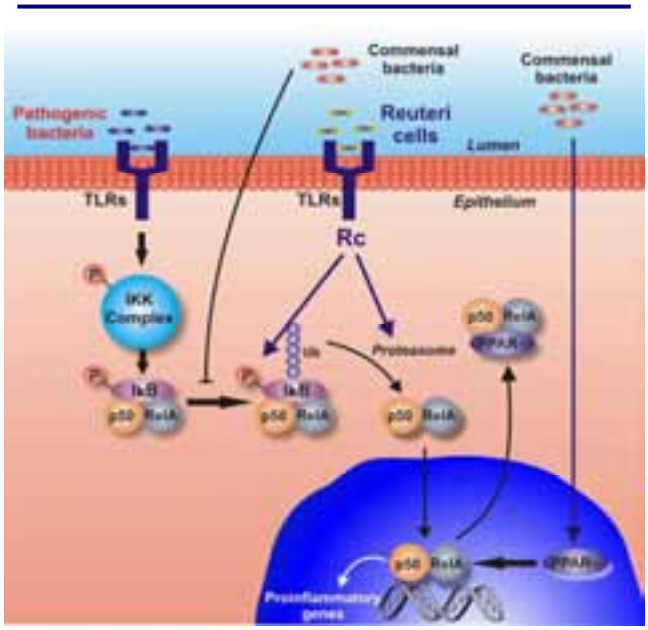


It has been shown that dendritic cell deactivation leads to a production of regulatory T cells and moderation of inflammation.
(Christensen et al. Immunol. 2002, 168:171/ Powrie&Maloy 2003 ; Science

This question can now be answered as follows: **Consumption of appropriate quantities of viable *L. reuteri* cells insures that the host's gut mucosal immune system is primed with T helper cells prepared to signal restoration of a healthy homeostatic state when confronted with microbial or chemical inflammatory challenges.**

- Recently published studies have provided valuable insights as to how *L. reuteri* is able to accomplish these tasks.

L. reuteri and Intestinal Epithelial Interactions



It has been shown that L. reuteri cells down-regolate intestinal cell inflammatory responses by preventing NK-kB access to the nucleus.
(Ma et al. Infect. Immune. 2004 : 72 :5308)

Briefly stated: Intestinal dendritic cells (DCs) and intestinal epithelial cells (IECs) possess receptors (pattern-recognition receptors, PRRs, and Toll-like receptors, TLRs) which recognize both pathogenic and beneficial microbes such as *L. reuteri*. Through these receptors, pathogens induce DCs and IELs to produce inflammatory substances.

To prevent an overproduction of these inflammatory substances, and thereby an unhealthy immunopathology, viable *L. reuteri* cells have been shown to **(a)** down-regulate production of inflammatory substances by IECs, and **(b)** convert DCs to a resting state required for conversion of naïve T cells into T regulatory cells (Tregs). Tregs function to down-regulate production of inflammatory substances by T effector cells which are produced in response to the pathogenic or other inflammatory stimuli.

Thus, *L. reuteri* (human strain ATCC 55730) once considered one of many commensal species found in the gut, and shown to have potent probiotic efficacy, has now been elevated to immunobiotic status, and it may now be viewed as a gut luminal regulatory agent shown to play an important role in maintenance of intestinal homeostasis and prevention of inflammation, and thereby accounting, at least in part, for its proven clinical efficacy.

A review of these new findings is in preparation (4), and the term immunobiotic rather than probiotic to better describe *L. reuteri* has been temporarily adopted as recommended by Clancy (5).

L. reuteri - A Unique Immunoprobiotic

Clancy (5) proposed that the term 'probiotics' was perhaps outdated and inappropriate to describe microbes believed to enhance human and animal health, but about which little is known as to their mode(s) of action other than an ability to colonize the GI tract, exhibit strictly local effects, and suppress and/or exclude putative pathogens via secretion of antimicrobial substances.

Clancy proposed the term 'immunobiotics' for those microbes shown to activate "the common mucosal system through the stimulation of gut antigen-presenting cells to both promote protection and to switch regulatory mechanisms".

Based on the studies described above, *L. reuteri* can now be designated an immunobiotic species. However, *L. reuteri* is also unique in that some strains may provide benefits to their host above and beyond their immunological role in maintenance of intestinal homeostasis and prevention of inflammation. For example:

- Strains of *L. reuteri* have been shown to be unique among *Lactobacillus* species in their ability to produce vitamin B_{12}. Whether or not it B_{12} is actually produced in the human gut ecosystem and available to the host has yet to be shown.
- Strains of *L. reuteri* are unique in their ability to produce the biologically active isomers of conjugated linoleic acid (CLA) - a microbial product shown to have remarkable health -enhancing effects in animal model studies.
- Strains of *L. reuteri* have been shown to produce prebiotics, namely fructan and glucan oligosaccharides. Production of these prebiotics by *L. reuteri* in the GI tract may promote development and sustenance of other health-enhancing microbiota in the gut environment.
- Strains of *L. reuteri* synthesize bile salt hydrolase (BSH). Strains having this activity administered to pigs fed a high fat diet promote a reduction serum levels of LDL/VLDL cholesterol.
- Most strains of *L. reuteri* are unique and distinguishable from other *Lactobacillus* species in their ability to produce and secrete the antimicrobials - reuterin and reutericyclin.

A review focusing on these non-immuinological, but possibly beneficial, activities of *L. reuteri* is currently in preparation (6).

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Clinical update with *Lactobacillus reuteri* and future perspectives

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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.

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 activates 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.

The use of modern genetic analysis of how specific strains of *L. reuteri* affect the human host, though the immune system will be an important tool in defining new, potentially more effective strains for clinical evaluation in the next few years.

L. reuteri ATCC 55730, a safe and effective probiotic

Wolf et al.[1] studied the safety and tolerance of 21 day of *L. reuteri* ingestion in healthy adult males in a randomized, double-blinded placebo controlled trial and showed that *L. reuteri* can be ingested at a level of $1x10^{11}$ CFU/day, gave clear colonisation of the gastrointestinal tract and was without any clinically significant safety or tolerance problems. The same investigators followed on with a similarly designed study[2] in immuno-compromised individuals (HIV infection) and confirmed that *L. reuteri* was without any clinical safety and tolerance problems even when the immune system is weakened.

Ruiz-Palacios et al.[3] established the tolerance and dose response of a probiotic mixture containing *L. reuteri* in children (n=72), aged 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 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 faecal *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 $1x10^{10}$ CFU/day.

Ruiz-Palacios et al.[4] further 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 significantly lower in the probiotic group compared to the control group and the incidence of diarrhoea per child was also lower in the probiotic group.

In a second blinded randomised study[5] compared the ability of two probiotic blends to prevent diarrhoea in healthy children (ages 12 to 32 months) was studied. One feeding group was given a blend of *L. acidophilis* and *B. infantis* (N=129). The second group received the same blend plus $1{,}5x10^8$ CFU *L. reuteri* ATCC 55730 per day (n=129). A third group received placebo (n=130).
The relative risk of contracting diarrhoea compared to placebo was significantly reduced to 0.67 for the *L. reuteri* fed group but not in the other group compared to placebo, thus indicating the specific protective effect of *L. reuteri* ATCC 55730 in children.

The probiotic *L. reuteri* in infants, babies and young children is becoming more commonly used as the benefits of supplementation of the diet with *L. reuteri* are being revealed.

The benefits of *L. reuteri* ATCC 55730 supplementation in both reducing the severity and extent of diarrhoea in young children are now well established. Two double-blind, placebo controlled studies examined the effects of *L. reuteri* on acute infectious diarrhoea in children[6,7].
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. A recent study of similar design was performed in Asian infants with acute diarrhoea given *L. reuteri* ATCC 55730 at 10^8 CFU/day and again, supplementation with the probiotic significantly increased the speed of recovery of the infants.[8]

Karvonen[9] 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.

Novel and effective formulation for paediatrics

Daily supplementation of infants and babies with *L. reuteri* ATCC 55730 was able to protect them from gastro-intestinal infections, which would otherwise have necessitated medical attention.[10] In a double-blind, placebo controlled trial in infant formulas were supplemented with either *L. reuteri* or *B. bifidum* (Bb12) and given to 4-10 month infants for 12 weeks.
L. reuteri was able to significantly reduce the incidence of fever, gastro-intestinal infections, visits to the doctor, day care absence and the use of antibiotics compared to placebo and *B. bifidum*. The authors noted that *L. reuteri* was found to be superior to both placebo and supplementation with *B. bifidum* (Bb12) in maintaining the gastro-intestinal health of the infants.

Conventional delivery systems for probiotics and *L. reuteri* for infants and babies include adding freeze-dried powders of the probiotic to mother's milk or milk substitutes prior to feeding the baby or addition of the bacteria to baby formulas. These modes of delivery are successful and are used today, but there is a need for further flexibility in young infants.
Adding freeze-dried powders to mother's milk is tedious for the parent whilst probiotic baby formulas only provide the full probiotic dose if the baby is fed exclusively with the formula. Thus, the nursing mother needs a convenient, complementary formulation, which will provide the probiotic without interfering with breast-feeding.

A novel and unique delivery system for *L. reuteri* has been developed by BioGaia AB (Sweden), where the freeze-dried probiotic is suspended in a mixture of food oils and this has now been tested clinically. Karvonen et al.[11] performed two randomised, double-blind and placebo-controlled clinical trials to look at tolerance of the formulation in both newborn, term infants and preterm, but otherwise healthy infants during 28-day supplementation at doses of 10^7 up to 10^9 CFU/day.

The *L. reuteri* drops led to a clear colonisation of the gastro-intestinal tract of the infants, showing that the oil formulation successfully releases live bacteria after ingestion by infants.
The investigators concluded that the product was well tolerated up to the highest dose of *L. reuteri*, in both newborn and premature infants, as expected from earlier studies.

These findings have been confirmed in a further study of 232 infants in a multi-centre, randomised, placebo-controlled, double blind clinical trial in Sweden. Here, infants were supplemented from birth up to 12 months of age. At 5-6 days of age, the prevalence of live *L. reuteri* in the stools of infants in the Reuteri Drops group was significantly higher than in the placebo group, 80% vs. 19% ($p<0.001$). The prevalence then declined to 63% at 12 months of age in the actively treated infants but remained at 23% in the placebo group ($p<0.001$)[12]. Thus, *L. reuteri* drops could increase the prevalence of *L. reuteri* in the gastrointestinal tract of infants from the natural levels of 17-20% up to 80%. *L. reuteri* drops were well-tolerated by the infants in long term use and no adverse effects were observed.[12]

A specific study of a randomly selected sub-group of the effect of supplementation with *L. reuteri* in infants on circulating D(-)-lactic acid levels showed that long-term supplementation with D(-)-lactic acid producing *L. reuteri* does not affect circulating D(-)-lactic acid levels at all and is safe for use in infants.[13]

In the same study, the mothers took either *L. reuteri* drops or an identical placebo formulation without the bacteria, for 4 weeks prior to giving birth and it was found that those mothers given *L. reuteri* had significantly higher levels of anti-inflammatory cytokines in the colostrum compared to those who received placebo drops.[14]

This provides key evidence that delivery of *L. reuteri* to the gastro-intestinal tract of the mother, leads to a stimulation of her immune system and the migration of immune signals (probably via circulating cells) to the mammary gland.

It is thus becoming clear that interaction between *L. reuteri* with human epithelial tissue can lead to alterations in immune function at other epithelia.

Recent clinical findings

Savino et al.[15] very recently presented a comparison of *L. reuteri* ATCC 55730 supplementation with simethicone for the alleviation of crying in infantile colic. They found that *L. reuteri* dramatically and significantly eliminated crying in all colicky infants studied whereas simethicone was essentially without effect.

This exciting finding, opens up new applications for clinical use of *L. reuteri* in infantile colic.

Further, the known protective effect of *L. reuteri* in older infants and children[10] will encourage continued use of the convenient new formulation to prevent gastrointestinal infections when the children enter day schools.

The protection of health in normal, healthy adults was recently shown in a double-blind, placebo-controlled, randomised clinical study performed at two work places in Sweden.[16]
Employees (n=219) were recruited and were asked to take L. reuteri ATCC 55730 (10^8 CFU/day delivered in a drinking straw) or placebo for 84 days. One hundred and eighty one subjects (*L. reuteri*, 94, placebo, 87) completed the protocol and it was found that *L. reuteri* supplementation significantly reduced short-term sick leave due to gastro-intestinal or respiratory symptoms (*L. reuteri* 11% vs placebo 26% sick leave; $P<0.01$).
This confirms the earlier finding in infants and children[10] and shows that regular use of *L. reuteri* maintains health in adults and children alike.

Ingestion of *L. reuteri* ATCC 55730 leads to a colonization of the human gastro-intestinal mucosa.[17] Colonisation and growth of *L. reuteri* in the gastric antrum and upper duodenum is particularly relevant since this is the site of infection for *Helicobacter pylori.* Clearly, since *L. reuteri* is effective in inhibiting the growth of *H. pylori* in vitro[18] it is of interest to determine the effect of *L. reuteri* in *H. pylori* infected subjects.
Imase et al.[19] used a double-blind, randomised cross-over design to study the effect of *L. reuteri* ATCC 55730 on the level of *H. pylori* infection in non-symptomatic but confirmed *H. pylori* positive volunteers.
A consistent reduction in *H. pylori* load was observed during *L. reuteri* supplementation. Saggioro et al.[20] examined the effect of *L. reuteri* ATCC 55730 in combination with omeprazole on *H. pylori* infection in symptomatic patients. Eradication of *H. pylori* was observed in 9 of 15 patients given *L. reuteri* compared to controls given omeprazole alone.
The interaction of adjustments in gastric pH, combined with the anti-pathogenic effects of *L. reuteri* may explain this exciting new finding.

Oral Health

Recent studies showed the efficacy of supplementation with this strain in reducing the oral load of *Streptococcus mutans* in healthy volunteers possibly indicating a possible role for this probiotic in reducing the risk of tooth decay.[21] During further studies to identify *L. reuteri* strains with protective properties in the oral cavity, a novel *L. reuteri* strain derived from the human oral cavity, *L. reuteri* ATCC PTA 5289, has been identified.
This strain predominated in, and was isolated from, the saliva of a young Japanese woman with exceptionally healthy teeth and gums despite neglect of her oral hygiene (due to mental disability). Identification of the strain after isolation showed it to be *L. reuteri* and this strain is thought to have protective effects on oral health.

Gingivitis is an inflammation of the gingiva caused by accumulation of bacteria in the gum pocket at the base of the tooth. Gingivitis is the most commonly occurring chronic inflammation and if the inflammation and degradation of collagen increases, it reaches further down into the gum pocket, the reversible gingivitis develops into irreversible periodontitis. Since gingivitis most often leads to periodontitis, treatment and prevention of the still reversible gingivitis are the main challenge.

Initial anecdotal and pilot study data supported the idea that daily supplementation with both *L. reuteri* strains in the oral cavity could positively affect the oral microfloral balance, which in turn reduced gingivitis and gum bleeding. A mixture of both strains of *L. reuteri* reduced the severity of gingivitis and extent of plaque formation and it was concluded that a combination of the two probiotic strains could potentially give significant benefit in human oral health. Further clinical studies have been initiated to further investigate this novel probiotic use of *L. reuteri*.

Future directions

Clear interactions between the human immune system and commensal bacteria through the intestinal epithelium and the immune cells that lie on the serosal side of the mucosal wall have been described.

Further, evidence clearly indicates that specific strains of, for example lactobacilli, possess probiotic effects which cannot be extrapolated to the whole species. Studies at the genome level show significant differences between strains of *L. reuteri* and allow the selection of potentially more effective strains for use as probiotics. These strains, for example, showing strong anti-inflammatory effects on intestinal epithelial cells and human macrophages, will be useful in future specific use in reducing or eliminating inflammation associated with *H. pylori* in the stomach or in the lower gastro-intestinal tract in inflammatory bowel disease. The use of microarrays of the *L. reuteri* genome will be an important tool in defining these new strains for clinical evaluation in the next few years.

Clinical studies with *Lactobacillus reuteri* ATCC 55730

Trial (ref)	Aim	Study design	Groups	Nr. of subjects	Duration	Main outcome
Wolf **(1)**	Safety Healthy adults	R, DB, PC	Placebo *L. reuteri* ($1x10^{11}$CFU/day)	15 15	21 days	No clinical safety or tolerance problems
Wolf **(2)**	Safety Immunocompromised adults	R, DB, PC	Placebo *L. reuteri* ($1x10^{10}$CFU/day)	20 15	21 days	No clinical safety or tolerance problems
Ruiz-Palacios **(3)**	Safety Children (1-3 years)	R, B, PC	Placebo *L. reuter* ($1x10^{10}$CFU/day)* *L. reuter* ($1x10^{8}$CFU/day)* *L. reuteri* ($1x10^{6}$CFU/day)*	20 18 16 18	21 days	No clinical safety or tolerance problems
Ruiz-Palacios **(4)**	Prevention of diarrhoea Children (1-3 years)	R, B, PC	Placebo *L. reuteri* ($5x10^{7}$CFU/day)*	120 119	14 weeks	Probiotic significantly reduced incidence of diarrhoea
Guerrero **(5)**	Prevention of diarrhoea Children (1-3 years)	R, B, PC	Placebo Probiotic mix without *L. reuteri*** Probiotic mix with *L. reuteri* ($1.5x10^{8}$ CFU/day)**	130 129 129	16 weeks	Only *L. reuteri* - containing probiotic mix significantly reduced incidence of diarrhoea
Shornikova **(6)**	Treatment of acute gastroenteritis in children (0.5-3 years)	R, DB, PC	Placebo *L. reuteri* ($1x10^{10-11}$CFU/day)	21 19	up to 5 days	*L. reuteri* significantly reduced duration of gastroenteritis
Shornikova **(7)**	Treatment of acute rotavirus gastroenteritis in children (0.5-3 years)	R, DB, PC	Placebo *L. reuteri* ($1x10^{10}$CFU/day) *L. reuteri* ($1x10^{7}$CFU/day)	25 21 20	up to 5 days	High dose *L. reuteri* significantly reduced duration of gastroenteritis Low dose tendency to similar effect
Eom **(8)**	Acute diarrhoea Children (6-36 months)	R, DB, PC	Placebo *L. reuteri* (10^{8} CFU/day)	25 25	5 days	*L. reuteri* significantly reduced duration of gastroenteritis
Karvonen **(9)**	Safety & colonisation Newborn term infants	R, DB, PC	Placebo *L. reuteri* ($1x10^{9}$CFU/day) *L. reuteri* ($1x10^{7}$CFU/day) *L. reuteri* ($1x10^{5}$CFU/day)	28 25 25 12	30 days from birth	No clinical safety or tolerance problems Significant reduction in watery stools with *L. reuteri*
Weizman **(10)**	Prevention of infection Day-care children (4-10 months)	R, DB, PC	Placebo *L. reuteri* ($3x10^{8}$CFU/day)*** *B. lctis* Bb12 ($1x10^{9}$CFU/day)	58 65 71	12 weeks	*L. reuteri* superior in significantly reducing incidence of gastrointestinal infection
Karvonen **(11)**	Safety & colonisation Newborn term infants Premature infants	R, DB, PC	Placebo *L. reuteri* ($1x10^{8}$CFU/day) Placebo *L. reuteri* ($1x10^{9}$CFU/day) *L. reuteri* ($1x10^{7}$CFU/day)	12 23 16 13 14	28 days from birth	No clinical safety or tolerance problems No clinical safety or tolerance problems
Connolly **(13)**	Safety Infants (0-12 months)	R, DB, PC	Placebo *L. reuteri* ($1x10^{8}$ CFU/day)	10 14	6 months from birth	No elevation of blood D(-)-lactic acid levels in *L. reuteri*-supplemented infants
Björkman **(22)**	Colonisation Healthy adults	Open	*L. reuteri* ($1x10^{9}$ CFU/day)	10	12 days	*L. reuteri* colonises the large intestine in healthy humans
Mikkonen **(23)**	Colonisation I Healthy adults Colonisation II Healthy adults Colonisation III Healthy adults	Open Open Open	*L. reuteri* ($1.3x10^{9}$ - $6x10^{10}$ CFU/day) *L. reuteri* ($4x10^{8}$ CFU/day) *L. reuteri* ($2.2x10^{8}$ CFU/day)	18 15 24	9-15 days 10 days 10 days	*L. reuteri* colonised the faeces and colon biopsies *L. reuteri* colonised the faeces but not the colon biopsies *L. reuteri* colonised the faeces and colon biopsies

Trial (ref)	Aim	Study design	Groups	Nr. of subjects	Duration	Main outcome
Hernell **(24)**	Safety & colonisation Day school children (1-3 years)	R, DB, PC	Placebo Probiotic milk with L. reuteri ($4.8x10^8$ CFU/day)**	20 14	14 days	Well tolerated and good colonisation
Arole **(25)**	Safety & prevention of diarrhoea Children (3-6 years)	R, DB, PC	Placebo *L. reuteri* ($1.3x10^9$ CFU/day)	152 159	35 days	No clinical safety or tolerance problems No diarrhoea incidence in study
Ouwehand **(26)**	Constipation Elderly (70-96 years)	Open	Control *L. reuteri* ($7x10^8$ CFU/day) Probiotic mix without *L. reuteri*	6 12 10	28 days	*L. reuteri* increased fecal output significantly compared to controls. Effect retained 3 weeks after *L. reuteri* stopped.
Weizman **(27)**	Safety Infants (3-65 days)	R, DB, PC	Placebo *L. reuteri* ($3x10^8$CFU/day)*** *B. lactis* Bb12 ($1x10^9$CFU/day)	17 17 16	60 days	No clinical safety or tolerance problems with either probiotic
Valeur **(17)**	Gastrointestinal colonisation Healthy adults	Open	*L. reuteri* ($4x10^8$CFU/day)	19	28 days	*L. reuteri* colonises the human stomach, duodenum and ileum
Nikawa **(21)**	Oral S mutans inhibition Healthy adults	R, DB Crossover	Placebo *L. reuteri* ($1x10^8$ CFU/day)	40	14 + 14 days	*L. reuteri* inhibits S. mutans growth in the mouth
Mertz **(27)**	Pilot effect on *H pylori* infection level Hp infected symptomatic adults	Open	*L. reuteri* ($4x10^8$ CFU/day)	7	6 months	*L. reuteri* colonised the human stomach Hp infection not affected by *L. reuteri*
Imase **(19)**	Effect on H pylori infection level in Hp infected non-symptomatic adults	R, DB Crossover	Placebo - Placebo *L. reuteri* ($4x10^8$ CFU/day) - Placebo Placebo - *L. reuteri* ($4x10^8$ CFU/day)	5 15 15	4 + 4 weeks	*L. reuteri* significantly reduced H. pylori infection levels
Tubelius **(16)**	Prevention of short term sickness Healthy adults	R, DB, PC	Placebo *L. reuteri* ($1x10^8$ CFU/day)	87 94	80 days	*L. reuteri* significantly reduced short term sick leave
Melin **(29)**	Nutrition and safety Healthy adults	Open	*L. reuteri* ($1x10^8$ CFU/day)	26	60 days	*L. reuteri* normalised low blood Fe in Fe deficient women
Niv **(30)**	IBS IBS suffering adults	R, DB, PC	Placebo *L. reuteri* ($2x10^8$ CFU/day)	27 27	6 months	*L. reuteri* tendencies to reduced gases and improved constipation in mixed IBS
Savino **(15)**	Colicky infants	Open random	Standard therepy (Simethicone) *L. reuteri* ($1x10^8$ CFU/day)	32 34	28 days	*L. reuteri* dramatically removed colic vs. standard therapy
Saggioro **(20)**	Hp infected symptomatic patients	R, B	Omeprazole (20mg/day) *L. reuteri* ($1x10^8$ CFU/day) + omeprazole (20mg)	15 15	30 days	*L. reuteri* + omeprazole eradicated *H. pylori*
Cirillo **(31)**	Atopic eczema and bovine milk allergy Children (3-5 years)	Open	Cetirizine (for 10 days) *L. reuteri* ($1x10^8$ CFU/day) + Cetirizine (for 10 days)	7 8	3 months	Atopic eczema relief in all children on *L. reuteri* Cetirizine alone all children relapse to eczema

Abbreviations: R, randomised; DB, double-blind; B, blind; PC, placebo-controlled
* also received *L. acidophilus* and *B. infantis* at constant dose
** Probiotic mix contained *L. acidophilus* and *B. infantis*
*** Personal communication

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Infantile colic in newborn and *Lactobacillus reuteri*

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Infantile colic is a very common problem in infancy with a frequency of 10-30% (1) and can be described as follows: an healthy infant with paroxysms of excessive, high-pitched, inconsolable crying, frequently accompanied by flushing of the face, drawing up of the legs, passing of gas and difficulties with the passage of stools.

The disorder begins before the sixth week of life in 90% of cases and generally resolves spontaneously by the fourth months. Typically the crying starts at the same time each day and is more intense in the afternoon, evening and night, during two or three hours (1).

Even if infantile colic is a common disorder, the aetiology is not completely understood. For severe infantile colics a food allergy has been remarked by several studies (2-8). Moreover, it has been considered, recently, the role played by the intestinal microflora in the etiopathogenesis of allergic diseases of which severe infantile colic may be the first manifestation. Bjorkstein et al. (9) reported that allergic 2-3 years-old children have an altered gut flora with a lower level of lactobacilli in comparison with healthy infants.

Lactobacilli consist of a diverse group of microorganisms forming part of a larger group of lactic acid bacteria, non-pathogenic, anaerobic and Gram-positive bacteria that produce lactic acid as a primary metabolic end product and are commonly found in humans as constituent of intestinal microflora.

This genus plays important physiological roles affecting the functions of the intestinal epithelium and of the mucosa, as well as local and systemic immune responses. Intestinal colonization acts as an effective antigenic stimulus for the maturation of the gut-associated lymphoid tissue (10).

The capacity to produce IgA-secreting cells increases progressively during the successive stages in the establishment of the gut microflora (11).

In particular, lactic-acid producing bacteria, such as *Lactobacillus spp.*, plays an important role for the immunological homeostasis of the host and for oral tolerance regulation (12).

Thus, qualitative differences of intestinal lactobacilli, occuring in the first months of life, may affect the immune responses of the host and could favour the development of different disorders such as infantile colic, that could represent a precocious clinical manifestation of atopic diseases (7).

Our data recently published show different patterns of intestinal lactobacilli in colicky and non-colicky infants, further in non-colicky infants we detected *L.acidophilus*. Some authors reported that the presence of *L.acidophilus* plays a protective role by stimulating the immune system (13,14).

This function is explained by the stimulation of production of secretory IgA and activation of non-specific immune response by enhancing macrophage activity (15). Lactic acid bacteria are also

able to degrade food antigens. In fact, it has been shown that cow's milk proteins, degraded by lactobacilli, may generate tolerogenic peptides from the native protein.

Literature data reported that lactobacilli can potentiate the production of interferon-γ (IFN-γ) and it has been shown that in early stage of gut maturation IFN-γ can promote the uptake of antigens in Peyer's patches (16).

Peyer's patches play an important immunoregolatory role in the mucosal immune system, implying suppression of T cell responses and induction of mucosal IgA responses, inducing a good maturation and functionality of the GALT (17).

In this way lactobacilli promote the balance Th1-Th2 by the production of IFN-γ, that is important in reduction of Th2 cytokines, which are involved in the production of IgE and allergy responses (18,19).

Thus close interaction between the intestinal immune system and gut microbiota is essential to maintaining a disease-free state in the host, as the gut microbiota provides the immune system with stimuli resulting in immunological maturation and tolerance.

More over our data show that infants with colics have a higher frequency of family history for atopy compared with control group, indicating a further data in favour of the allergic hypothesis in the pathogenesis of colics, according an our previuos observation (4) .

Our findings show that an alterated balance of intestinal lactobacilli in infants with colics could lead to an immaturity of gut barrier and to aberrant antigen transfer and immune responses, thus explaining increased vulnerability to breaking down of oral tolerance, as reported by many Authors (2,6,7, 12, 20, 21).

These findings are therefore in agreement with literature data (6,9) and support the hypothesis that an alteration in intestinal microflora could be responsible of severe infantile colic and food intolerance.

Our results associate with our previous observations (20,21) and data of Bjorksten et al. (9) in atopic infants, suggest the etiopathogenetical hypothesis that colics are often related to a food allergy and confirm the presence of an interaction between altered gut microflora and development of oral tolerance or atopy.

The results of our findings suggest that an alteration on intestinal lactobacilli may be involved in pathogenesis of infantile colic and, for this reason, we performed a prospective randomised study to investigate the efficacy of *Lactobacillus reuteri* in the treatment of infantile colic.

Our results on 66 breastfed colicky infants show that symptoms improved within one week of treatment with the probiotic *L.reuteri*.

Daily average crying time (in minutes) in colicky infants treated with *L. reuteri* and simethicone

Colicky infants (n = 66)	*L. reuteri (n = 34)*	*Simethicone (n = 32)*	*t-test*	*CI 95%*
Day 0	**205.08** (SD 27.13)	**206.30** (SD 26.91)	p = 0.855	-14.52;12.08
Day 1	**191.35** (SD 35.61)	**195.75** (SD 41.60)	p = 0.645	-23.41;14.61
Day 7	**140.89** (SD 47.96)	**169.45** (SD 45.73)	p = 0.016	-51.63;-5.49
Day 14	**74.76** (SD 38.03)	**158.24** (SD 40.76)	p < 0.05	-102.85;-64.11
Day 21	**38.02** (SD 28.87)	**150.82** (SD 38.34)	p < 0.05	-129.43;-96.17
Day 28	**17.18** (SD 12.33)	**147.75** (SD 20.87)	p < 0.05	-138.94;-122.20

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Helicobacter pylori infection in children: new therapy

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Clinical use of probiotics

Probiotics were recently redefined by Guarner as "live micro organisms which, when administered in adequate amounts, confer a health benefit on the host" (1). Indeed, there is an increasing amount of evidence indicating health benefits by their consumption (2,3). Temporary colonising of the gut with an appropriate probiotic strain not only promotes the state of "eubiosis" (favorable balance of the gut flora), but may have a favourable immunomodulatory effect (4). Controlled clinical trials have shown beneficial outcomes for the use of probiotics in different conditions such as rotavirus infections, antibiotic-associated diarrhea, irritable bowel syndrome, inflammatory bowel disease and prevention of atopic diseasey in at risk infants (5-6).

Microorganisms most commonly used as probiotics are lactic acid-producing *Lactobacilli* and *Bifidobacteria*. Both bacterial genera belong to the normal microflora and several strains produce not only lactic acid but also other antimicrobial substances such as hydrogen peroxide and bacteriocins (7). Moreover, probiotic agents compete with pathogens for microbial adhesion sites and are claimed to modulate the immune response of the host. The specific effects on the immune system are, however, still unclear (8). Other less commonly used probiotic microorganisms are *Saccharomyces boulardii* and strains of *Streptococcus* and *Escherichia coli*.

Research efforts into the clinical effects of probiotics in man are rapidly increasing. One field of particular interest is Helicobacter pylori (*H. pylori*) infection.

Helicobacter pylori

H. pylori (originally called Campylobacter pyloridis), first recognised in 1982, plays a crucial role in the pathogenesis of both chronic active gastritis and peptic ulcer disease in children and adults (9,10). An increasing amount of evidence also supports the hypothesis that *H. pylori* is an important cofactor in the development of gastric cancer (11,12). The microrganism can be found in 70-90% of the population in developing countries and in 25-50% in developed countries (13). In most subjects, its presence may be regarded as carriage rather than infection, as it is usually asymptomatic. Nevertheless, *H. pylori* causes an enormous burden of morbidity and is the reason for prescription of many courses of antibiotics (4).

Combination therapy has been shown to be necessary to eradicate *H. pylori* from the stomach. Antimicrobial agents such as amoxicillin, clarithromycin, bismuth salts and nitroimidazoles (metronidazole or tinidazole) together with proton pump inhibitors (e.g. omeprazole) or H2 - receptor antagonists (e.g. ranitidine) are used, in triple or quadruple combinations. These regimens have the disadvantages of being expensive, risking poor compliance, causing side-effects and in particular encouraging resistance emergence, both in *H. pylori* and commensal organisms exposed

gratuitously. There is thus considerable interest in measures capable of reducing some or all of these drawbacks, such as alternative therapies (e.g. targeting urease, a known virulence factor) or adjunctive treatment. A considerable amount of work has been done on the possible role of probiotics in the treatment and prophylaxis of *H. pylori* infections (4).

Interactions between probiotics and *H. pylori*

A study was performed to test whether bacterial products of *Lactobacillus johnsonii* La1 could be used against *H. pylori;* the work shows that the supernatant from La1 culture inhibits the growth of *H. pylori* in vitro whether or not *H. pylori* was bound to epithelial cells (14). In addition, also in vivo the supernatant of La1 was assessed to be able to interfere with *H. pylori* in infected subjects (14); this effect could be reproduced by lactic acid. Another lactic acid-producing strain was however unable to inhibit *H. pylori* growth, suggesting other possible interactions (15).

Similar results have been shown in a gnobiotic murine model in which *L. salivarius* was able to inhibit *H. pylori* both in vitro and in vivo (16). This effect was due to the production of a large amount of lactic acid since the inhibitory effect could be reproduced after the incubation of *H. pylori* in the presence of lactic acid. Lorca et al have also investigated the ability of lactobacilli to inhibit *H. pylori* in vitro and in vivo (17). They tested the antibacterial activity of 17 strains of lactobacilli against 10 strains of *H. pylori* and showed a different antagonistic effect of lactobacilli-spent broths on *H. pylori*. The inhibitory effect was again associated with acid production, but for one strain, *L. acidophilus* CRL 639, it was also related to an intracellular proteinaceous component.

Another probiotic, *Weissella confusa* PL9001, was shown to inhibit the binding of *H. pylori* to the human gastric cell line MKN-45 (18). The probiotic strain *Bacillus subitilis* 3 has also been shown to inhibit the growth of *H. pylori*. Pinchuk et al found that the anti-H. pylori activity present in the cell-free supernatant of *Bacillus subtilis* 3 was not related to pH or organic acid concentration (19).

We have recently studied the interaction between *L. reuteri* SD2112 and *H. pylori* by evaluating the ^{13}C-urea breath test (^{13}C-UBT) and the bacterial stool antigen (HpSA) before and after probiotics administration; both tests are a semi quantitative measure of the bacterial load. To this aim, 20 adults infected by *H. pylori* were blindly randomised to receive either *L. reuteri* SD2112 or placebo for 28 days. We were able to show a significant decrease of both ^{13}C-UBT delta (that moves from 31 to 22) and of HpSA (that goes from 673 to 427) only in patients receiving the probiotic indicating its ability to reduce the bacterial load (unpublished data).

Probiotics and *H. pylori* adhesion to epithelial cells

H. pylori can bind tightly to epithelial cells via multiple bacterial surface components (20). There is increasing evidence in animal models that this adhesion is important in determining outcome in *H. pylori*-associated disease (21). In this context, Mukai et al's study is particularly interesting (22). These investigators showed that two out of nine *L. reuteri* strains, JCM 1081 and TM 105, were able to bind to asialo-GM1 and sulphatide and to inhibit binding of *H. pylori* to both glycolipids. These results suggest that selected *L. reuteri* strains could help to prevent infection in an early stage of colonization of the gastric mucosa by *H. pylori* (15).

Probiotics and *H. pylori*-induced gastritis in man

Felley et al demonstrated that strain La1 was able to diminish the density of *H. pylori* colonization, inflammation of the antrum and the activity of the inflammation in both the antrum and the corpus (23). Similar results were obtained by Sakamoto et al using *L. gasseri* OLL2716 (LG21) resulted effective in both suppressing *H. pylori* and reducing gastric mucosal inflammation (24).

Probiotics and *H. pylori* eradication rate

A number of clinical studies on the effects of probiotics on the eradication rates of *H. pylori* have been carried out (25). Subjects attending a screening programme for the assessment of prevalence and risk factors for *H. pylori* have been enrolled in a study on the effects of L. GG in combination with standard triple eradication therapy (26). No significant differences were observed between the supplemented and the placebo group in eradication rates. Canducci et al (27) evaluated an inactivated preparation of *L. acidophilus* (LB) in conjunction with standard triple therapy on *H. pylori*-positive patients. A significantly increased eradication rate was observed in the group receiving the supplement. In another study, 85 asymptomatic patients were examined and randomized into four groups (28). All patients received a 1-week triple therapy in combination with L. GG or *S. boulardii,* a combination of *L. acidophilus* and *Bifidobacterium lactis* or a placebo product. The *H. pylori* eradication rate did not differ between the groups. *H. pylori*-infected volunteers were given either a fermented milk product-containing *L. johnsonii La1* or placebo for 3 weeks (23). During the last 2 weeks, all subjects also received clarithromycin. The eradication rate was not improved by the probiotic administration. The effect of *L. johnsonii La1* supernatant on *H. pylori* was evaluated in infected subjects (14). The subjects were randomised to receive a concomitant treatment with omeprazole or placebo tablets. Four weeks after the end of treatment, the urea breath best (^{13}C-UBT) values were still significantly below the pretreatment values regardless of treatment group. This finding is in contrast with a study where *H. pylori*-positive subjects ingested a milk product added with *L. acidophilus* (NAS) as the only therapy during 8

weeks; *H. pylori* was eradicated in six out of the 14 patients (29). Patients receiving triple therapy for eradication of H. pylori were randomly assigned to receive a supplement of Lactobacillus and Bifidobacterium-containing yoghurt (30). By intention-to-treat analysis, the probiotic group had a higher eradication rate than the group receiving only the triple therapy (91% vs. 78%). Per protocol analyses yielded no differences between the groups. Asymptomatic women positive for *H. pylori* were recruited and administered a yoghurt-containing *L. casei 03*, *L. acidophilus 2412* and *L. Acidophilus ACD1* and a commercial starter culture (containing *L. bulgaricus, S. thermophilus* and *L. acidophilus*) (31). One month after ingestion of the yoghurt, the ^{13}C-UBT values remained positive in the majority of women although the probiotic strains were shown to be effective in the inhibition of H. pylori growth in vitro.

Overall so far, there has been no convincing evidence to support the use of probiotics as the main treatment or an adjunct with the aim of increasing the *H. pylori* eradication rate and further studies are needed to clarify their role in this particular issue.

Probiotics and *H. pylori*-related dyspeptic symptoms

In the aforesaid study, we were able to demonstrate a favourable effect of *L. reuteri* SD2112 on dyspeptic symptoms induced by *H. pylori* in adults. In this study, subjects randomised to receive *L. reuteri* significantly improved their symptoms during the four weeks of treatment, while only a small improvement was seen in those receiving placebo.

Probiotics and antibiotic-associated gastrointestinal side-effects during *H. pylori* eradication therapy

H. pylori eradication fails in about 25-30% particularly because of the occurrence of resistance to antibiotics and/or their side-effects (26). Three studies evaluated whether probiotic supplementation might help to prevent or reduce drug-related side-effects during *H. pylori* eradication therapy. The first study used L. GG (26), the second used differnt probiotic preparations (*L. GG* or *Saccaromyces boulardii* or a combination of *L. acidophilus* and *Bifidobacterium lactis*) (28) and the last used *Bacillus clausii* (33). All found that probiotics were superior to placebo for the prevention of side-effects.

Although diarrhea may be considered a reasonable consequence of dysbiosis caused by the antibiotic and probiotics may correct of this condition, this logical explanation remains unproven (4). No suggestions have been made by which mechanism probiotics can reduce the incidence of nausea and/or vomiting (brought about by a direct effect on the vomiting centre) or the unpleasant taste associated with combination treatment. Such phenomena could possibly arise if the absorption of antibiotics were modulated in some way, by analogy with, for example, the situation

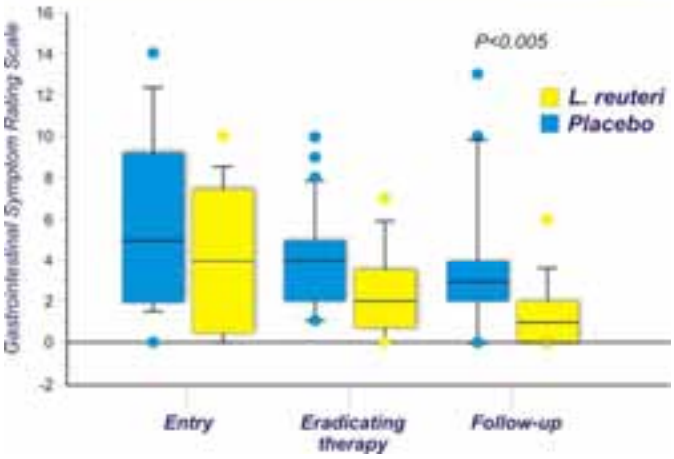
for the micro and macro-crystalline forms of nitrofurantoin (34). However, this is pure speculation, and while co-administration of yogurt, as done by Sheu et al (35), might possibly affect the absorption of antibiotics, it is very difficult to see how the same could occur when the probiotics were given as freeze-dried powders (4).

We have recently conducted the first trial in children to determine whether adding the probiotic *L. reuteri* to an anti-*H. pylori* regimen could help to prevent or minimize the gastrointestinal side-effects burden in children.

Twenty-five *H. pylori*-positive children were consecutively treated with 10-day sequential therapy [omeprazole (1 mg/kg/day) + amoxycillin (50 mg/kg/day) for 5 days, and omeprazole (1 mg/kg/day) + clarithromycin (15 mg/kg/day) + tinidazole (20 mg/kg/day) for other 5 days] and blindly randomised to receive either *L. reuteri* SD2112 (10^8 CFU) or placebo for 20 days starting from the first day of treatment. In order to determine the type and severity of side-effects, all children completed the Gastrointestinal Symptom Rating Scale (GSRS) at entry and on day 5 and 10 of treatment and follow-up. *H. pylori* status was assessed after 8 weeks by ^{13}C-UBT.

At entry, children in both groups were homogeneous for demographic variables and GSRS score. Overall, in all probiotic supplemented children as compared to those receiving placebo there was a significant reduction of GSRS score during eradication therapy (2,4 ± 2,2 vs. 4,1 ± 2,5; p<0,008) and follow-up (1,4 ± 1,8 vs. 3,7 ± 3,6; p<0,005), the effect being more pronounced on day 20 when they refer less frequently abdominal distension (31% vs. 0%; p<0,03), eructation (38% vs. 0%; p<0,016), hard stools (54% vs. 17%; p<0,05) and inappetence (33% vs. 0%; p< 0,04).

Severity of dyspeptic symptoms and side-effects assessed by the Gastrointestinal Symptom Rating Scale at entry, during treatment and at follow-up.



There were no differences in adherence to treatment schedules (97% in both groups) and *H. pylori* eradication rates (92,3% vs. 100%; p= NS) between the two groups. Therefore, our study clearly shows that *L. reuteri* given during and after anti-*H. pylori* eradication therapy significantly reduces frequency and intensity of antibiotic-associated side-effects.

Conclusions

Investigation of a possible role of probiotics in the prophylaxis and treatment of *H. pylori* infections is clearly in its infancy. Results so far are encouraging and further clinical trials are called for. The design of such studies should be such as to clarify which probiotic strains are suitable, in what form, in what dose and for how long. The types of patient should be clearly defined and the method of deciding outcome should also be standardised as far as possible.

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Symposium " Highlight On *Lactobacillus reuteri*"
Rome, September 5th 2005

Helicobacter pylori eradication with *Lactobacillus reuteri*

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Summary

The gastric acid secretion is the most important mechanism to control the growth of microbes in the human stomach.
The acid secretion in man may change due to gastric surgery, medical procedures, diseases, anti-secretory drugs, etc. and that allows different microbes to colonise the stomach. The variation of bacterial contents in a healthy stomach before and after a meal is also related to the pH of the gastric juice.
Immediately after a meal the microbial concentration is around 10^5 bacteria per ml of gastric juice but as the pH falls the bacterial count declines and when the pH is lower than 3 the stomach content is usually almost sterile[1].
Lactobacilli, streptococci and staphylococci have been isolated in the stomach of healthy persons and patients with gastrointestinal diseases[2].
Even though gastric acid play an obvious role for inhibiting the growth of microbes in the stomach other mucosal factors must not be forgotten.
The specific adhesion of gastrointestinal pathogens to mucosa is a well established virulence factor. Bacteria of various morphological types have been studied and it has been found that adhesion to a surface is the normal state for the great majority of microbes[3].
The adhesion of lactobacilli to epithelial cells and mucus, has been described by various authors. Adhesion of *L. fermentum*, *L. acidophilus*, *L. plantarum*, *L. casei* and *L. reuteri* to stomach squamous epithelial cells of mouse, mucus-secreting cell line, human colonic cell line and glycolipid structures of the mucosa are some examples of this [4,5,6,7,8,9].
Competition for adhesion sites between lactobacilli and some pathogens, that seem to use the same ligands for adhesion, have been observed.
Even components secreted from lactobacilli have been described to inhibit the adhesion of pathogens to mucus or cells[10,11]. Various lactobacilli can produce antimicrobial substances such as organic acids, bacteriocines, and reuterin. Gram-positive bacteria are often more sensitive for bacteriocines from lactobacilli than Gram-negative bacteria.
In contrast the Gram-negatives seems to be more sensitive for reuterin[12]. Reuterin is a broad-spectrum antimicrobal substance produced by *Lactobacillus reuteri.*
Reuterin affects viruses, fungi, protozoa and bacteria[13,14,15,] and have an antagonistic effect on the growth of *Helicobacter pylori* [12].

Helicobacter pylori is a curved, Gram-negative, microaerophyllic, oxidase, catalase and urease-positive bacillus presenting at one of its poles a bundle of four to six sheathed flagella conferring motility.
H. pylori is the main aetiologic agent of peptic ulcer and is also involved in the pathogenesis of non-atrophic and multifocal atrophic gastritis and in the risk of developing gastric cancer and gastric lymphoma.
H. pylori is exquisitely adapted to the gastric cavity environment. Its spiral shape is important for penetrating and traversing the gastric mucus layer and for achieving colonisation of the underlying mucosa.
Many factors involved in *H. pylori* virulence have been studied in detail, including urease, the VacAcitotoxin, CagA, the neutrophil-activacting protein NapA and lipopolysaccharide[16].

Bacterial adhesion to the surface is an initial important step for colonization and infection. It was widely accepted that *H. pylori* adheres to receptors in gastric epithelium by means of specific adhesions. Sialyl glycoconjugates, phosphatidylethanolamine and extracellular matrix proteins have been reported as putative receptors for *H. pylory* adhesion[17].
It was also demonstrated that *H. pylori* binds to glycolipids, including gangliotetraosylceramide (asialo-GM1) and sulfatide, and that adhesion to a human gastric epithelial cell line, MKN cells, was inhibited by an anti-sulfatide antibody[18]. High level of sulfatide expression in the gastric mucosa allows abundant colonization by *H. pylori*, resulting in the development of gastric lesions in animal models[19].
These findings imply that sulfatide is the main receptor molecule and that other glycoconjugates such asialo-GM1 also partially participated in the *H. pylori* adhesion.

L. reuteri has shown to possess the cell surface protein that inhibits the binding of *H. pylori* to receptor glycolipids in *vitro*[20].
L. reuteri is known to produce the antimicrobial compound 3-hydroxy propionaldehyde (reuterin), trough the metabolism of glycerol. The effect of reuterin on the growth of H. pylori has been tested.
The presence of 100 mM glycerol in the overlay assay resulted in a strong enhancement of the antagonistic activity which resulted in complete inhibition of *H. pylori* growth[12].

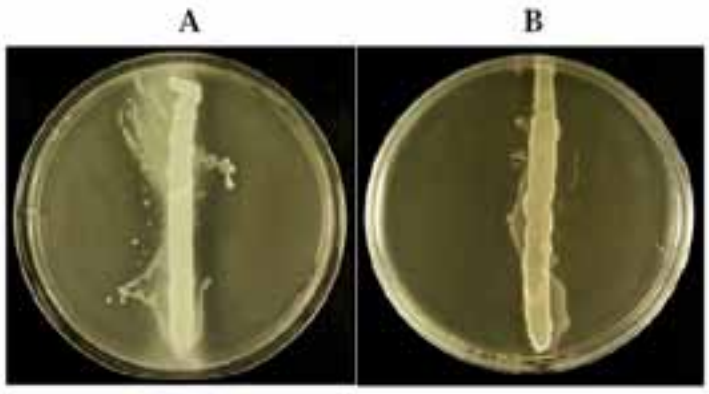


Fig. 3. Overlay assay for anti *H. pylori* activity. Lactobacillus strains were grown for 2 days on buffered Brucella agar plates and then overlaid with softagar containing *H. pylori* strain NCTC11637.
In experiments with L. reuteri, incubation was also performed on plates containing 100 mM glycerol.
A: Example of a positive (+) *L. gasseri.*
B: *L. reuteri* SD2112 on a plate with glycerol (+++).

The biological significance of reuterin activity is uncertain, since triglycerides are not fully degraded to free glycerol and fatty acids in this environment. However, many foodstuffs including bakery, ice-cream and wine contain considerable amounts of glycerol.

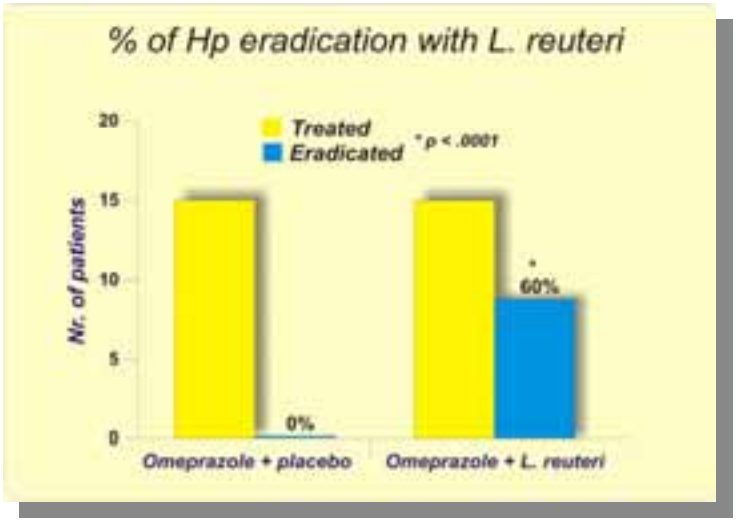
Moreover, Valeur has recently shown the *Lactobacillus reuteri in situ* colonisation of stomach (corpus and antrum) in heathy healthy volunteers and volunteers with ileostomy underwent gastroscopy or ileoscopy[21].

The need for alternative treatment strategies for *Helicobacter pylori* infections has created an interest to control this pathogen with probiotics.

We carried out a double-blind placebo-controlled study, to evaluate the effect of *Lactobacillus reuteri* administration on *H. pylori*-infected adults[22].

30 patients, aged 25-56, suffering from dyspepsia, and positive to UBT (urea breath test), were randomly and blindly located on either omeprazole 20 mg plus *L. reuteri* (ATC 55730) 10^8 cfu b.i.d. before breakfast and dinner or omeprazole 20 mg plus placebo b.i.d. for 30 days, after the infection with *Helicobacter pylori* was confirmed by endoscopy and CLO-test plus histology. 4 weeks after the end of therapy all patients were again controlled with all the three tests.

Patients receiving omeprazole plus *L. reuteri* were eradicated (3 negative tests) in 9 out 15 treated patients (60%), while no eradication occurred in the control population (omeprazole alone). Data were significant with $p<0.0001$.



Conclusions

The gastric pathogen *Helicobacter pylori* is the principal cause of peptic ulcers and the major risk factor for gastric cancers in humans.
From this study, it seems that probiotic supplementation by *L. reuteri* has a beneficial effect on *H. pylori* infections in humans being by itself able to eradicate the bacteria. It was demonstrated, in fact, that *L. reuteri* possesses the cell surface protein that inhibits *in vitro* the binding of *H. pylori* to glycolipids receptor and *in vivo L. reuteri* strains might be an effective competitor to *H. pylori* at the receptor site.

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Inhibition of Cell Adherence of *Clostridium difficile* by *Saccharomyces boulardii*

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Saccharomyces boulardii is a non pathogenic yeast originally isolated from lychee fruit related to *Saccharomyces cerevisiae. S. boulardii* administration significantly reduces the frequency of diarrhoea in patients administered antibiotic therapy. *S. boulardii* also protects against diarrhoea induced by a variety of enteric pathogens and reduces the risk of frequent relapses in *Clostridium difficile* infections. Recent studies have established that the yeast synthesizes proteins able to neutralize the effects of *Vibrio cholerae* and *C. difficile* toxins A and B. *C. difficile* is the etiological agent of 90% of cases of pseudomembranous colitis and 30% of cases of post antibiotic diarrhoea linked to the release of toxins A and B. However, colonization is likely to have an important role in the infectious process with adherence playing a key role due to the presence of adhesins on *C. difficile* surface.

The aim of this study was to assess the impact of the yeast on *C. difficile* cell adherence. Therefore we conducted inhibition adherence assays using Vero cells in the presence of whole yeast and its cytoplasmic and cell wall fractions.

In vitro and *in vivo* adherence assays

Adhesion assays revealed that *S. boulardii* does not adhere *in vitro* to Vero cells. In a human flora associated mouse model, *S. boulardii* was not associated to the mucosa but with the dominant microbial flora in the luminal part of the intestine during the administration of the yeast. In this model, the yeast did not quantitatively alter the total anaerobic flora nor the dominant bacterial groups. After, the end of the administration of *S. boulardii*, the yeast was cleared from the stools rapidly showing that it does not colonize the intestinal tract of mice.

In contrast, *C. difficile* attaches to Vero cells in strict anaerobiosis or after heat shock in aerobiosis. The mean number of *C. difficile* adhering to cells represents a positive control for adherence (100%. Figure 1). *C.difficile* adheres *in vivo* to the caeca of axenic mice as well.

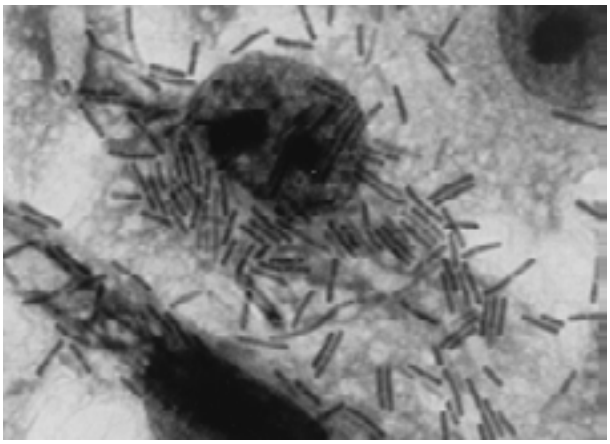

Figure 1: Adherence of *C. difficile* to Vero cells (Adherence 100%).

Different mechanisms could be responsible for the activity of *S. boulardii* against *C. difficile* in the gut, for example adherence of *C. difficile* to *S.boulardii*. To assess this hypothesis, bacteria and yeast were mixed and incubated under anaerobic conditions. A microscopic observation did not reveal bacterial adherence to the surface of yeast cells. Thus, the yeast does not appear to possess surface receptors to allow *C. difficile* attachment.

Adherence inhibition assays

S. boulardii* (whole yeast or yeast fractions) pre-incubated with *C. difficile. In order to determine the impact of the yeast and its cell fractions on adherence of *C. difficile* to Vero cells, *C. difficile* cells were pre-incubated with different fractions of the yeast prepared with or without the serine protease inhibitor PMSF before contact with Vero cells.

In the presence of the whole yeast cells, the level of adherence reached only 34% as compared with control ($P < 0.001$). The cell wall fraction of *S. boulardii* also reduced *C. difficile* adherence to Vero cells. When bacteria were pre-incubated with increasing quantities of the cell wall fractions (10 and 50 mg), the level of adherence reached 45% ($P = 0.009$) and 14% ($P = 0.004$), respectively, compared with the control. Furthermore, with PMSF in the yeast preparations, levels of adherence were close to that of the control, 92% and 85% with 10 mg and 50 mg of membrane extracts, respectively. The yeast culture supernatant did not interfere with *C. difficile* adherence to tissue culture cells. In the presence of the cytoplasmic contents of *S. boulardii* prepared with or without PMSF, the level of adherence was close to that of the control (91% and 109%, respectively) .

Only the cell wall fraction of *S. boulardii* displayed inhibition similar to that of whole cells. Moreover, the inhibitory effect of the membrane fraction was dose-dependent. These results

suggest that one or several proteins of the cell wall of the yeast could be responsible for the inhibition of adherence of *C. difficile* to Vero cells. These proteins could be proteases, because with the serine protease inhibitor PMSF, the effect was eliminated. However, we cannot exclude the hypothesis of steric hindrance as the mixture of *C. difficile* and whole yeast or membrane extract was incubated with the cultured cells during the assay.

***S. boulardii* (whole yeast or yeast fractions) pre-incubated with Vero cells.** To determine whether *S. boulardii* and its cellular fractions act on the Vero cell receptors mediating *C. difficile* adherence, target cells were pre-incubated with whole yeast or its cell fractions prepared with or without of PMSF prior to bacterial contact.

Pre-incubation of target cells with whole yeast cells did not interfere significantly with *C. difficile* adherence. When Vero cells were preincubated with 50 mg of *S. boulardii* cell wall fraction, a pronounced reduction of *C. difficile* adherence was evident (36% of control level, $P < 0.001$). As in the previous experiment, the level of adherence did not decrease in the presence of PMSF. Incubation of target cells with the yeast culture supernatant had no influence on levels of *C. difficile* adherence (126% and 84% with the non concentrated and concentrated supernatant, respectively). When Vero cells were preincubated with the intracellular contents of the yeast, the level of bacterial adherence was 71% as compared with the positive control (not significantly different).

These results suggested that the yeast cell wall appears to be able to modify the surface receptors involved in the adherence of *C. difficile*. Again, in presence of PMSF, the inhibitory effect of the extracts is abolished, suggesting a role for proteases. Moreover, the hypothesis of competition between the yeast and the bacteria can be excluded because *S. boulardii* does not adhere to Vero cells. Thus, the proteolytic enzyme(s) could act both on the receptors of the bacteria and the Vero cells and thereby inhibit the adherence.

In conclusion, these results show that *S. boulardii* could inhibit the adherence of *C. difficile* to cells and suggest that a membrane proteolytic enzyme could be responsible for destroying the adhesins in *C. difficile* and also the cell surface receptors. However, the hypothesis of steric hindrance by the yeast on the adherence of *C. difficile* to Vero cells could not be eliminated.

Horizontal transfer of antibiotic resistance genes between *Clostridium difficile* and commensal bacteria.

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Gene transfer events have been documented among bacteria belonging to different species and genera and also to different hosts. The widespread use of antibiotics has been responsible for the emergence of antibiotic resistance in both human and veterinary pathogens and it has been indicated as the main cause favoring the horizontal transfer of antibiotic resistance genes among various ecosystems. Since the occurrence of horizontal gene transfer between bacteria colonizing humans and those colonizing livestock is of great interest, this study was carried out to examine the *in vitro* transfer of one of the most widespread resistance genes, the macrolide-lincosamide-streptograminB (MLS$_B$) resistance gene *erm*(B), from the human intestinal pathogen *Clostridium difficile* to a commensal bacterium, abundant in the cattle rumen, *Butyrivibrio fibrisolvens.*

C. difficile is a spore-forming Gram-positive bacillus, opportunistic pathogen, that is responsible for many cases of antibiotic-associated diarrhea in humans and animals, and it has been recognized as one of the major causes of nosocomial diarrhoeic diseases. *C. difficile* strains are frequently resistant to macrolides and this resistance seems to underline the circulation of strains more prone to cause epidemics. *Butyrivibrio fibrisolvens* is one of the most abundant bacteria isolated from the rumen and has been identified also in the human gastrointestinal tract. It is a small, motile, curved rod with tapered ends bacterium, that, although currently classified as Gram-negative, analysis of both cell wall structure and 16S rRNA gene sequences indicates as Gram-positive. Previous studies demonstrated that some tetracycline resistance determinants could be transferred, under laboratory conditions, from different rumen and human microorganisms to *B. fibrisolvens*. On the basis of this evidence and considering the importance of gene horizontal transfer between bacteria colonizing

humans and those colonizing livestock, for example in a farm environment, we examined the possibility of the *erm*(B) gene transfer between *C. difficile* and *B. fibrisolvens*.

The transfer of erythromycin resistance from the *erm*(B)-positive *C. difficile* CD51, a clinical isolate, and the rumen commensal *B. fibrisolvens* 2221^{R} and *B. fibrisolvens* 1.230 strains was demonstrated. The transfer occurred at high frequency rates and *B. fibrisolvens* transconjugants showed high and stable resistance to erythromycin. Furthermore, onward transfer of erythromycin resistance determinant from *B. fibrisolvens* 2221^{R} transconjugants to the tetracycline resistant strain *B. fibrisolvens* 1.230, was also observed. The *erm*(B) gene transfers were confirmed by PCR, pulsed field gel electrophoresis (PFGE) and hybridization assays. The *in vitro* transfer of the MLSB resistance gene *erm*(B) between two obligate anaerobes, the human spore-forming *C. difficile* and the animal commensal *B. fibrisolvens*, suggests this event might occur also in the natural environment more commonly than previously suspected.

The conditions present in the gastrointestinal tract are recognized as strongly favoring the intra or interspecies gene transfer, so commensal and anaerobic bacteria can represent an important reservoir of antibiotic resistance genes and play an important role in antibiotic resistance spreading. The data presented in this paper provide the first evidence of transfer of erythromycin resistance between the human pathogen *C. difficile* and the rumen commensal *B. fibrisolvens* by conjugation *in vitro*, supplying an additional proof that the resistance gene horizontal transfer among gastrointestinal anaerobic bacteria could involve bacteria belonging to different ecosystems and normally found in different hosts.

The probiotic bacterium *Lactobacillus plantarum* as a model system to study biofilm formation and bacterial adhesion to epithelial host cells

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The idea to use commensal microorganisms to influence positively the course of an illness caused by pathogens has been pursued in the last years and increasing clinical evidence are now in favor of the initial hypothesis (1, 2, 3). Lactic acid bacteria of the genus Lactobacillus and Bifidobacterium, traditionally present in the dairy products, have been used for treatment and prevention of gut diseases since long time ago (4). *Lactobacillus plantarum* is among the Lactobacillus species encountered in the human intestinal tract (5). It is a member of the facultative heterofermentative group of lactobacilli, often isolated from plant material and fermented food. Probably due to its ecological niches, *L. plantarum* is able to hydrolyze various substrates of vegetable origins, showing strong ability to preserve food and prevent spoilage. For these metabolic characteristics *L. plantarum* is largely used in food industry as starter in vegetable, meat, fodder and beverage fermentation. Among Africans and Asians, whose diet is based on large quantities of *L. plantarum* fermented food, almost 100% of the population harbour this bacterial species in their gut (5). Among the features necessary to provide health benefits, commensal microorganisms must have the ability to adhere to human intestinal cells and consequently be able to colonize the gut. Strains of *L. plantarum* have been positively tested for their ability to adhere to cells of human colonic cell lines and as an antigen delivery system to elicit local immune responses (6, 7, 8, 9).

We tested the ability of *Lactobacillus plantarum* LM3, an isolate from plant material, to bind to human intestinal cells. For adhesion tests Caco-2 cells, a well-characterized cellular model deriving from human colon adenocarcinoma, were used. Experimental data showed that the number of LM3 cells adhering to random microscopic fields, allowed us to score this strain as strongly adhesive, according to the classification proposed by Jacobsen and co-workers (10) (fig. 1). Furthermore these experiments showed that the adhesion of *L. plantarum* to Caco-2 cells does not depend on secreted factors.

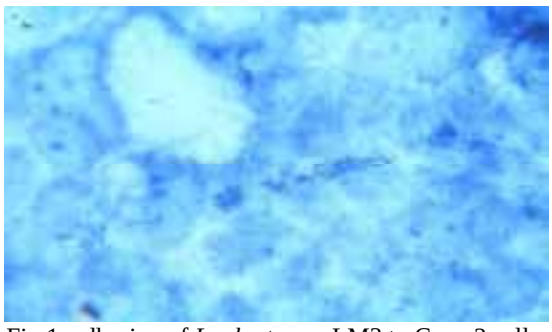

Fig.1: adhesion of *L. plantarum* LM3 to Caco-2 cells

Adhesion mechanisms of other bacteria belonging to the genus Lactobacillus involve non specific factors, particularly hydrophobicity and surface charge, as well as specific components such as carbohydrates and proteins on the cell surface. Concerning the target molecules on the human cell surface, some reports suggest that extracellular matrix and mucus proteins are possibly involved in the colonization of

the gut by commensal bacteria. A study performed on strains 299 and 299V of *L. plantarum* suggested that the binding to HT-29 cell line is mediated by mannose-specific adhesins (6). This kind of adherence mechanism is common among Gram-negative bacteria, but is rare for Gram-positive bacteria. This could be the reason for the strong ability of *L. plantarum* to compete with potentially pathogenic microorganism for receptors on the surface of human intestinal mucosal cells. More recently, Kleerebezem and coworkers identified 72 proteins from *L. plantarum* WCFS1 specifically expressed in the mouse gastrointestinal tract by the resolvase-based in vivo expression technology (11). Due to the specific selection adopted in this kind of technique, only differentially expressed genes were detected.

By ligand immunoblot assay we found two surface proteins involved in binding of *L. plantarum* LM3 to the fibronectin, with apparent molecular mass of 40 and 48 kDa. MALDI-TOF analysis of the latter, together with the availability of the *L. plantarum* WCFS1 genome sequence, allowed us to identify it as the product of the *enoA1* gene, encoding an alpha-enolase. Surface alpha-enolases of *Streptococcus mutans* and *Streptococcus pneumoniae* have been recently shown to bind to human plasminogen, while alpha-enolase of *Streptococcus aureus* was shown to be a laminin-binding protein (12, 13, 14). To demonstrate the role of alpha-enolase in binding to extracellular matrix proteins, attempts to construct strains carrying a null mutation in the *eno* gene have been made in *S. pneumoniae*, with no success due probably to the essentiality of the gene. Analysis of the complete genome sequence of the *L. plantarum* WCFS1 strain revealed the presence of a second gene coding an alpha-enolase, named *enoA2*. Microarrays analysis of global gene expression in *L. plantarum*, showed expression of both genes *enoA1* and *enoA2* (Muscariello, personal communication). We have successfully constructed the *L. plantarum* LM3-C1 carrying a null mutation in the *enoA1* gene. Characterization of the *L. plantarum* LM3-C1 phenotype is in progress.

The ability of the *L. plantarum* LM3 strain to form biofilm and the regulatory pathway of biofilm formation has been investigated as well. Biofilm microtitre plate assays showed that the *L. plantarum* LM3-2 strain, carrying a null mutation in the *ccpA* gene, encoding the catabolite control protein A (CcpA), was defective in biofilm production compared to the wild type strain (LM3) (fig. 2).

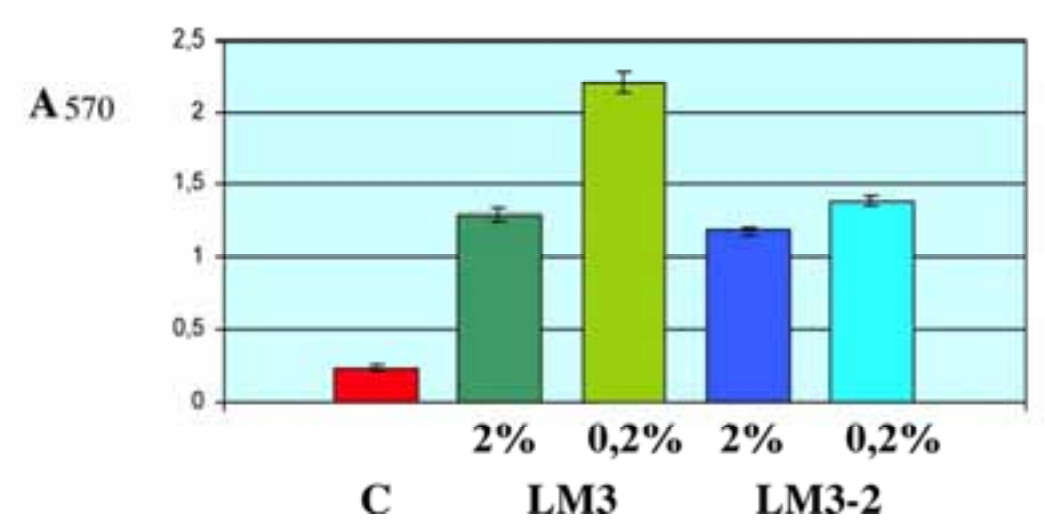

Fig. 2: quantitative assay of biofilm formation in *L. plantarum* LM3 (wt) and LM3-2 (*ccpA*$^-$) strains grown up to stationary phase in MRS broth containing 2% or 0.2% glucose. Control (C) consists of non-inoculated medium.

This result strongly suggests the involvement of the master regulator protein CcpA in biofilm biogenesis. Moreover, by sequence analysis, we found two loci in the *L. plantarum* genome, hereby named *flm1*and *flm2*, whose deduced amino acid sequences show 35 and 38 % identity with the *S. mutans* BrpA (biofilm regulatory protein A) (15). We investigated the role of the Flm1 and Flm2 proteins in biofilm formation by isolating two strains carrying null mutations in the two corresponding genes. Our results suggest involvement of both proteins in the regulatory pathway leading to the switch from the planktonic to the sessile form.

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Influence of iron and lactoferrin on aggregation and biofilm formation in *Lactobacillus* GG

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Thanks to its unique chemical properties, iron plays a pivotal role in biology. Although iron is vital for life, it is highly reactive and so can be toxic when in excess.

On the other hand, lactoferrin, highly conserved among human, bovine, mouse, and porcine species, is a glycoprotein of about 690 amino acid residues belonging to transferrin family, able to reversibly chelate two Fe(III) per molecule with high affinity (K_D ~10^{-20} M) retaining ferric iron to pH values as low as 3, whereas transferrin to pH of about 5.5 (Mazurier and Spik, 1980; Baker, 1994).

The iron-binding affinity is high enough that, in the presence of lactoferrin or transferrin, the concentration of free iron in body fluids cannot exceed 10^{-18} M, thus preventing the precipitation of this metal as insoluble hydroxides.

In addition, iron limitation (10^{-18} M) is considered in the healthy humans a physiological status hindering microbial growth and toxic reactive oxygen species. Conversely, an increase of iron concentration, as a consequence of some pathologies, favors microbial virulence.

Forty-five percent of the late deaths in neonatal intensive care units [NICUs] are caused by infection (Gaynes *et al*. 1996, Stoll *et al*. 1996). These deaths occur primarily in extremely preterm infants. The intestine is an important source of the pathogenic bacteria that cause either necrotizing enterocolitis [NEC] or gut-related sepsis. It is proposed that abnormal bacterial colonization of the intestine causes NEC in preterm infants (Claud 2001).

It is reported that pregnant women have an enteric bacterial flora that has genetic compatibility with their intestinal epithelia (Hooper 2001). These Authors suggest the maternal flora is best-suited to colonize the sterile gut of their newborn infant. This maternally-acquired gut flora provides commensal bacteria that promote growth and maturation of the neonatal intestine. Breastfeeding allows close contact between mother and infant and human milk has biofactors that

help establish bifidobacteria as a predominant intestinal bacterium (Harmsen *et al*. 2000). Bifidobacteria have been used to mitigate NEC in a neonatal rat model (Caplan *et al*. 1999). The healthy term neonate has close maternal contact, leaves the hospital shortly after birth, and is breast fed. The birth of an extremely preterm infant interrupts early nutrition with maternal milk and close maternal contact, and the intestinal flora of this infant is acquired from the NICU.

It is well known that lactoferrin acts as a bacteriostatic or bactericidal agent against several pathogens and these effects are mediated by iron sequestration and by the binding of this protein to microbial surfaces (Valenti et al 2004 and references therein).

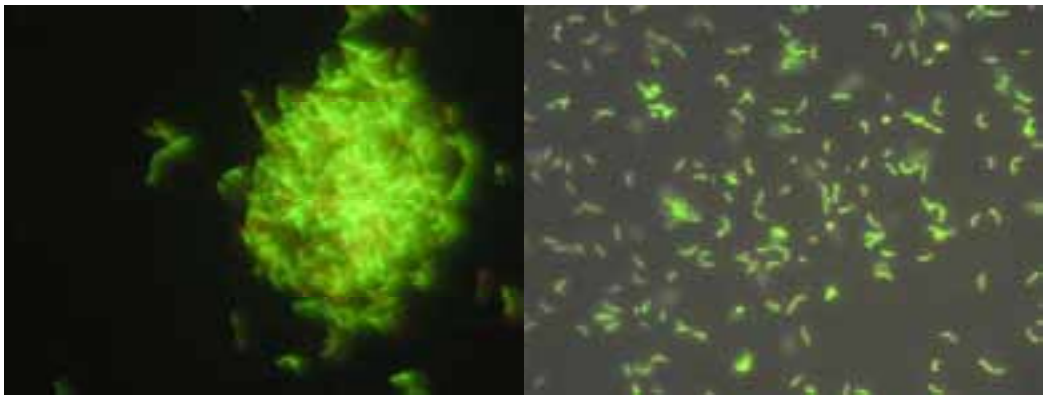
Here, we report that lactoferrin does not inhibit *Lactobacillus* GG growth and its adhesion to cultured intestinal cells. Lactobacilli are another member of the "bifidus flora" in the stool of breast-fed term infants (Harmsen *et al*. 2000).

However, the different iron availability influences *Lactobacillus* GG morphology. In particular, iron deprivation stimulates *Lactobacillus* GG to aggregate and form biofilm (Figure 1A), while iron load decreases aggregation and biofilm formation (Figure 1 B).

Moreover, *Lactobacillus* GG aggregates and biofilm exert an higher adhesion capability on cultured intestinal cells with respect to that showed by free-living forms.

Therefore, the iron-withholding capability of apo- or native-lactoferrin is an important signal to which *Lactobacillus* GG counteracts by leaving the free-form state and entering into a new lifestyle, aggregation and biofilm, to colonize and consequently to persist in the human intestine.

Figure 1. Morphological aspects of *Lactobacillus* GG induced by iron deprivation (A) and iron load (B)



A B

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Adhesion properties of *Bacillus clausii* probiotic strains

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The use of probiotics to enhance human health has been proposed for many years. A significant number of studies have demonstrated the therapeutic efficacy of probiotics when applied to the treatment of several gastro-intestinal microbial disorders (7, 8).
It has been suggested that the possible action mechanisms of probiotics involve pH reduction in the gut (from LAB), direct antagonism by production of antimicrobial compounds, viability and persistence in the gastro-intestinal tract, competition for the adhesion sites and stimulation of the immune system. Adhesiveness may be considered as a criterium to choose a probiotic strain (9). Adhesion to intestinal mucus and epithelial cells could confer a competitive advantage probably important for its beneficial health effect (2). Furthermore, probiotics should be able to compete with pathogens for the same receptors and to occupy their potential binding sites in the gut contributing to the competitive exclusion (Figure 1) (5, 6). Adhesion is also considered necessary for the probiotic to display immunomodulation properties (3).
The mechanisms responsible for the beneficial effects of *Bacillus* species, have remained relatively unexplored. The probiotic Enterogermina® that includes four *B. clausii* strains with a low level of intraspecific diversity, has been reported to exert beneficial clinical effects, notably in the treatment of diarrhea (4, 7). However, the study of the mechanisms responsible for these effects remain to be completed.
The present work was aimed to analyze the adhesive properties of Enterogermina® *B. clausii* strains and others *Bacillus* probiotics, to pre-characterize some proteins that could be implicated in adhesion and to investigate whether *B. clausii* could inhibit the ability of pathogenic adherent-invasive- *E. coli* to adhere to *in vitro* models.

Materials and methods

Bacterial strains and culture media.

The *Bacillus clausii* strains O/C, N/R, SIN and T (Enterogermina®), *B. subtilis* 3 (Biosporine®), *B. licheniformis* 2336 (Biosporine®), *B. subtilis* DSM 5750 (Bioplus 2B®), *B. subtilis* 534 (Sporobacterin®), *B. pumilus* Nha (Biosubtyl®), *B. cereus* (Prob.China), *B. cereus* (Neutralin®), and *B. cereus* DM-423 (Cereobiogen®) were used in the present study. *E. coli* EPEC O111 was used as positive control and in co-incubation experiments. All strains were grown routinely in Muller Hinton (MH) medium. To increase sporulation MH medium was supplemented with various mineral compounds.

Adhesion tests.

The difficulties in assessing adherence of probiotic strains *in vivo* have led to the development of *in vitro* adherence assays. Various *in vitro* models have been proposed to test the adhesion. In this study three models were used, involving different substrata : (i) Mucin, a glycoprotein which is the main component of mucus, (ii) extracellular matrix (ECM) proteins or Matrigel and (iii) Caco-2 intestinal epithelial cells. Bacterial adhesion to mucin and differentiated Caco-2 cells were assessed by counting Colony Forming Units (CFU). Bacterial adhesion to Matrigel was determined by microscopic counting of stained bacteria.

Protein extraction and analysis.

Bacterial surface proteins were extracted from vegetative cells by using three different methods based in the utilization of 5 M LiCl, 2 M guanidine hydrochloride or 10 mM NaOH. Spore proteins were extracted using alkaline reducing buffer, as detailed by Sylvestre *et al*

(10). The extracted proteins were separated by SDS-PAGE. Some electrophoretic bands were analyzed by nanospray-MS/MS.

Results and discussion

Bacterial adhesion to porcine mucin, Matrigel and Caco2 cells.

The results showed that, among all *Bacillus* species tested, vegetative cells of *B. clausii* strains were the most adhesives to mucin (approx. 3000 CFU/well) while three other strains presented moderately adhesion capacities and the remainder strains were very poorly or not adhesive (Figure 2). However, in comparison with *E. coli* EPEC and *Lactobacillus plantarum* 299v, which are highly sticky strains (approx. 14000 CFU/well), vegetative cells of *B. clausii* strains were moderately adhesive. In contrast, some *Bacillus* spores, including those of *B. clausii*, displayed higher adhesion to mucin than the control positive strains (Figure 2). Concerning Matrigel adhesion, strains were moderately adhesive compared with *E. coli* EPEC (3100 bacteria per microscopic field). Vegetative cells of *B. cereus* (Prob. China) were the most adhesives. With spores adhesion values were in the same range than values with vegetative cells, unlike what was observed in adhesion to mucin (Figure 3). Adhesion to Caco2 cells demonstrated that spores were in general highly adhesives. Spores of *B. cereus* (Prob. China) showed capacities of adhesion similar to those of *Lb. plantarum* 299v (9.2 CFU/cell). Vegetative cells excepted were poorly adhesive to Caco2 cells, excepted for two probiotics strains (Figure 4).

Characterization of compounds implicated in the adhesion.

Vegetative cells of *B. clausii* NR and *B. cereus* (Prob. China) and the Matrigel test were used in this study. Attachment of strains to Matrigel was reduced by treatment with LiCl and trypsin. *B. cereus* (Prob. China) attachment was completely abolished after a treatment using mannose and α-methylmanoside. This strain was also the only one capable of agglutinating yeast cells in a mannose-sensitive manner. This strain could thus posses adhesins specific for mannose receptors (1). The reduced adhesion of strains after protease treatment suggests that bacterial proteins were essential for adhesion to extracellular matrix proteins.

Extraction of surface proteins from vegetative cells and analysis by SDS-PAGE exhibited several proteins. A protein with a MW approx. of 30 Kd from *B. clausii* NR was extracted using NaOH 10mM together with two minority proteins (82 and 86Kd). *B. cereus* (Prob. China) showed various proteins extracted by LiCl or guanidine hydrochloride. Extraction of proteins from spores revealed one unique protein by strain.

Competition assay *E. coli / Bacillus*.

The adhesion of *E. coli* strain to mucin was inhibited in a competitive assay in presence of vegetative cells of *B. clausii* NR and OC strains. This reduction of adhesion was approximately of 75%.

Conclusions

The results obtained with *B. clausii* strains in the different adhesion models used in this study indicate that these strains are capable of adhering efficiently. Some candidate proteins were identified. Further studies are needed to characterize them and to demonstrate the implication of these surface proteins in the adhesion mechanism.

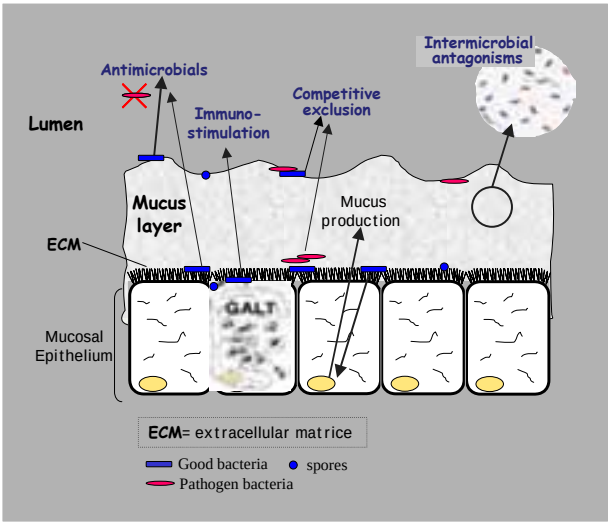

Figure 1. Gastrointestinal tract and bacterial interactions.

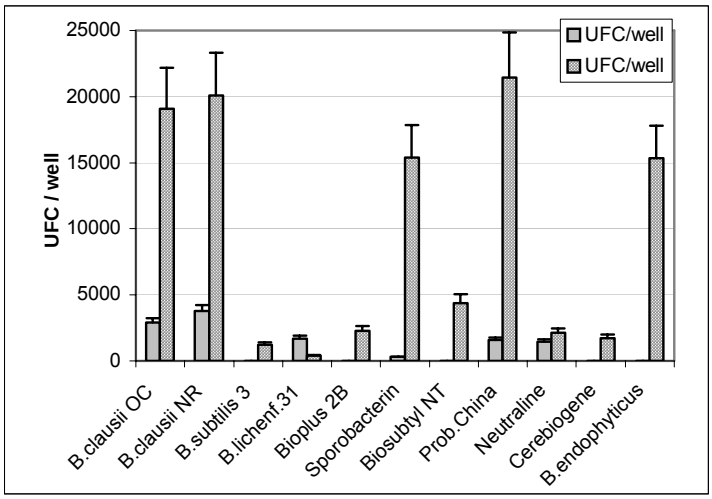

Figure 2. Bacterial adhesion to Mucin.

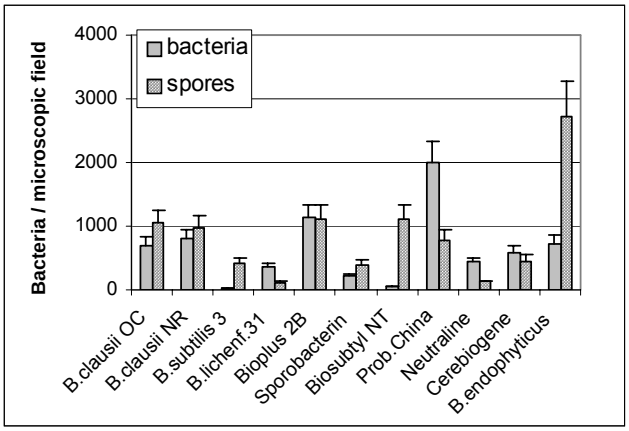

Figure 3. Bacterial adhesion to Matrigel.

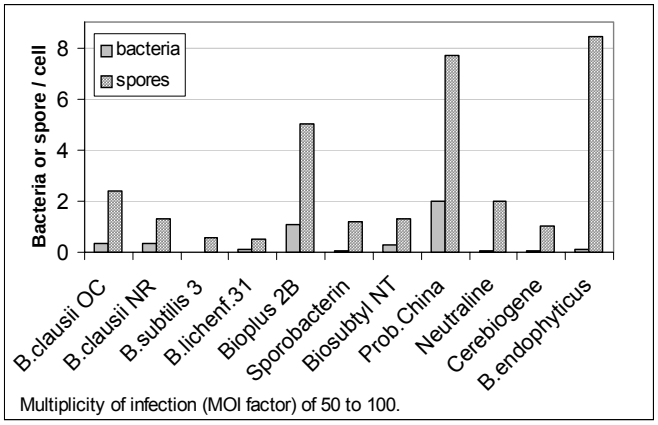

Figure 4. Bacterial adhesion to Caco2 cells.

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Bacillus clausii: new clinical immunological evidence.

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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 colonize 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).
Probiotics may stimulate immune system at all mucosal surfaces (6). In this regard, it has been demonstrated that probiotics exert a primary prevention of atopic diseases (7).
Gastroenteric microflora promote potentially antiallergic processes: T helper-1 (Th1) immunity (8), generation of TGFβ (9), and IgA synthesis (10). 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 subjects are characterised by a Th2 polarization (11). Th2 cells play a critical role in the pathogenesis of allergic rhinitis and asthma (12). However, the immunological mechanisms that downmodulate and protect against the development of them are poorly understood. A spectrum of CD4+ T cells might exert a role in regulating allergic reaction (Treg). Recently, a role for Treg has been evidenced in patients treated with specific immunotherapy for grasses and house dust mites (13,14). Moreover, a specific defect of Treg has been demonstrated in allergic patients in comparison with normal subjects. Thus, this evidence demonstrates that normal subjects are protected by allergies as they have normal T regulatory response to allergens.
In this regard, probiotics could represent an important

therapeutic advance concerning the prevention and therapy of atopy. *Bacillus clausii* is a safe and frequently prescribed probiotic. There are some studies showing its effects in preventing gastroenteric and respiratory infections in children. In addition, its effects on immune response have been reported in *in vitro* and *in vivo* studies. Recently, it has been provided evidence that *B clausii* is capable of reducing IL-4 levels and increasing IL-12, IFNγ, IL-10 and TGFβ levels in the fluids recovered from nasal lavages of both allergic children with recurrent respiratory infections and atopic adults (15,16). Moreover, it has been reported that *Bacillus clausii* is able of reducing the symptomatic use of antihistamines and eosinophilic infiltration in children with seasonal allergic rhinitis (17). Finally, preliminary evidence reports that *Bacillus clausii* might significantly reduce the number of upper respiratory infections in children in comparison with placebo.
In conclusion, it seems to be a body of evidence supporting a positive role of *Bacillus clausii* in regulating the immune response.

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PROBIOTICS FOR TREATMENT AND PREVENTION OF DIARRHOEAL DISEASES

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OBJECTIVE

To systematically evaluate the effect of probiotic administration for treating and prevention of diarrhoeal diseases.

SEARCH STRATEGY

Medical subject headings and free-language terms were used to search the MEDLINE (1966 – May 2005) and The Cochrane Library (Issue 2, 2005) for studies relevant to probiotics and diarrhoeal disorders; additional sources were obtained from reference lists of review articles. Only randomized controlled trials (RCT) or their meta-analysis were considered.

RESULTS

Treatment of acute infectious diarrhea

The rationale for the use of probiotics to treat or prevent acute infectious diarrhoea is based on the assumption that they modify the composition of the colonic microflora and act against enteric pathogens. The evidence from four meta-analyses[i] [ii] [iii] [iv] consistently show statistically significant effect and moderate clinical benefit of *some* probiotic strains in the treatment of acute watery diarrhea, mainly rotaviral, in infants and young children mainly. So far, the beneficial effects of probiotics in acute infectious diarrhea seem to be: (1) moderate; (2) strain-dependent; (3) dose-dependent (greater for doses $>10^{10}$–10^{11} CFU); (4) significant in watery diarrhea and viral gastroenteritis, but not existing in invasive, bacterial diarrhea; (5) more evident when treatment with probiotics is initiated early in the course of disease; (6) more evident in the developed countries. Discouraging results from several recent studies[v] [vi] (not included in any of the meta-analyses) should be considered.

Prevention of nosocomial diarrhea

Nosocomial diarrhoea defined as any diarrhoea which a patient contracts in a health-care institution may prolong hospital stay and increase medical costs. Two studies on *Lactobacillus* GG in young children admitted for relatively short stay showed conflicting results.[vii] [viii] Of two RCTs addressing the efficacy of *Bifidobacterium bifidum* (recently renamed *B. lactis*) and *Streptococcus thermophilus,* one trial suggests the benefit of *B. bifidum* in sick infants admitted to hospital,[ix] and one no such benefit in healthy children in residential care settings.[x] Clearly, these interventions need to be studied further before they are routinely recommended for prevention of nosocomial diarrhea in children.

Prevention of antibiotic associated diarrhea

Antibiotic-associated diarrhea (AAD) is defined as an acute inflammation of the intestinal mucosa caused by the administration of broad-spectrum antibiotics. The rationale for the use of probiotics in AAD is based on the assumption that the key factor in the pathogenesis of AAD is a disturbance in normal intestinal microflora. Two systematic reviews of RCTs were found.[xi] [xii] The authors of both concluded that there is evidence of a benefit of some probiotic strains in the prevention of antibiotic-associated diarrhoea. However, the evidence for

beneficial effects is still not definitive. Meta-analysis of the various studies assessing different probiotic strains in preventing AAD is difficult, as the studies are heterogeneous and the definition of diarrhea is not consistent. There was also a variability in the antibiotic type, dose, and duration of different studies. With few exceptions, the trials included in meta-analyses were mainly carried out in adults, and thus the authors' conclusions may not be directly applicable to children. In the latter population, two RTCs provided evidence of a moderate beneficial effect of *Lactobacillus* GG in the prevention of antibiotic-associated diarrhea, but results in adults are conflicting.[xiii] [xiv] [xv] One recent large RCT[xvi] provided evidence that *S. boulardii* is effective in preventing AAD and this result is consistent with data in adults. Data on the efficacy of other probiotic strains in children are very limited. Further research is needed.

***Clostridium difficile* diarrhoea**

The bacterial agent most commonly associated with antibiotic-associated diarrhoea is *Clostridium difficile*. One systematic review on the use of probiotics in the prevention and treatment of *C. difficile* diarrhoea was found.[xvii] All studies reviewed were conducted among adult subjects. The authors concluded that there is very little evidence relating to the use of probiotics for either prevention or treatment of *C. difficile.* Available evidence does not support the administration of probiotics with antibiotics to prevent the development of *C. difficile* diarrhoea, and is inadequate to justify its introduction as a treatment for developed *C. difficile* diarrhoea.

Prevention of community acquired diarrhea

Prevention is the most important challenge posed by childhood diarrheal diseases, particularly in the developing countries. There has only been one randomized controlled trial evaluating the beneficial effect of probiotics on prevention of community acquired diarrhea. The study involving 204 undernourished infants living in a community with a high burden of diarrheal diseases showed fewer episodes of diarrhea in children who received *Lactobacillus* GG. This benefit was particularly evident in non-breast fed children.[xviii]

Prevention for acute diarrhoeal illnesses in otherwise healthy children

Children attending day care centres are considered to be at high risk of gastrointestinal and respiratory infections. The successful prevention of these events could be useful for families and for society in general. Fours RCTs evaluating the beneficial effect of probiotics on prevention of acute infectious illnesses, including diarrhoeal diseases, in otherwise healthy children were found. [xix] [xx] [xxi] [xxii] These RCTs provided evidence of very modest effect (statistically significant but of questionable clinical importance) of some probiotic strains on prevention of diarrhoeal diseases in otherwise healthy infants and children. Future large-scale and long-term studies should establish preferred modes of therapy (ie, duration, dosage, etc) for better clinical effects. Whether or not this effect size has a substantial public-health effect remains to be determined.

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Fecal microbiota in IBS

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Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders, since approximately 5-20% of the western population is estimated to suffer from it. IBS is characterised by abdominal pain, variable bowel habit, bloating and flatulence. IBS is neither life threatening nor does it predispose to any other diseases. The syndrome has, nonetheless, a considerable effect on the quality of life. The pathogenesis of IBS remains unknown, but current evidence suggests that altered gut motility, visceral hypersensitivity and dysregulation of the brain-gut axis play a crucial role. There is also a growing body of data showing that the gastrointestinal (GI) microbiota may be associated with IBS.

An imbalanced microbiota could contribute to GI symptoms through several mechanisms. Altered colonic fermentation may result in increased formation of gas and an abnormal pattern of short chain fatty acids. Evidence from inflammatory bowel disease also shows that enteric bacteria can trigger mucosal wall inflammation. Studies in animal models show that mucosal inflammation can result in IBS-like symptoms, such as altered gastrointestinal motor function and visceral hypersensitivity. The possible role of low-grade mucosal inflammation in IBS has recently attracted growing attention. A raised number of inflammatory cells in mucosal biopsies suggest the presence of inflammation in some IBS patients. Studies in animal models clearly show that a causal relationship exists between mucosal inflammation, altered gastrointestinal motor function and visceral hypersensitivity.

Early studies by conventional methods suggest that IBS patients have increased numbers of facultative organisms and decreased numbers of lactobacilli and bifidobacteria compared to healthy controls. The patient populations have, however, been rather small in these studies. We have studied the faecal microbiotas in 27 IBS patients fulfilling the Rome II criteria, and in 22 healthy age- and gender-matched control subjects (Malinen et *al*. 2005, Mättö et *al.* 2005). Subjects gave three faecal samples (0, 3 and 6 months) during a six-month follow-up. Total bacterial DNA was analysed by 20 quantitative real-time PCR assays covering approximately 300 bacterial species. In addition, DGGE and culturing methods were applied. Considerable individual variations in the GI microbiota

were found both in the patient group and among the healthy controls. Since IBS is a heterogeneous condition the analyses were performed for all the patients, and separately for the diarrhoea-predominant, the constipation-predominant and the mixed subgroup. We found that especially diarrhoea-predominant patients have a decreased amount of *Lactobacillus* spp., whereas constipation predominant patients carry an increased number of *Veillonella* spp. Average results from the three faecal samples suggested differences between IBS patients and controls also in the *Clostridium coccoides* subgroup and the *Bifidobacterium catenulatum* group. We also saw temporal instability in the predominant bacterial populations among the IBS patients, while the composition of the GI microbiota is known to be relatively stable in healthy subjects.

The alterations seen in the GI microbiota suggest that a probiotic therapy approach is justified in irritable bowel syndrome. Thus we further investigated whether a probiotic mixture containing *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus rhamnosus* LC705, *Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* JS (Valio Ltd, Helsinki, Finland) is effective in alleviating IBS symptoms. One hundred and three patients fulfilling the Rome criteria I or II took part in a 6-month, randomised, double-blind placebo-controlled trial. The patients received a probiotic capsule or a placebo capsule daily. Gastrointestinal symptoms and bowel habits were recorded. The results are given for the per protocol population (n=81). During the last month of the study the treatment difference in the baseline-adjusted symptom score (abdominal pain + distension + flatulence + borborygmi) was -7.7 points (95% CI -13.9 to -1.6) when the probiotic group was compared to placebo (p=0.015; figure 1). This is comparable to a median reduction of 42% in the symptom score for the probiotic group versus a 6% reduction for the placebo group. In individual symptoms, borborygmi was milder in the probiotic group (p=0.008), and for the rest of the symptoms there was a non-significant trend. The defecation data were analysed for all the patients, and separately for the diarrhoea-predominant, the constipation-predominant and the mixed subgroup, according to the Rome II criteria. A trend towards increased weekly frequency in the probiotic group was noted in the constipation and mixed subgroups during the last three months of the study. There were, however, no significant differences between the treatment groups. When the probiotic group was compared to the placebo in the constipation subgroup, the proportion of hard stools had decreased with a treatment difference of -25% (95% CI -52.5 to 2.0; p=0.067).

Our findings imply that alterations in the GI microbiota are associated with irritable bowel syndrome. A probiotic supplementation with LGG, *L. rhamnosus* LC705, *B. breve* Bb99 and *P. freudenreichii* JS could be a safe and easy way of alleviating IBS symptoms. Considering the high prevalence of IBS and the lack of effective therapies, even a slight reduction in symptoms could have positive public health consequences.

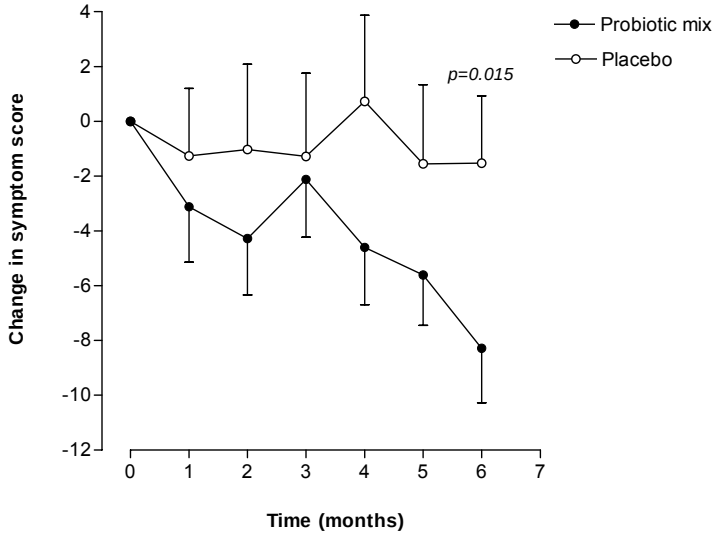

Figure 1. Change (mean ± SEM) in total symptom score (abdominal pain + distension + flatulence + borborygmi) during the six-month intervention (p=0.015 at six months; n=81).

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POST-INFECTIOUS IRRITABLE BOWEL SYNDROME

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Acute infectious gastroenteritis is a common event with in western countries with an estimated frequency of 1.4 episodes per person per year. Although the vast majority of subjects recover completely following resolution of the acute infection, a significant subgroup of subjects (up to 30%) go on to develop long-lasting digestive symptoms. In most cases, these symptoms fulfill the criteria for the irritable bowel syndrome (IBS), the so called post-infectious IBS (PI-IBS). In a classical study on IBS published some 40 years ago, Chaudary and Truelove underscored the importance of infectious gastroenteritis in the etiopathogenesis of IBS. In their series, a proven or highly presumptive episode of acute gastroenteritis preceded the onset of symptoms in 34 out of 130 patients (about 25%). More recently, the link between gastrointestinal infection and the development of prolonged IBS-like symptoms has been confirmed in several prospective studies showing that PI-IBS symptoms develop in 7-32% of subjects after acute enteritis. In these patients the most frequent complaints are diarrhea, urgency, abdominal discomfort or pain and abdominal bloating.

There are several risk factors for the development of PI-IBS. These include, the virulence of the pathogen (the risk for the developing IBS after *Campylobacter* and *Shigella* is similar, but greater than that for *Salmonella*), the severity and duration (more than 2 week) of the acute enteritis, younger age, female sex and the concomitance of significant psychological disturbances (including hypochondria, anxiety, depression and adverse life events) around the time of the infection. The use of antibiotics during acute bacterial gastroenteritis is also thought to increase the risk of development of long-term digestive symptoms. Unpublished observations suggest that

probiotics may reduce the long term development of symptoms, however controlled studies are needed. The prognosis of PI-IBS does not appear to be different from that of non-specific IBS.

Data indicate that patients with PI-IBS develop gut functional abnormalities including motility disturbances and visceral hypersensitivity. Animal models in which to test pathophysiological hypotheses ad pharmacological intervention have been recently developed. Recent work demonstrated that following acute infectious gastroenteritis whole gut transit was accelerated in all subjects. However, the faster transit times were observed in those who developed IBS. Furthermore, in the same study it was shown that PI-IBS patients developed a reduced threshold for discomfort during balloon distension of the rectum. The mechanisms underlying the post-infective gut dysfunction are yet to be elucidated, however, the persistence of a low grade inflammatory response in the colonic and ileal mucosa (albeit undetectable with colonoscopy or routine histology), suggest that immunological/inflammatory mechanisms may be involved. Increased numbers mast-cells, T-lymphocyte, macrophages, as well as increased expression of pro-inflammatory cytokines within the colonic and ileal mucosa have been described. Immune/inflammatory cells may perturb the function of neurons within the intestinal wall leading to changes in motor function as well as that of sensory nerves conveying information to the central nervous system, evoking visceral hypersensitivity and abdominal pain. We have recently observed that mast cells are significantly increased in both patients with non-specific as well as PI-IBS. Interestingly, mast cells were located in close proximity to fibers innervating the colonic mucosa and released an increased amount of mediators (e.g. histamine and tryptase) known to affect the function of gut intrinsic and sensory nerves. Thus immune/inflammatory mechanisms may well be involved in sensory and motor dysfunction and participate to symptom generation in patients with PI-IBS. In addition to immune/inflammatory changes also serotonin-containing enteroendoccrine cells were increased 3 months after infection in PI-IBS patients. Serotonin regulates several important digestive functions by acting, mainly on several receptors located on intrinsic and extrinsic nerves. A number of effective drugs acting at serotonin receptors have been developed for IBS, further supporting the importance of

serotonin in IBS patophysiology. Thus, changes in serotonin metabolisms has been recently suggested to participate to the pathophysiology of PI-IBS.

For the time being the management of patients with PI-IBS should not differ consistently from that of non-specific IBS. The diagnosis should be based on current symptom-based criteria in most cases, with careful attention to the eventual presence of alarm features or age above 45 years that impose further testing. Testing for persistent infection (particularly Giardiasis), celiac disease, microscopic colitis or bile salt malabsorption can be considered in selected cases. The pharmacological treatment is based on current management strategies applied for non-specific diarrhea predominant IBS. Regarding the specific issue of probiotics, there are no controlled studies available on their role in PI-IBS. Nonetheless, recent studies in animal model of PI-IBS have shown that *Lactobacillus paracasei* normalized persistent post-infective motility dysfunction, suggesting that probiotics may be useful to improve post-infective bowel disturbances. Further work is now required to assess whether probiotics may be useful in PI-IBS.

Suggested reading

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Bifidobacterium - rifaximin combined therapy for the treatment of IBS

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Bifidobacteria form one of the major groups of the intestinal bacteria that play beneficial effects for the health of the host. Due to their probiotic properties, some *Bifidobacterium* species have become common components in many fermented dairy products and pharmaceutical formulas. The efficacy of pharmaceutical probiotics in the management of intestinal disorders, including IBS, has been demonstrated by recent clinical controlled trials (1, 2). Several mechanisms exerted by probiotics have been suggested to explain their efficacy: competitive exclusion; modulation of the immune response of gut-associated lymphoid and epithelial cells; antimicrobial activity and suppression of pathogen growth; enhancement of barrier function; and induction of T cell apoptosis in the mucosal immune compartment. Furthermore, the importance of bacteria in sustaining intestinal disorders such as IBS is supported by the clinical experience that antibiotics reduce disease activity. Due to their complementary mechanisms of action, effective therapeutic approaches for intestinal diseases alternate between antibiotic and probiotic treatments.

Rifaximin is one of the antibiotics used within the treatment regimens for intestinal disorders (3). It belongs to the rifamycin family and is characterized by a low gastrointestinal absorption and good antibacterial activity against Gram-positive and Gram-negative aerobic and anaerobic organisms. Rifaximin, in common with its structural analogue rifampicin, acts on the RNA polymerase enzyme of bacteria to inhibit RNA synthesis.

In a previous clinical trial performed with patients affected by ulcerative colitis, it was demonstrated that the administration of high doses of antibiotic did not permanently alter the microbiota equilibrium, but allowed the selection of a significant number of rifaximin-resistant *Bifidobacterium* (4). Interestingly, these mutants disappeared during the “wash-out” cycle suggesting a possible resistance reversion and/or possible metabolic disadvantages in competition with the wild-type clones without selective pressure.

The idea at the basis of the present study is to propose the simultaneous use of the antibiotic rifaximin and probiotic bifidobacteria for the clinical treatment of several intestinal disorders, including IBS. In this perspective, we tried to understand, by using both genetic and proteomic approaches, the molecular factors determining the rifaximin resistance in *Bifidobacterium* and the interactions occurring in the gut between these bacteria and the drug.

The first step of our investigation was the *in vitro* demonstration that a representative number of intestinal bifidobacteria, showing a high sensitivity to rifaximin (MIC values range from <0.0625 to 0.5 μg/ml), spontaneously assumes the resistant phenotype when cultured in presence of rifaximin (100 μg/ml).

Rifaximin forms a complex with the β subunit of the RNA polymerase (*rpoB*) resulting in blockage of the transcription process. Since point mutations in *rpoB* have been indicated as the principal factor determining the rifampicin resistance in *E. coli* and *M. tuberculosis* (5, 6), we analysed a 129-bp *rpoB* core region (codons 508-550) for several wild-type and rifaximin-resistant bifidobacteria. Even if a high frequency of silent mutations was a peculiar genetic feature of *Bifidobacterium* rifaximin-resistant clones, five different types of miss-sense mutations were found in codons 513, 516, 522 and 529. The possibility that other resistance mechanisms take place in *Bifidobacterium* is supported by the observation that only silent nucleotide changes, and no functional mutation, were found in the rifaximin-resistant mutant of *B. bifidum* ATCC 29521. Related to this result, a significant number of rifampicin-resistant mycobacteria did not show mutations targeted to *rpoB* gene.

Further physiological and biochemical aspects of the rifaximin resistance were taken into account using as model system the strain *B. infantis* BI07, component of the probiotic VSL#3 preparation. We showed that BI07-res did not present any cross-resistance to different antibiotics, except to rifampicin, and it was genetically stable, as no reversion phenomenon was observed after 400 bacterial generations in absence of selective pressure.

Comparison of the growth characteristics of BI07-wt and BI07-res was performed in order to understand whether structural modifications of RNA polymerase could be mirrored in changes of growth and metabolism of resistant bacteria. Interestingly, no significant difference was observed between time courses of BI07-wt and BI07-res in single batch fermentations, whereas in a co-culture process without selective pressure BI07-wt was advantaged in competition with BI07-res. These data support the hypothesis that metabolic changes occur as a consequence of the antibiotic resistance acquisition and can explain the disappearance of the rifaximin-resistant bifidobacteria from the gut of patients after the interruption of the antibiotic treatment.

To elucidate the physiological changes correlating to the rifaximin resistant phenotype and to determine novel molecular mechanisms related to the *rpoB* gene mutation, a proteomic analysis of *Bifidobacterium* rifaximin resistance was carried out. The comparative proteomic mapping of BI07-wt and BI07-res revealed a linkage between the rifaximin-resistant phenotype and increased expression levels of stress proteins. Over-expression of stress proteins was expected as they represent a common unspecific response of bacteria when stimulated by different shock conditions,

including the exposure to toxic agents like heavy metals, oxidants, acids and antibiotics. Also positive transcription regulators were found over-expressed in BI07-res, suggesting that bacteria could activate compensatory mechanisms to assist the transcription process in presence of RNA polymerase inhibitors. Other differences in expression profiles were related to proteins involved in the central metabolism; these modifications could explain the kinetic advantages shown by the wild type bifidobacteria in comparison with the resistant mutants, without selective pressure, in *in vitro* co-cultures and *in vivo* gut environment.

In conclusion, the genetic and proteomic characterization of the rifaximin resistance in *Bifidobacterium*, excluding any risk factor for the horizontal transmission of resistance elements between the intestinal microbial species, supports the idea for the development of an antibiotic-probiotic combined therapy for the treatment of the gastrointestinal diseases.

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Probiotics and Irritable Bowel Syndrome

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Abstract

This talk will review the current evidence of the efficacy of probiotics, including lactobacilli, bufidobacteria, and VSL#3, in the treatment of irritable bowel syndrome. The mechanisms potentially important will be evaluated, with specific reference to immune function, effects on motility and intraluminal milieu.

Introduction

Probiotics are preparations that contain viable microorganisms that confer potential health benefits by preventing or treating specific pathologic conditions[1,2]. They are currently easily available, do not require prescription, and are used extensively for the relief of abdominal symptoms[1-6]. The scientific basis of probiotic use has been investigated recently, and clinical studies have documented efficacy in treatment of inflammatory bowel disease or pouchitis[6-8] or irritable bowel syndrome[9-11]. The mechanism of such benefit is unclear; in the latter study, improvement in symptoms with bifidobacteria was associated with changes in the relative production of anti-inflammatory to pro-inflammatory cytokines. Other effects of probiotics may conceivably account for the clinical benefit. Colonic bacteria normally metabolize nutrient substrates reaching the colon with the formation of gas and production of short chain fatty acids. The latter may induce propulsive contractions[12] and accelerate transit, or enhance fluid and sodium absorption in the colon[13].

We recently compared the effects of a specific probiotic combination, VSL#3 and placebo, on IBS symptoms and colonic transit in patients with IBS and significant abdominal bloating in a parallel-group, double-blinded, placebo-controlled study with a total of 48 patients being randomized to either placebo (N=24) or VSL#3 (N=24). Participants were required to pursue a baseline 2-week run-in period, recording daily symptoms by means of a diary, and followed by a treatment period. At the end of run-in, and of the treatment phase, all patients underwent scintigraphic measurement of colonic transit.

VSL#3 is a composite probiotic and each sachet contains 450 billion viable lyophilized bacteria: *Bifidobacterium* (*B. longum, B. infantis,* and *B. breve*); *Lactobacillus* (*L. acidophilus, L. casei, L. delbrueckii* subsp*. bulgaricus,* and *L. plantarum*); and *Streptococcus salivarius* subsp. *thermophilus*. The study preparation was in a powder form which is miscible in yogurt, which facilitates the dissolution and enhances palatability of the preparation. The matching placebo powder was supplied by the manufacturer (VSL Pharmaceuticals Inc., Gaithersburg, MD). The powder preparations were dissolved in 6 ounces of commercially available pasteurized yogurt and immediately ingested. Others have shown PCR detection of *Bifidobacterium* strains and *Streptococcus thermophilus* in feces of human subjects (healthy and inflammatory bowel disease) after VSL#3 and the recovery of *S. thermophilus* by PCR was around one log higher with VSL#3 than with yogurt[14].

Results

The prior medical histories and two-week run-in period were used to classify bowel function predominance of the 48 participants: 16 were constipation-predominant, 20 were diarrhea-predominant, and 12 were alternators. Of the 48 patients, 24 were randomly assigned to VSL#3 and 24 to placebo. The demographic data were similar between the two groups.

The mean post-treatment scores (Table 1) were numerically lower for the VSL#3 treatment group for virtually all symptoms, with the score for flatulence being statistically significant [(p=0.01) Figure 1]. The score for bloating was also reduced though it was not statistically significant [(p=0.11) Figure 1].

The proportion of the two groups who reached the threshold of 50% weeks with satisfactory relief, thus fulfilling the *a priori* set criterion for responders for abdominal bloating were 46% (11/24) for the VSL #3 group vs. 33% (8/24) placebo group (Fisher's exact test, p=0.27).

The colonic geometric center (GC) at 24 hours (primary transit endpoint) obtained for the two groups (n=45 with complete data) showed that colonic transit was retarded with VSL#3 relative to placebo (n=45). The mean GC (adjusted for baseline transit) was 2.76±0.20 for placebo and 2.17±0.20 for the VSL#3 group (p=0.05). The Pearson correlation coefficient between the change in flatulence score (baseline to average on treatment) and the change in colonic transit (GC24 at baseline and end of treatment) was 0.19 (p=0.21).

There were no adverse effects attributable to treatment with either VSL#3 or placebo.

Discussion

The data presented here extend information from three recently published studies. In a 4-week randomized, controlled trial, $5*10^7$ cfu/ml of *Lactobacillus plantarum* and 0.009g/ml (3.6gm) of oat flour in a rose hip drink were compared to placebo (rose hip syrup) in 60 patients with IBS[9]. Flatulence was significantly lower in the Lactobacillus-treated group compared to the placebo-treated group. Abdominal pain was lower than at baseline in both groups, but no significant difference between the two treatments was observed. Overall gastrointestinal

function at one year was reported to be significantly better in the active treatment group. In this study, the test product contained fiber and this may have confounded the observed results. Further evaluation, without the addition of fiber is warranted to assess the effect specifically attributable to the probiotic in the improvement of symptoms.

We have previously conducted an 8-week randomized, placebo-controlled trial comparing VSL#3 to placebo in 24 diarrhea-predominant IBS patients and identified a trend towards improvement in abdominal bloating[10].

The largest trial to date demonstrated a significant effect of *Bifidobacteria species* but not with *Lactobacilli* species or placebo[11]. The latter study also provided intriguing mechanistic information about changes in inflammatory cytokines, which might help explain the beneficial treatment effect observed. It is unclear why *Lactobacilli* were ineffective in the Cork study, given the experimental data (discussed below) and the efficacy of *Lactobacilli* species in the study by Nobaek et al[9].

The primary endpoint used in the trial from Cork was novel, a composite Likert score of pain/discomfort, bloating and difficulty with bowel movements. The secondary endpoints were significantly different for Bifidobacteria vs. placebo in different weeks during the 12-week trial.

The mechanism of the benefit observed with probiotics remains a matter of significant interest. The observations from Cork suggest that probiotics have an anti-inflammatory effect[11], and this is consistent with the demonstrated benefit in inflammatory bowel disease including pouchitis. Animal model and human studies have evaluated the immunologic modulation with specific probiotic bacteria. In brief, the potential anti-inflammatory effect of *Lactobacillus reuteri* in an experimental rodent study demonstrated an inhibition of tumor necrosis factor-α (TNF-α) induced production of IL-8[15]. *Lactobacillus casei,* which is found in the probiotic combination VSL#3, significantly decreased TNF-α release in ileal tissues from patients with Crohn's disease[16]. Other papers[17-21] that evaluated the anti-inflammatory effects of probiotic organisms are summarized in Table 2.

A second potentially beneficial mechanism is that probiotics may alter the colonic milieu or motility. We observed a significant retardation of colonic transit with VSL#3, though this was not associated with worsening of bowel function. Further studies are now indicated to explore the mechanism of the retarded transit of stool, and potential effects on colonic sensation, and colonic fermentation of nutrients reaching the colon.

An alternative explanation for the observed retardation in colonic transit is that *Lactobacilli* and *Bifidobacteria* subspecies are able to deconjugate and absorb bile acids[22-26]. This may result in a reduced bile salt load to the colon.

Another mechanism may be considered, based on the demonstration by Bazzocchi et al. that the colon's reflex motor responses to balloon distension were reduced during an open treatment study with VSL#3[27].

In summary, the literature confirms the potential benefit of *bifidobacteria* alone or the specific probiotic combination, VSL#3, on symptoms in IBS without induction of significant changes in bowel function.

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Table 1A. Analysis of Symptoms Analyzed over Entire Treatment Period (Mean ± SEM)

VAS Data	Urgency	Pain	Bloating	Flatulence
Placebo	22.7 ± 2.1	26.9 ± 2.2	38.5 ± 3.1	39.5 ± 2.6
VSL #3	19.7 ± 2.1	23.0 ± 2.2	31.3 ± 3.1	29.7 ± 2.6
p-value	0.32	0.22	0.11	0.01

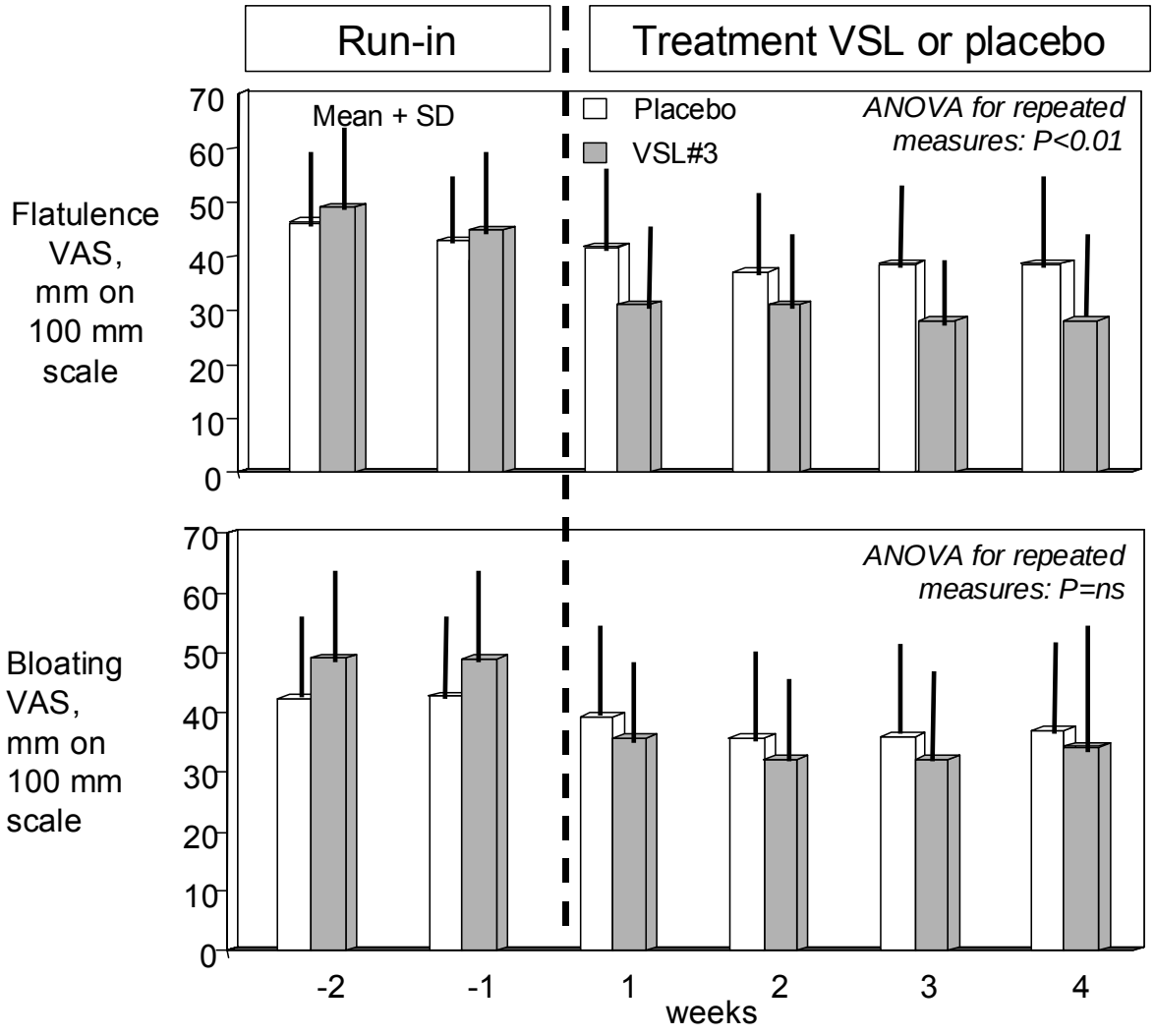
Table 1B. Analysis of Stool Data Analyzed over Entire Treatment Period (Mean ± SEM)

Stool Data	Ease of Passage	Number of stools per week	Stool Form	Percentage (%) with Incomplete Evacuation
Placebo	4.1 ± 0.1	12.2 ± 0.7	3.8 ± 0.1	45 ± 3
VSL #3	4.2 ± 0.1	11.3 ± 0.7	3.7 ± 0.1	50 ± 3
p-value	0.54	0.39	0.49	0.28

Table 2. Summary of Literature on Effects of Bacterial Species on Inflammatory Responses

Species	Cytokines	Reference	Present in VSL#3
Lactobacillus			
plantarum	↑ IL-10	Pathmakanthan S, et al.[17]	+
casei	↓ TNF-α	Borruel N, et al.[16,18]	+
bulgaricus	↓ TNF-α	Borruel N, et al.[16,18]	+
reuteri	↓ IL-8	Ma D, et al.[15]	-
salivarius	↓ IFN-γ	McCarthy J, et al.[19]	-
Bifidobacterium			
breve	↓ TNF-α, ↑ IL-10	Menard S, et al.[20]	+
infantis	↓ TNF-α, ↓ IL-12, ↑ IL-10/IL-12 ratio	McCarthy J, et al.[19] O'Mahony L, et al.[11]	+
Streptococcus thermophilus	↓ TNF-α	Menard S, et al.[20]	+

Figure 1. Weekly scores of flatulence and bloating during run-in and in response to placebo or VSL#3



PROBIOTICS FOR ANIMAL NUTRITION, CONCEPT AND EVIDENCE

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Primordial Probiosis: between empirism and intuitions

Probiotics were already famous in their effectiveness to the Greeks and the Romans who recommended the consumption of fermented milks above all for the children and the convalescents. Some thousand years later, the Russian biologist Metchnikoff, Nobel prize for the studies on the role of the phagocytes in the defence from infections, asserted that the assumption with the food of alive micro-organisms can improve the health of the intestine and the well-being of man. The scientist supposed indeed that the longevity verified in the eastern European countries and in particular in Bulgaria, was in great part tied to the consumption of fermented milk produced in the native place. In his studies on the aging he stated in fact that the yoghurt was source of robustness and reason of "healthy" aging and gave out the hypothesis that the responsible bacteria of the putrefaction processes can provoke a poisoning that the micro-organisms fermenting sugars and producing lactic acid succeed to hinder inducing what the scientist defined a "healthy aging". These hypotheses, basing both on scientific observations and brilliant intellectual speculations, open the road to what, in times much nearer to ours, will be the *probiotics, prebiotics and new foods*.

From intuition to science

Setting aside all brillant intuitions of Metchnikoff, it has been observed that, beyond the nutritional contributions of proteins, amino acids, vitamins and sugars, the bacteria introduced with food can carry out important functions reflecting on the consumer's health. In the course of the '60^{s}, the term **probiotic** was invented, in order to indicate the micro-organisms alive able to express, if eaten with food, positive effects thanks to the activity of a specific microbial mass. In the term probiotic indeed the concept of promotion (in favour of) life is inborn, opposite to that of antibiotic, reserved to those active antimicrobial principles able to contrast life.

To this indication followed, in the last thirty years, several definitions for probiotic such as: "food/feed preparations made up of alive micro-organisms able to favourably affect the host, improving the balance of the intestinal bacterial flora (Fuller, 1999)" or "alive micro-organisms that, ingested in sufficient amount, induce a beneficial effect on the health of the individual (man or animal) that goes beyond the simple nutritional properties (Guarner e Schaafsma, 1998)".

Basing on these considerations and reflections one cannot but agree with the definition approved from the FAO (Food Agricultural Organisation of the United Nations) and the WHO (World Health Organisation) in Rome in 2001, according to which **"probiotics are alive micro-organisms, generally bacteria but also yeasts that, when ingested alive in sufficient amount, they have a positive effect on the health going beyond the nutritional ones commonly known"**.

Probiotics would have therefore a role, on the balance of the intestinal flora, so to increase the resistance to the infectious diseases, both through a strengthening of the intestinal barrier, both stimulating directly the immune system.

For all this reasaons the benefits are discontinuous and can interest various expressions, physiopathologic and nutritional.

During the intestinal transit the probiotics come in contact with the flora, with the eventually pathogens present and the cells of the host. The interaction between probiotics and this complex ecosystem represents the fulcrum of many researches of functional and clinical nutrition finalized to the understanding of those multiple mechanisms responsible for the action of the healthy benefits observed in the practice (Veillet, 2004). Indipendently from such mechanisms whose acquaintance is still incomplete, it must be specified that probiotics defend the enterocites tying some

glycoproteins, competing directly with the potentially pathogenic micro-organisms and opposing one physical barrier to their adhesion to the mucosa.

Recent History of probiotics in animal feeding

In the course of the last thirty – fourty years, frequent pharmacologic participations have allowed to solve some of the sanitary disadvantages that are found in the intensive breeding. In these conditions the pharmacologic intervention, masking many environmental and nutritional deficiencies, has concurred to "limit" many igienic problems without removing the causes at the origin.The use of drugs with productive purposes subsequently evolved in "growth enhancers" or "auxinics". In 2005 however the antibiotics used as growth enhancers will be definitively prohibited with all the theoretical, psychological and practical benefits deriving, but also with all the problems that the provision, unavoidably, will raise. It is necessary therefore to find other types of solution.

Can probiotics represent a valid alternative to the zootechnical use auxinic antibiotics? In a detailed review (Mordenti and Martelli, 1997) they specified that, in order to have a correct use of probiotics in animal feeding, it is necessary to know their behaviour in the digestive tract and first if they are micro-organisms sporigen or not, of an in transit or resident flora and other characteristics of resistance towards stress of various nature and origin to which they are unavoidably submitted.

"Resident" or "in transit" flora - aerobic sporigen bacteria of the genus *Bacillus* are considered as "in transit" as they do not establish in the digestive tract, while clostridia as well as , firstly, the bacteria forming lactic acid of the genus *Lactobacillus (acidophilus* in particular) and *Bifidobacterium* are to be considered as "resident", being usually components of the intestinal bacterial flora.

Sporigen and not sporigen probiotics - Probiotics exert their effects in quality of alive micro-organisms after having reached the specific sites of action in the digestive tract. If given with feed they should therefore result stable, alive and effective. As a consequent they should be resistant to antimicrobial substances possibly present in food, to the physical treatments to which feed are submitted (pelletting, extrusion, etc.) and also to the gastric acidity and bile action. Uneasily the non sporigen probiotics succeed to survive in the feed. For these reasons the sporigen micro-organisms are preferred when they must be used with feed while the lactic bacteria (non sporigen) are preferred for the specialties to be given directly to the animal.

Since the authorised sporigen probiotics do not colonize the digestive tract it appears logical that their use is continuous (it is therefore natural their addition in feed), while for those not sporigen, able to stably colonize in digestive tract, their use can be limited for the moments of stress (birth, after massively therapeutic treatments, during the weaning, etc.) for direct way or with the drinking water.

Prebiotics and Syimbiotics

The substrate on which micro-organisms develop is determining for the proliferation of the indigenous bacterial species or the probiotics given: it is obvious that, at least theoretically, through the control of the principles contained in food it should be possible, in some way to orient the development of the ecosystem of the digestive tract. Based upon this principle is the interest to favour the development of the "good micro-organisms" supplying them with a suitable fermentative medium.

Therefore **prebiotics** are food ingredients not hydrolysed neither absorbed by the upper intestine and therefore available in the caecum-colon where they are fermented from a limited number of "indigenous" bacteria.In order to be clear and correct it must be specified that prebiotics have been defined (Gibson and Roberfroid, 1995) as "not digestible food ingredients influencing the host favourably, stimulating in a selective way the growth and/or the activity of one or a limited number of bacteria in the colon, with positive effects on the health of the host".At present prebiotics are identified in food principles mainly of glucidic nature, natural principles or of syntheses constituted

from glucose, fructose, galactose and mannose with a degree of polymerization from 2 to 20 monosaccharides linked in between them.
Sources of natural prebiotics are the seeds of leguminosae (soya, peas, broad beans, lupins), yeasts in whose walls MOS are widely represented (mannano-oligosaccharides), well-known for their prebiotic activity. The prebiotics of synthesis are obtained both for polymerization of the dysaccharides or from complex carbohydrates (fructo-oligosaccharides, galacto-oligosaccharides: GOS and FOS) via enzymatic hydrolysis.If probiotics can improve the healthy status and prebiotics can favour (accelerate and amplify) the effects, it is at least legitimate to assume that their association can carry out interesting synergistic effects.
Starting from these theoretical suppositions and taking advantage of the association between probiotics (lactic bacteria us), milk whey and protein hydrolyzed in the role of probiotics, twenty years ago we evidenced, in newborn swines, some synergistic effects showed through meaningful reductions of mortality, of diarrhoeas and improvements of growth (Table 1).It was, as per what we know, the first step of the road then covered by numerous investigators who, in the '90s, invented the term symbiotic assigning it, in particular, to the associations between prebiotics and probiotics with the purpose to favour, electively, at intestinal level, the useful microbial flora.
In fact the **symbiotics** have been defined (Salminen et al., 1998) as "one mixture of probiotics and prebiotics influencing favourably the host, improving the survival and the colonization of the probiotics in the gastrointestinal tract through one selective stimulation of the growth and/or activating the metabolism of one or of a limited number of bacteria promoting the well-being and health of the host". In fact, at least theoretically, if in the direct combination of prebiotics and probiotics exploited for this symbiotic approach, the prebiotic shall stimulate the survival and the metabolic activity of the probiotic species, it should derive an exaltation of the probiotic effect of the bifidobacteria, of the lactic bacteria and also of yeasts.
The experimentation on animals often gives interesting results but, because of the elevated variability, it is not easy to distinguish if the effectiveness is related to one or to the other of the principles studied or their association.
In any case an aspect is sure and important: neither the prebiotics nor the probiotics and their association are source of worry for man and animals: they are safe.

Conclusions
The observations and brilliant intuitions of the Nobel prize Metchnikoff marked, at the beginning of the past century, the starting point of the epoch of "probiotics". The scientist in fact, in his studies on the aging, observed that the populations of the Eastern Europe, consuming fermented milk, were sturdy and aged in a "healthy" way.
The evocative hypotheses issued in order to justify the phenomenon, were involving directly the activity of the micro-organisms in the digestive tract, their biological balance and their ability to neutralize the toxic substances arising, in particular, from the degradation of proteins.
Some of his thesis have found confirmation in the studies done in the years to come, but it is only over half a century later and more precisely in the '60s that the term **probiotic** was invented in order to refer in general to "microbial food with beneficial effects for the health". Several definitions have been given in the end of the past century to probiotics, but one generally approved is that of the FAO in Rome in 2001, being one of the most complete "*probiotics are alive micro-organisms, generally bacteria but also yeasts that, when ingested alive in sufficient amount, have a positive effect on the health, that goes beyond the traditional nutritional effects*".
The definition does not make differences - and it could not be otherwise - between indigenous flora and in transit, sporigen bacteria and not. Under a practical point of view these distinctions deserve the maximum attention if one wants to avoid disagreeable failures.
Then in the '90s the term **prebiotic** appeared by the side of probiotic, that is *non digestible substances mostly of glucidic nature able to stimulate the development and proliferation of the useful bacteria and, in particular, of the probiotics themselves*. To the prebiotics the research and

the zootechnical practice have deserved and are addressing a great interest due to the benefits non digestible they can bring.

So it was born, at the end of the century, the concept of **symbiotic**, we yet acquired experimentally in the '80s, according to which the *association of prebiotics and probiotics* gives, in many cases, synergistic effects.

In the certainty, in the doubts and in the inconstancy of effectiveness today, one thing is however sure: neither prebiotics, nor probiotics and their associations are source of any minimal worry. The fundamental principle *primum non nocere* is therefore respected.

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Table 1: Effects obtained by giving single or associated probiotics (lactic bacteria), prebiotics (whey) and peptides (protein hydrolyzed) to newborn swines. (Mordenti, 1985).

Treatments [(1)]		**Control (placebo)** (0,2 g milk whey)	**Placebo + peptides** (0,2 g whey + 0,2 g protein hydrolyzed)	**Placebo + probiotics** (0,2 g whey + 200 million cells of *Streptococcus faecium*)	**Placebo + peptides + probiotic** (0,2 g whey + 0,2 g protein lisates + 200 million cell of *Streptococcus faecium*)
Piglets	n°	471	480	484	499
Weight when born	kg	1,22	1,21	1,21	1,22
Growth/d	g	186^{D}	195^{C}	207^{B}	221^{A}
Difference with the control	%	--	4,3	11,3	19,4
Incidence diarrhoea					
1st week	%	$20,4A^{a}$	$16,4^{ABa}$	$9,1^{Bb}$	$6,0^{Bb}$
2nd week	%	28,8	28,8	21,4	14,7
Average (1-34 days)	%	$10,9^{ab}$	$14,5^{a}$	$7,9^{bc}$	$5,3^{c}$
Mortality					
Total	%	$11,7^{a}$	$12,7^{a}$	$11,5^{a}$	$8,4^{b}$
For diarrhoea	%	$6,7^{ab}$	$7,3^{a}$	$5,9^{b}$	$3,2^{c}$
Others	%	5,0	5,4	5,6	5,2

Average values of two trials made in autumn (35 days) and winter (33 days).
[(1)] Treatments made when the swines were born (1st day life) respectively with the principles indicated in 2cc water.

LEGISLATION OF PROBIOTICS FOR ANIMAL NUTRITION IN THE EUROPEAN UNION.

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Probitics as feed additives.-

Probiotics are live micro-organisms been used in animal nutrition. Their use was linked with a proven efficacy on the gut microflora resulted in improved health status. The positive effect on gut flora resulted in improved health status, especially for young animals, but also in improved animal performance such as growth or feed conversation rate. Micro-organism used in animal feed are mainly bacterial strains of Gram-positive bacteria belonging to the types *Lactobacillus*, *Enterococococcus*, *Pediococcus*, *Streptococcus* and *Bacillus*. It is noticed that *Bacillus* and *Lactobacillus* differ in many characteristics. Some others probiotics are microscopic fungi such as strains of yeast belonging to the *Saccharomyces cerevisae* species and kluyveromyces. Furthermore, *Lactobacillus* and *Enterococcus* are bacterial families present in great quantities, $10^7/10^8$ and $10^5/10^6$ per gram, respectively, in the digestive microflora of animals. On the other hand, the *Bacillus* and the yeasts are not usual components of the gut microflora. While most of the species and genera, particularly lactobacilli and bifidobacteria are apparently safe, certain micro-organisms may be problematic, particularly the enterococci, which are associated with nosocomial infections and harbour transmissible antibiotic resistance determinants.

History and legal basis in the EU on probiotics in feed.-

The microbial feed additives were covered by Council Directive 70/524/EEC of 23 November 1970 concerning additives in feedingstuffs (OJ No L 270, 14.12.1970). Directive 70/524/EEC was amended five times; the last amendment was by Council Directive 96/51/EC of 23 July 1996 (OJ No.L 235, 17.9.96). In 2003, these Directives were repealed by the new Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (OJ No L 268, 18.10.2003) which sets out the rules for its authorization, use, monitoring, labeling and packaging. In the Regulation 1831/2003 the micro-organism are included in the category 'zootechnical additives' as funcional group on 'gut flora stabilisers: micro-organism or other chemically defined susbstances, which, when fed to animals, have a positive effect on the gut flora'. Under this Regulation specific labelling requirements are needed for micro-organisms such as the expire data of the guarantee or storage life from the data of manufacture, the directions for use, the strain identification number, and the number of colony-forming units (cfu) per gram.

With respect to the guidelines for the assessment of micro-organisms, the Council Directive 87/153/EEC of 16 February 1987 established the composition of the submission dossier for all feed additives (OJ No L 64, 7.3.1987). The Commission has updated these guidelines in 1994 introducing specific requirements for enzymes and micro-organisms (Commission Directive 94/40/EC, OJ No L 208, 11.8.1994). In 2001, the Directive 87/153/EEC was amended by the Commission Directive 2001/79/EC of 17 September 2001 fixing guidelines for the assessment of additives in animal nutrition (OJ No L 267, 6.10.2001). Currently is in preparation a Commission Regulation on implementing rules concerning applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003; this new Regulation shall contain specific guidelines for the authorisation of feed additives (Article 7 of the Regulation).

In the meantime, Scientific Committee on Animal Nutrition (SCAN, Oct 2001) has published its opinion concerning guidelines for the assessment of additives in feedingstuffs, Part II: enzymes and micro-organisms. The guidelines impose the layout of the submission dossiers based on six sections: (1) summary of the data in the dossier; (2) Identity, characterisation and conditions of use

of the additive. Methods of control; (3) Studies concerning the efficacy of the additive, (4) Studies concerning the safety of the use of the additive; (5) Form of monograph; (6) Form of identification note.

Identity, characterisation and conditions of use; methods of control: This section relates to the identity of the additive (proposed proprietary name, type of additive according to its main function, qualitative and quantitative composition of any impurities, physical state of each for of the product and manufacturing process); characterisation of the active agent(s) (nomenclature, biological origin, genetic modification, compliance with release Directive for GMOs, toxin production and virulence factors, antibiotic production and antibiotic resistance, other relevant properties); characterisation of the additive: physico-chemical and technological properties (stability of the additive, other physico-chemical or biological properties, incompatibilities with other feed ingredients); conditions of use of the additive (technological and zootechnical additives, safety data sheet). The proposed method and level of inclusion in premixtures, feedingstuffs or raw material should be stated. This should include the minimum (and maximum) inclusion levels expressed as cfu per gram of final product.

Efficacy**:** studies on efficacy for probiotics strains must be performed in target species/animal categories. The claims for microbial products are: improved performance and feed conversion of the target species; reduced morbidity or mortality which improves the welfare of the target species; benefits of the consumer through improved product quality and benefits to the wider environment. According to the current guidelines demonstration of these effects is based on a minimum of three trials demonstrating a statistically significant ($P<0.05$) on the specific animal categories, where an effect is claimed. The three significant studies preferably should be done in at least two different locations. Current categories comprise, as examples piglets (suckling, weaned), pigs for fattening, sows (for reproduction, and in order to have benefit in piglets), poultry [chickens for fattening and reared for laying hens, turkeys (for fattening, for breeding purposes and reared for breeding)], calves (for fattening, for rearing), cattle for fattening, dairy cows (milk production, for reproduction), lambs (for rearing, for fattening) dairy sheep (milk production), ewes for reproduction, kids (for rearing, for fattening), dairy goats, goats for reproduction, rabbits for fattening, and breeding does. Trials should be compliant with the criteria established by a recognised, externally-audited, quality assurance scheme. The trial protocol should be carefully drawn up by the Study Director with respect the data as follows: herds: location and size; feeding and rearing conditions. For aquatic species, size and number of tanks or pens at the farm and water quality. For all trials, the conditions including: animals (species, breed, age, sex, initial weight, identification procedure, physiological stage and general health), description of manufacture and quantitative composition of the ingredients used, concentration of the micro-organism in the diet, date and exact duration of testing, the timing and prevalence of any undesirable consequences of treatment in individuals or groups shall be described in details in order to allow proper scientific assessment.

Safety under the condition for use**:** The microbial feed additives regulated by Regulation No. 1831/2003 and in accordance with the previous guidelines are subjected to detailed safety assessment with the intention of ensuring that they are innocuous to target animals, users and consumers. Particular attention is focused on the presence of transmissible antibiotic resistance markers, and to the potential for production of harmful metabolites. The guidelines do not differentiate between species and strains with long histories of safe use and other micro-organisms.

- Studies on target species:

Tolerance testing on target species/animal categories: For each animal category, a target species tolerance testing shall be designed to determine a safety margin. The aim of such trial is to evaluate the risk for the animals of an accidental overdosing during feed production. The trial shall be conducted at a dosage being at least 10-fold the maximum recommended dosage proposed by the

applicant. In farm animals an experimental duration period of one month for young, fast-growing animals and three month for adults such as dairy cattle in lactation. For breeding animals, the experimental period should be the total length of a reproductive cycle. The tolerance test requires at least the assessment of clinical signs (i.e. morbility, mortality,) and other zootechnical parameters (i.e. weight gain, feed intake, feed conversion ratio, laying rates, egg mass, milk production). When safety margin is less than 10, to demonstrate safety of the additive data on haematology, blood chemistry, and histopathology must be available.

Other studies on the effects on the microflora of the digestive tract are required.

• Consumer safety assessment:
Genotoxicity studies including mutagenicity: at least two different genotoxicity tests (a bacterial reverse mutation assay and an *in vivo* assay for clastogenicity in mammalian cells (e.g. a methaphase cytogenetic assay).
Oral toxicity studies: The duration of the tests must be at least 90 days. The preferred mode of administration is by incorporation into the feed, but if this is impractical, administration in drinking water or by oral *gavage* may be used. For additives intended for used in food producing animal species, the studies should be carried out in at least one laboratory species (usually the rat).
Toxin production and virulence factors: as, under certain specific conditions, some *Bacillus* species have shown to be able to produce toxins. SCAN (2000) has issued an opinion concerning the safety of the use of *Bacillus* species in animal nutrition. This Opinion examines the extent to which toxin production may be an unrecognised problem amongst some species of *Bacillus* and the implications this may have for their continuing commercial use. Knowledge of the genetic and biochemical basis for toxin production and methods for the detection of *Bacillus* toxins are reviewed and recommendations made for how best to ensure the absence of toxins (or a capacity for toxin production)].
Antibiotic resistance profile and transferability of resistances: bacteria may bear transferable resistances. Some enterococcal strains have shown a resistance to vancomycin and were shown able to transfer this kind of resistance to other species. The SCAN has adopted an opinion on this matter in July 2001, which was updated on 24 January 2003. In 2005 a new opinion of FEEDAP Panel of EFSA has been given. The aim of this opinion is to provide guidance to develop studies in order to show the potential of each bacterial strain (and not yeast) to bear resistances and to transfer them. The basis of such an evaluation starts with the assessment of MIC for a large range of antibiotics. When a strain is known to be resistant to a specific antibiotic, while the species normally is susceptible to this antibiotic, the applicant should evaluate the reason for such a resistance. If such an acquired resistance may transferred or it known exogenous resistance genes are present, the probiotic strain is not considered as suitable for use as feed additive.

• Worker safety assessment: The worker safety of the formulated product should be addressed. For formulated commercial product should be examined for irritancy using validated laboratory animal tests for skin irritation and for eye irritation (liquid products), the skin sensitisation potential and the toxic effects on the respiratory system.

• Environmental risk assessment: The impact on the environment of microbial additives can be considered negligible and to require assessment only in exceptional cases. Genetically modified micro-organism within the meaning of article 2(1) and 2(2) of Council Directive 2001/18/EC on the deliberaterelease of genetically modified organisms (OJ L 106, 17.4.2001) must first satisfy the requirement of the release Directive, which includes an assessment of ay risks for the environment related to the GMO(s) (Anadón *et al.*, 2005).

Probiotics authorised in the EU
The probiotics authorised in the EU, are the following: *Bacillus cereus* var. *toyoi* (NCIMB 40112/ CNCM I-1012); *Bacillus licheniformis* (DSM 5749); *Bacillus subtilis* (DSM 5750); *Enterococcus*

faecium (ATCC 53519; ATCC 55593; NCIMB 10415; DSM 5464; DSM 10663; NCIMB 11181; DSM 7134; DSM 7133; NCIMB 30098; CETC 4515; DSM 3530; DSM 7134);Kluyveromyces marxianus vr. Lactis k1 BCCM/MUCL 39434; *Lactobacillus acidophilus* (D2/CSL CECT 4529; DSM 13241); *Lactobacillus casei* (NCIMB 30096); *Lactobacillus farciminis* (CNCM MA 67/4R); *Lactobacillus plantarum* (CNCM I-840); *Lactobacillus rhamuosus* DSM 13241; *Pediococcus acidilactici* (CNCM MA 18/5M); *Saccharomyces cerevisiae* (NCYC Sc 47; CNCM I-1079; CBS 493.94; CNCM I-1077; MUCL 39885); *Streptococcus infantarius* (CNCM I-841).

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New topics and limits related to the use of probiotics in animal feeding

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Introduction – Since the first proposal of the concept of probiosis was advanced by Metchnikoff in 1908, after studies in laboratory animal models and in vitro tests, evidences of positive effect of probiotics on human health have been more and more increasing in number. For animal productions, the interest on probiotics is rising after the EU ban of the use of growth promoting in-feed antibiotics, particularly for weaning-growing subjects. However it is our perception that the use of probiotics in feed compounds has a slow diffusion, compared to other dietary solutions, such as organic acids. In this discussion, focused mainly on problems related on weaning-growing animals, we try to underline some aspects that can refer to possible insufficient responses to probiotics in producing growing animals.

Growth promoting effect in stressed animals – It is generally assumed that the use of probiotics in the species where they are often isolated can improve the probability of their positive action. Indeed we observed that the supplementation of the diet with a strain isolated in the University of Wageningen in pigs fed a diet based on fermentable fiber (*Lactobacillus sobrius* strain 001^T), improved the live weight gain of weaning pigs orally challenged with an enterotoxigenic *Escherichia coli* K88 (ETEC*)* (Konstantinov et al., 2005). It can be supposed that in case of the use of probiotics not well adapted to the gut environment of swine, no effect is observed. However, after the supplementation with a "human" well-studied probiotic (*Lactobaciluls rhamnosus* GG) to weaning pigs orally challenged with ETEC, a reduced growth and a trend of more ETEC excretion in faeces were observed, in confront to control pigs (Bosi et al., unpublished). In dogs, *Lactobacillus rhamnosus* GG did not prevent the relapse of tylosin-responsive diarrhoea (Westermarck et al., 2005).

The action mechanisms of probiotics demonstrated by studies are mainly related to the competitive exclusion of pathogens in the lumen and on the intestinal surface, to the preservation or stimulation of barrier functions of the host, and to the immune modulation.

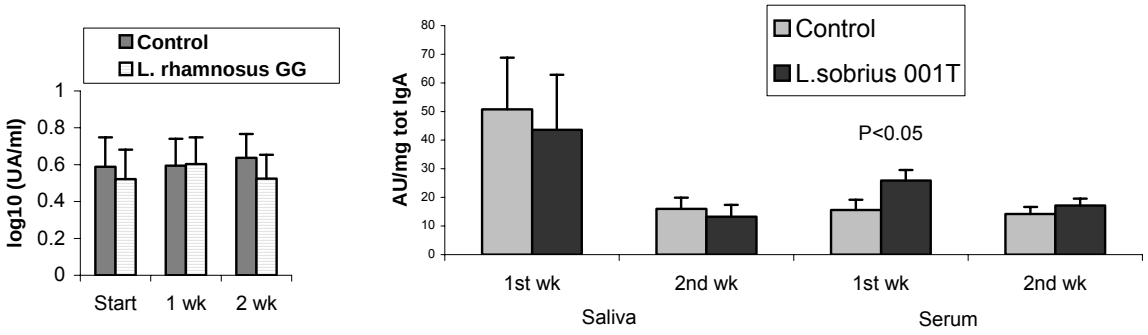
Receptors, competition and translocation - Commensal bacteria and probiotics compete with pathogens in gut content by subtracting nutrients and by producing various metabolites. However there is more and more evidence of the importance of interaction of commensal and pathogens with the intestinal surface. Glycosylation pattern of the intestinal cell layer is typical of different species, and probably of individuals, but microbial-host interactions specifically control this parameter, for example by the secretion of soluble factors or the action of bacteria on galactosyl moieties in host surface (Freitas et al., 2002). In other cases the competition is directly on mucus or on cell receptors. In pigs, the presence of intestinal receptors for some pathogen *Escherichia coli* - such as K88 or F14 - is genetically determined. So, the competition of probiotics against pathogen on gut wall should be related to genotype too. Various molecules from the surface of favourable bacteria have been proposed for their adhesion to the gut wall. An S-layer protein from *Lactobacillus fermentum* 104R that binds porcine small intestinal mucus and gastric mucin has been identified (Rojas et al., 2002). However we do not know if there is any difference between individuals in the predisposition of probiotic to the adhesion to the intestinal surface. We can presume that there is, and, in this case, this could explain possible differences in the exploitation of probiotics, in animal feeding. The specific ability of commensal and pathogen bacteria living in the gut, or the disruption of the intestinal wall integrity or functionality, give to the microorganism the opportunity to pass from the epithelial mucosa into the *lamina propria* and then to mesentheric lymph nodes and other tissues (translocation). Usually commensal bacteria are confined by the immune system within the

area of mesenteric lymph nodes, however host needs time to control their diffusion in other compartment of the body. Translocation of the intestinal resident microflora or of the supplemented probiotics has been used in mice as a measure of possible secondary effects, and to fix the optimal probiotic oral dose (Vinderola et al., 2004). However, in recent trials with weaning pigs supplemented with different Bifidobacteria we found species specific DNA in liver in many subjects (control fed pigs included), and no relationship between oral dose and strain has appeared up to now.

Immune-modulators suppression, tolerance or activation - Many experiments in vitro and on laboratory animals demonstrate that overall health of the host is improved, when probiotics down-regulate or, at the opposite, up- regulate the gut immune system. However, we can assume that in typical animal production system, young animals differ from children and young laboratory animals for the degree of development of immune system. The presence of commensal microflora is essential for an early maturation of the local immune system (Pabst et al., 1988), and the higher is overall bacteria load in the environment, the higher is the presence of microflora in the gut and the stimulation of local immune compartment of young producing mammals. Indeed 21-days weaned pigs challenged with ETEC are able to mount rapidly an immune response against it (Bosi et al., 2004), and have detectable amounts of specific immunoglobulins in the blood. Besides this, the piglet seems to be not already able to distinguish between harmful and not harmful antigens (Stokes et al., 2004). We have poor information about how the immune system of normally reared young animals reacts against a probiotic supplemented in the diet and about the possible development of specific immune response towards commensal bacteria and probiotics. This is important for the energetic efficiency of the host, due to the cost of immune globulins production and secretion, and probably for the survival of the probiotic. Mice mono-associated with one of two lactobacilli, having similar in vitro adhesion patterns (*L. johnsonii* NCC 533 or *L. paracasei* NCC 2461), had different levels of *Lactobacillus*-species specific IgA (Ibnou-Zekri et al., 2003).

It is interesting to note that IgA anti-*L.rhamnosus* GG were present in blood serum of weaning pigs before treatment with this probiotic and also after 1 or 2 weeks in control pigs (Fig.1) (Casini et al., 2005), notwithstanding the absence of DNA from *L.rhamnosus* GG in colon samples of all the subjects, except for pigs fed the probiotic. Also weaning pigs fed or not *Lactobacillus sobrius* strain 001^T for 1 or 2 weeks, had IgA specific for the strain supplied, both in saliva and in serum. However, in this case we found also in control subjects DNA from this microbe species in colon contents. We hypothesized that part of this IgA strain-specific activity is partially related to immune cross-reactivity (CRI) between two different Lactobacillus-species. To test CRI between different *Lactobacillus* species, we implemented the procedure of Shu et al. (1999), doing an ELISA test on

Figure 1. Effect of dietary supplementation with *L.rhamnosus* GG on seric IgA anti-*L.rhamnosus* GG (Casini et al., 2005), and of *Lactobacillus sobrius* strain 001^T on seric and salivary IgA specific for this strain (Konstantinov et al, 2005).



blood serum or saliva pre-incubated with one or the other Lactobacillus. We found that in both trials, each *Lactobacillus* blocks a relevant part of seric and salivary IgA specific for the other (Casini et al., 2005). Gram+ bacteria share some amino acid and sugar motifs in the structure of the

S-layer that protects their cells, and this similarity can explain the presence of IgA reacting against different microbes. However the presence of "polyreactive" or "natural" IgA is also claimed. Nevertheless, the functional importance of this response by the host against commensal microorganisms is not clear. In mice, the long-term colonization of *Morganella morganii* in the gut was compatible with the coating of this bacterium by IgA for at least 314 days (Shroff et al., 1995). The aggregation can be a positive factor to improve the adhesion to mucous and the persistence in the gut. However it can be important to confine the commensal in the digestive compartment. It is not enough investigated, if the presence of these IgA contributes to reduce the chance of colonization of the probiotics fed with the diet, but in any case the continuous production of IgA must be considered a permanent cost for the growing animal. The presence of an active response against the probiotics can also explain some unsuccessful results in the practice of probiotic supplementation.

Conclusions – The practice of use of probiotics in animal feeding is strategic for a healthy production well accepted by the consumer. This short presentation reports some evidences that induce a careful evaluation of some parameters before transferring the results obtained in well-controlled experiments to the heterogeneous situations in the farms. More comprehensions about the role of commensal microflora in the gut and in the whole organism of growing producing animals, could help the identification of probiotics suitable for specific purposes.

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Probiotics for ruminant: action, effects

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Introduction

Use of probiotics in animal feeding is now a common practice, and probiotics have been presented as a way to improve animal health and performance by a more natural way than antibiotics. In ruminant species, the most widely used probiotics are live yeast, and different strains of *Saccharomyces cerevisiae* have been extensively studied. In the European Union, live yeast are feed additives, and the authorisation needs proofs about efficiency and safety for animals and human consuming animal products. Yeast cultures, which do not always contain live yeast but contain the culture media, are also widely used.

Ruminal digestion

In ruminant animals, most of dietary constituents are digested in the rumen. Ruminal milieu is characterised by constant temperature, lack of oxygen except oxygen arriving with food, continuous mixing of contents due to rumen contractions, and a pH which normally is maintained between 5.6 and 6.8. These conditions favour an intense microbial activity, mostly by bacteria (more than 10^{10}/mL), protozoa (more than 10^{5}/mL), and some fungi. All these microorganisms are strictly anaerobic.
Both fibre and non-fibre carbohydrates can be fermented in the rumen. This fermentation is slow for fibre carbohydrates, and rapid for non-fibre carbohydrates, particularly for sugars. Bacteria that utilise fibre and non-fibre carbohydrates are different, which means that the ruminal ecosystem has to be in equilibrium with the diet. This fermentation produces volatile fatty acids, mainly acetic, propionic and butyric acids, which are absorbed by the rumen wall. In normal conditions, acetic acid is the most abundant, but the ratio acetate / propionate decreases when starch is added to the diet, which is common on high performance animals.
Part of dietary crude protein is degraded in the rumen, the main degradation product being ammonia. If sufficient energy supports microbial growth, ammonia is used by microorganisms for the synthesis of their own amino-acids, included in microbial proteins. These microbial protein have a better equilibrium of amino-acids than food proteins, and are partly digested in the small intestine together with undegraded dietary protein.
Ruminal digestion is quite important for ruminant: nearly all energy and two thirds of amino-acids used by the ruminant are the result of this ruminal digestion.

However, disturbances of the digestion can be observed. The most common is subacute ruminal acidosis. This disorder typically arises when an excess of non-fibre carbohydrates is given to animals, which is common for high producing animals, dairy cows or growing cattle. In a first time, the hastened fermentation increases the production of propionate to the expense of acetate. Higher concentration of volatile fatty acids and lower rumination lead to a decreased ruminal pH, which in turn lowers the digestion of fibre, by inhibition of fibrolytic bacteria. Further, subacute ruminal acidosis can lead to a production of lactic acid, as a final fermentation product in the rumen, and lactic acid still decreases ruminal pH. Subacute ruminal acidosis can result in various disorders: decreased appetite, lameness, diarrhoea, decreased milk fat content.

Mode of action of yeast in the rumen

Yeast cells are unable to develop in a durable manner in the ruminal milieu, so that they have to be fed every day. However, they can stay alive in the rumen during about 30 hours (Durand-Chaucheyras et al., 1998).

Several modes of action have been demonstrated. Yeast respiratory activity lowers the redox potential. Because main ruminal microorganisms are strictly anaerobic, this protects anaerobic rumen bacteria from damage by O2, increases the number of cellulolytic bacteria (Dawson et al., 1990), and improves ruminal digestion (Newbold et al., 1996). Moreover, yeast action can utilise part of free sugar in the rumen, and limit a fermentation shift due to rapid degradation of these compounds.

Only live yeast can use ruminal sugars and oxygen. However, some comparisons of live yeast with yeast culture have shown similar effects on fermentation in vitro (Lynch and Martin, 2002). Similarly, autoclaved *Saccharomyces boulardii* resulted in the same stimulation of microbial metabolism than live yeast from the same strain (Oeztuerk et al., 2005). *Saccharomyces cerevisiae* can carry some metabolites that are useful for ruminal microorganisms. Yeast cultures contain B vitamins, amino acids and organic acids, particularly malate, which stimulates growth of ruminal bacteria that digest cellulose (Callaway and Martin, 1997). Malate has been shown to be a potent growth promotor for lactate-fermenting bacteria in vitro (Nisbet and Martin, 1991), but is not sufficient to increase the number of ruminal bacteria in vivo (Newbold et al., 1996).

The main effects of yeast on ruminal metabolism are:

- increased digestibility of fiber.
- changes in the production of volatile fatty acids. Distribution of yeast in some experiments increased the concentration of total VFA in the rumen. Among VFA, the ratio of acetic to propionic acid usually decreases, mainly due to a higher production of propionic acid.
- less variation in ruminal pH, after a meal of concentrate, as a result of a reduced concentration of lactic acid. Effect on ruminal pH is more important when the ruminal pH of controls is low.

These improvements of ruminal digestion have been largely shown in vitro. Their are not systematically confirmed in vivo.

Finally, yeast has often been reported to reduce ammonia concentration in the rumen. This can be due either to a reduced degradation of dietary protein, or to an enhanced use of ammonia by bacteria resulting in an enhanced production of microbial protein, or both.

Effects on production

Production effects of dietary live yeast have mainly been studied in dairy cows and young calves, but positive effects have been reported on growing cattle (Williams and Newbold, 1990).

In lactating dairy cows, the effects of yeast addition are very variable among experiments. A recent meta-analysis based on published data demonstrated non significant effects of yeast on dry matter intake, milk protein content or body weight change, but a trend toward increased raw milk production (+1.3kg/day ; Sauvant et al., 2004). Milk fat content was increased when the value for control cows was under 33 g/kg of milk.

Effects on milk production can depend on numerous factors, such as lactation number, period of supplementation relative to calving, diet, and the type and dose of used yeast. In a review of literature data, Ali-Haimoud et al. (1999) outlined that yeast addition had positive and significant effects when given in the beginning of lactation (+0,7 kg dry matter intake per day,

+1.3 kg milk per day, +0.9 g of milk fat per kg of milk). On the contrary, effects were not significant when yeast was given during mid- or late-lactation. Action of yeast in the rumen improves pH stability, which is much more interesting when the diet leads to a risk of subacute ruminal acidosis. Du to the changes of diet between pre-calving and post-calving periods and due to the high requirements in the beginning of lactation leading to high-concentrate diets, the risk of acidosis is much more important during this period than in mid- or late-lactation. Similarly, experiments with low milk fat content in control cows suggest a subacute ruminal acidosis in the experimental herd. Improvements due to yeast observed in early-lactation cows or herds with low milk fat are consistent with the ability of yeast to prevent low ruminal pH.
The type of yeast used can also be of importance. In the literature reviews of Ali-Haimoud et al. (1999) and Sauvant et al. (2004), results obtained with live yeast or yeast cultures were both taken into account.

In ruminant animals, successful weaning needs a colonisation of rumen by micro-organisms, because the new-born calve or lamb is, on a physiological point of view, a monogastric. Use of live yeast have been shown to stimulate the development of cellulolytic microflora and enhance microbial activity in the rumen of young lambs (Chaucheras-Durand and Fonty, 2001). Similarly, yeast culture addition in a calf diet during their 6 first weeks of life increases dry matter intake, growth, and rumen development (Lesmeister et al., 2004). Other probiotics than yeast (*Lactobacillus, Enterococcus faecium, Steptococcus bovis*) have been shown to improve digestive health around weaning.

Conclusions

The action of probiotics in ruminant animals is mainly due to improved and more stable ruminal fermentation, and production effects are very variable among experiments, making further research necessary to specify the optimal conditions of use. If live yeast are the most widely and studied probiotics for ruminants, other microorganisms, such as *Aspergillus oryzae, Enterococcus faecium, Propionibacterium, Lactobacillus*, also can exhibit interesting effects.

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Probiotic on monogastric: effect on gut structure

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Introduction

Piglets undergo a stress-related growth check at weaning, often associated with anorexia and undernutrition, with disposition to diarrhea and infection. Several alternatives to antibiotics have been proposed for managing piglet gut health at this crucial period, including the administration of probiotics. Probiotics are preparations of non-pathogenic micro-organisms, prepared for animals and human use, that may have beneficial effects on digestive ecosystem and confer resistance to infection (Fuller et al., 1992; Mathew et al., 1998; Nousiainen and Setälä, 1993). Two studies aimed to evaluate the effects on selected histometrical and histochemical aspects of piglets ileum after weaning as consequence of: i) live yeast (trial 1) and ii) *Pediococcus* (trial 2) dietary administrations are presented.

Materials and methods

Trial 1

A total of 352 piglets from weaning (25 d of age) until a month after weaning were used. Piglets were randomly allotted to control (Ctr) or yeast supplementation (Y). All piglets received a dry starter diet that was either control (no added yeast) or contained 0.01 % added live yeast. The yeast supplement used was a concentrate of live *S. cerevisiae* spp. Boulardii. Live yeast content was over 2 x 10^6 CFU/g of feed. Live weight, feed intake, and feed efficiency were recorded for the 30-day post-weaning study. At d 30 post-weaning, 10 female piglets per group (total 20) were sacrificed for histological and histometrical analysis of the gut.

Trial 2

A total of 200 piglets from weaning (25 d of age) throughout forty-two days after weaning were used. Piglets were randomly allotted to control (Ctr) or lactobacilli supplementation (P). All piglets were ration-fed an early weaner diet (Ctr), supplied as a meal and mixed with water to provide a dry matter concentration of 255 g kg^{-1} (water feed ratio of 2.5:1). The lactobacilli supplement used was a single strain of *Pediococcus acidilactici* which was included in the appropriate diets at 0.10 % and was calculated to provide 1 x 10^9 CFU/g of feed. Live weight, feed intake, and feed efficiency were recorded for the 42-day post-weaning study. At the end of the experiment, 8 female piglets per group (total 16) were sacrificed for histological and histometrical analysis of the gut.

Microscopic anatomy of piglet gut

Histological and histometrical analysis

- Serial microtome sections were stained with Hematoxilin-Eosin (HE)
- Assessment of morphological structure
- Histometry
- Ten villi and crypts for section were measured to evaluate:
  - -Villi height (V)
  - -Crypts depth (C)
  - -V:C ratio

Histochemical analysis

- Serial microtome sections were stained with Alcian blue 8GX pH 2.5/Periodic Acid Schiff (AB/PAS) to reveal:
    a. Neutral/Acidic mucins
    b. goblet cells
    c. adherent mucous gel
- Thickness of the adherent mucous gel was measured at ten points in each section
- Serial microtome sections were stained with High Iron Diamine/Alcian Blue 8GX pH 2.5 (HID/AB) to demonstrate:
    a. Acidic solphomucin
    b. Acidic sialomucin

Immunohistochemical analysis

- To visualize mucosal cells in S phase sections from both ileum and ciecum were immunostained by:
    a. Monoclonal anti-Proliferating Cell Nuclear Antigen (anti-PCNA, Sigma, Italy)
    b. The proliferative index was determined by counting epithelial cells with PCNA-positive nuclei in ten well-oriented villi/crypts for each section
- To identify mucosal macrophages sections from both ileum and ciecum were immunostained by:
    a. Monoclonal anti-macrophage (Sigma, Italy)
    b. The number of positive mucosal cells was counted in ten fields of lymphatic tissue for each section (tissue section area of about 0.015 mm^2)

Results and discussion

Growth performance

Trial 1

Control piglets were heavier ($P < 0.001$) than treated piglets at weaning. Piglets fed yeast had significantly greater average daily gain from weaning throughout 30 days post-weaning ($P < 0.001$) than non-supplemented piglets. Piglets fed yeast also grew more efficiently (better feed:gain ratio) than those fed basal diet from weaning through 30 days post-weaning, but the difference was not significant.

Trial 2

Piglets from P group revealed to be significantly heavier than Ctr animals at the end of the trial ($P=0.006$). Similarly, ADG revealed to be significantly higher in the P animals ($P=0,009$) than in Ctr animals.

Microscopic anatomy of piglet gut

Histological examination showed that the ileum of the treated piglets maintained its normal structural aspect after both type of supplementation (tab 1,2). Histometrical analysis of the ileum of the Y e P animals resulted in an increase in villi height (V) and crypt depth (C) ($P<0,01$) as well as in the V:C ratio ($P<0,01$) compared with the controls according to Baum et al. (2002). A thicker mucous gel layer was observed in the controls than the Y and P piglets ($P<0,01$) as reported by Tang et al. (1999). The percentage of mitotic cells in the intestinal mucosa was higher both in piglets fed yeast and piglets fed *Pediococcus.*

Table 1. Effect of live yeast on villus height, crypt depth, V:C ratio; mucin profile; mitotic cell counts; mucosal macrophage counts in piglets ileum (means ± pooled SE)

	Ctr	Yeast	*P*
Villi height µm	214 ^{A} ±6.23	242 ^{B} ±6.23	*0.0013*
Crypts depth µm	147 ^{A} ±4.67	177 ^{B} ±4.67	*<0.001*
V:C ratio	1.48±0.03	1.39±0.03	*0.088*
Adherent Mucous Gel layer µm	2.79 ^{A} ±0.07	1.76 ^{B} ±0.07	*<0.001*
Mitotic cells %	43.50 ^{a} ±2.05	48.18 ^{b} ±2.05	*0.03*
Macrophage %	4.02 ±0.07	4.62 ±0.07	*0.57*

^{A,B}= P< 0.01 Within rows, means lacking a common superscript differ significantly (P<.01)

^{a,b}= P< 0.05 Within rows, means lacking a common superscript differ significantly (P<.05)

Table 2. Effect of *P. acidilactici* on villi height, crypts depth, V:C ratio, adherent mucous gel, mitotic cell count and mucosal macrophage counts (means ± pooled SE).

	Ctr	*P. acidilactici*	*P*
Villi height µm	300 ^{A} ±7.52	327 ^{B} ±7.52	*0.010*
Crypts depth µm	247 ^{A} ±10.31	287 ^{B} ±10.31	*0.009*
V:C ratio	1.25±0.03	1.15±0.03	*0.063*
Adherent Mucous Gel layer µm	2.95 ^{A} ±0.07	2.35 ^{B} ±0.07	*<0.001*
Mitotic cells %	39,85 ^{A} ±2.35	45,37 ^{B} ±2.35	*<0.001*
Macrophage %	5.05 ± 0.64	4.34 ± 0.64	*0.43*

^{A,B}= P< 0.01 Within rows, means lacking a common superscript differ significantly (P<.01)

These findings may signify that both live yeast and *P. acidilactici* have potentially positive effects on piglets intestinal mucosa as also reported by Mathew et al. (1998) and Nousiainen and Setälä (1993). This is important in the view of food safety and consumer health. This work may contribute to focus on how probiotics locally act in certain species, taking into account that the full efficacy of the diets treatments is strictly dependent upon the knowledge of the mechanism of action.

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EPA-SYMPOSIUM

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PROBIOTICS : HOW TO USE IT ?

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INTRODUCTION

Probiotics : how to use it ? That is the question I'll try to answer…

First of all, what is the goal : it's simply to ensure that the Feed diet shall contain the expected amount of live probiotics, as decided by the Formulation department. To achieve this objective, the probiotics shall be treated through the following Processing solutions :

- Weighing or Proportioning, per batch or on a continuous way, to get the right incorporation rate of probiotics in the Feed
- Mixing, also per Batch or continuously, to get the right distribution in the Feed,
- Gentle Processing, not to damage the stability of this living material.

We know that there is no way to change the conditions of the Process and to adapt it to the probiotics : it is logically driven to optimise the Quality and the capacity of the production. Therefore, our aim shall be to adapt the probiotics and their application way to the existing tools. To achieve this, we first need to learn which mistakes to avoid.

The actions generating a negative effect on the probiotics stability are :

- Moisture (an excess of water can activate the probiotics much too early before its consumption),
- Chemicals (such as organic acids, mold inhibitors),
- Pressure & Shearing (mechanical treatment),
- Temperature (probiotics are heat sensitive living material),
- Oxido-reduction agents (such as Zinc or copper),
- And TIME, which shall increase the effect of all these above mentioned actions.

How to use probiotics means also which probiotics to use… Roughly we may separate the probiotics available in the Market into 2 main categories : standard

powder form products and micro-encapsulated products, these ones presenting a nicely improved resistance to the action of chemicals, oxidation agents, moisture, temperature and mechanical treatment. The choice, with its economical consequences, shall be driven by the study of the Process and its actions.

PROBIOTICS APPLICATION

4 main ways to apply probiotics can be foreseen :

- Batch Inclusion, where the probiotics are incorporated into the main Mixer through the Premix (if not, under “pure” form, a premixing with a carrier is needed to allow the right mixing homogeneity). Only recommended when the downstream Process is not supposed to damage the probiotics stability, this is surely the simplest way, using existing equipment and generating no extra work and control in the factory ;
- Continuous inclusion, solution which can allow to by-pass some too strong Processing steps, and consists in dosing and mixing the probiotics and the meal in a continuous way. This may request some extra equipment and a fine regulation system ;
- Liquid coating, using liquid form probiotics (or powder probotics diluted in a liquid carrier) to be sprayed into various coating systems, is a nice solution to by-pass the whole Process.
- Powder coating, another solution to bypass the damaging Processing steps, where the powder is distributed and sticked to the pellets by the mean of a glue (generally oil). Caution : any downstream ventilation, sifting, strong handling or discharge into high silos may separate the powder from the product…

Batch coaters, whether there are running under atmospheric or vacuum conditions, are considered as the most flexible way for liquid and/or powder coating.

PROBIOTICS & FEED PROCESSING

There are many different kinds of Animal Feed, such as Minerals produced under powder form, semolinas, pellets, blocks or in buckets, or complete Feed under mash form, pelleted or extruded pellets, crumbles, and so on. There are also many kinds of relevant Process : Feedmills are all the same, but different ! Let's now check the most popular solutions used in the Feedmilling Industry, step by step, and the best way probiotics can be used accordingly.

The Batching Unit is generally common for all types of Feed, and may contain the following steps :

- Storage : please refer to the recommendations made by the probiotics supplier such as storage temperature & humidity, closed bags after partial use, expiry date, etc ;
- Weighing : to respect the right incorporation rate ;
- Grinding or Pulverising (fine grinding), a Process step which must be absolutely by-passed to avoid probiotics destruction ;
- Mixing (per batch and/or continuous), where the goal is to get homogeneous and stable batches but where some critical ingredients can be found.

Generally, standard powder form probiotics are strong enough to go through this Process, except if any thermal treatment is considered just after…

The thermal treatment Process is combining first a long time Preconditioning Unit (steam addition & retention), often followed by a drying-cooling system prior to a final Batch Mixer, where standard powder form probiotics can be easily added.

The expanding Process can be used instead of the previous one, adding a thermo-mechanical treatment through an expander. As no post mixing is required, the application of probiotics may be done through coating to produce expanded mash Feed.

These three ways of producing Meal Feed vary according to their destruction power : all of them can be damaged by chemicals or oxidation agents present in the recipe. The thermal treatment can be by-pass in most of the well designed factories, but the expanding process requests an extra coating or continuous liquid mixer to accept liquid or diluted probiotics.

To produce pellets can be done through several ways. The most common one is called simple Pelletising, where the unit contains a Preconditionner (steam addition, various retention time), a Pellet-Mill (pressure & temperature), a dryer-cooler and possibly a sifter and a continuous coater. Micro-encapsulated probiotics are generally suitable to go through this Process.

Double Pelletising is another variance, where the mechanical action is reinforced and can be more damageable than simple Pelletising. According to the processing conditions, micro-encapsulated probiotics or liquid solutions can be used.

An expander can replace the first Pellet-Mill of the double Pelletising Unit : in such a case, the probiotics must by-pass the expander and can so be applied on a continuous way into the pellet-Mill (if possible), or sprayed into a coating system.

Some specific Feed request a post-conditionner after Pelletising, such as Shrimp Feed. This unit supposed to maintain the Pellets under high temperature and moisture content during a long retention time (20 to 40 minutes) is too strong for probiotics : application into a coater system is clearly advised…

The Extrusion Process is also a truly damaging one for probiotics : applying more shearing and pressure than a standard expander and requesting real dryers (high teperature / long time), only coating can be accepted as a solution for probiotics.

After extrusion or Pelletising, a crumbler can be used to produce small sized Feed. The crumbler itself do not generate any problem, but when a sifting unit is used to select the particles size, then a big amount of recycled products can be reprocessed, with the relevant probiotics destruction that can be expected…

Out of these 5 main ways to produce Pellets, only simple Pelletising and sometimes double Pelletising can accept probiotics inclusion. For the others, powder distribution or liquid coating is requested.

CONCLUSION

The golden rules of the probiotic application are :

- To respect the recommendations of the probiotics supplier in terms of storage and handling
- To identify the possible risks due to chemicals or oxidation agents in the recipes
- To identify the real risks generated by the Process in terms of probiotics stability
- To combine the right choice of probiotics (standard, micro-encapsulated, liquid) and the right Processing application and equipment.

The two last pages of this presentation are showing the different types of minerals and Feed which can be produced, the main Processing effects in terms of probiotics stability, and the advised technological solutions…

Probiotics: back to basics

Bruno ROCHET
EPA chairman

The empirical use of microbial fermentation goes back to the early Antiquity, when it was widely used to transform and preserve ingredients such as milk, grape, meat or seeds. It is only with the advent of microscopy and the observations of the invisible world by Leeuwenhoek that microorganisms were finally seen and identified. Later on, the works of Louis Pasteur and other scientists of his time finally shed the light on the biology and mode of actions of those microorganisms, allowing to distinguish between helpful and pathogen microorganisms, depending on their activity. Since Pasteur's revolution, microorganisms have been used in an increasingly rational manner and their domestication has grown.

The potential usage of microbes seems today infinite. Their applications can be found in various fields of today's life, such as human nutrition (bread, wine, sauerkraut...), animal nutrition (silages), pharmaceuticals (vaccines, antibiotics...), environment (depollution) and high technologies such as nanotechnologies or electronic. This fad for those microscopic beings could lead to excess and we have to be careful not to confer to microbes more power than they actually have.

If we are now certain that life on earth comes from microorganisms, their origins are unclear and still fuel some debates. But what is certain, is their ubiquitous presence in our environment, playing a part even in our most intimate functions: billions of bacteria are present in our digestive tract, on our skin, but also in our mouth and any compartment of our body that is in contact with the external environment. These microorganisms are not evenly distributed, either in terms of time, space, density or species. Their regulation is complex and often very sensitive to many parameters such as temperature, pH, presence of oxygen, type of nutrients, to cite just a few. Microbial ecology allows understanding the complex interactions between microbes and their environment, other microbes, or their host.

With Pasteur, the link was finally drawn between harmful microorganisms and pathologies, and for most of the public, microbes took on a negative image: a good microbe was a dead microbe. This lead to confusion and the distinction between hygiene and disinfection easily vanished. In order to protect ourselves form microbes, they had to disappear, and it was the era of bleach and stainless steel. In animal production, this gave rise to the extensive use of antibiotics. Now, we know by experience that these disinfection techniques have some limits, such as, in particular, the selection of pathogen strains resistant to antibiotics. This shows that hygiene is not just disinfection, but should incorporate many other aspects, such as the integration of the microbial ecology. Since it is not possible to live in a sterile environment, we have to deal and live with microorganisms, in particular when they are a part of our body.

In recent years, many solutions came on offer from ingredients producers, with a view to regulate the microflora of farm animals. Some of them act by modifying physicochemical conditions of the environment, such as:

- Acidifying compounds, that decrease the intestine pH, selectively preventing the growth of some pathogen bacteria;
- Enzymes, that transform raw elements into more digestible nutrients that can be used by a certain population of the gut microflora, thus displacing the flora balance;
- Plants natural extracts, that will act by eliminating or controlling specific populations of intestine microorganisms.

The supplementation with specific nutriments or ingredients such as sugars (FOS) or yeast extracts will promote the selective growth of certain population of microbes, such as bifidobacteria, creating a flora profile beneficial for the host.

Gut microflora can also be balanced by directly adding live microorganisms into the animals diet. This concept is known as Direct Fed Microorganisms (DFM), whereby microorganisms thought to be beneficial for the animal are mixed with the feed, it is mainly in use in the US.
It is in Europe that has been developed the probiotics concept for animal production: it is also based on the administration of one or several live microorganisms, yeast or bacteria, but, contrarily to DFM, the microorganisms used are well characterised and identified and continuously administered either through feed or drinking water in determined quantities. In fact, microorganisms define as probiotics are fully registered in the EU. Their safety, for the target species, but also for human and the environment, is proven, as well as their efficacy, through numerous and elaborated trials and testing by independent experts. The safety guaranteed by the homologation files is strengthen by the continuous questioning of the products in the light of the latest advances of the research. This ensures optimum safety for both the consumer and the environment. The numbers of products that have been granted such guarantee in Europe is limited, and important financial, human and time resources are necessary form the industrials who file the applications.

It is sometimes argued that some non-registered microorganisms or techniques show better performances level that registered probiotics, as shown in some field trials or laboratory experiment. But, the guarantee of registered probiotics is unique, due to the complexity of the file and its strict scrutiny by the European authorities. Safety and efficacy of registered products are guaranteed for a range of conditions of use possible in Europe. In this case, isn't it better to work with well known and safe products, with a defined level of performances, economically viable and with controlled effects, rather than relying on techniques that are not fully proven, being either empirical or based on poor technical and scientific references? Should some anecdotic efficacy results prevail over a wealth of data allowing to guarantee total traceability of the products as well as maximum safety in line with today's knowledge? It is the responsibility of the industrial that is engaged. He will have to make the decisions, under the control of legal authorities, the consumers and the media. In the end, it is the market that will be the final judge. Probiotics that respect the physiology of the animal correct the effects of rational farming upon the animal microflora. This biological approach is acceptable and accepted by today's consumer. This could lead to a better acceptation of animal products, which in turn, can only

increase their consumption, with an expected effect on the prices of the whole animal production industry.

To summarise, probiotics represent today a solution of choice to balance animals gut microflora, an aspect as truly important as we know that “animals live around their flora”. As a result, the overall farming environment, the animal health and well being, as well as the animal product quality will be enhanced. Nevertheless, the probiotic solution should not replace good management practices that respect the animals conditions and hygiene rules, but should be used together as it acts more like a performances revealing agent.

Probiotics can be applied to any type of animal feed, from meal, to liquid feed and even extruded pellets, thanks to powder and spraying application techniques. They answer the needs of the feed producer, the farmer and the animal. Probiotics are also well received and demanded by consumers, who have been increasingly including them in their own diet in the past years. In conclusion, probiotic, in its European understanding, is the guarantee of safety and efficacy for animal productions, and represents a major contribution to today’s farming, that respects the animal, the human and their environment. It fits perfectly in the development of a sustainable agriculture.

CHARACTERISATION OF THREE PROBIOTIC STRAINS OF LACTOBACILLUS RHAMNOSUS PRESENT IN LAKCID

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Lakcid is a probiotic preparation produced by the Biomed in Lublin widely used in Poland during antibiotic therapy and protects against undesirable effects of bacterial diarrhea. This preparation has a long and safe history of use. However, strains constituting Lakcid have not been intensively studied, yet. Therefore, we tested whether the preparation contains 3 different strains, and if their taxonomic classification and antibiotic resistance patterns are in agreement with those declared by the producer. Using classical, microbiological methods as well as PCR, DNA sequencing and agar disc-diffusion technique we found that Lakcid contains 3 different strains, which belong to the species of Lactobacillus rhamnosus. They were found to differ one to another in colony and cell morphology, carbohydrate utilization pattern (API tests), nucleotide sequence of their DNAs coding for 16S rRNA and in antibiotic sensitivity. Lakcid as a whole preparation was shown to be resistant to 42 among 47 chemotherapeutics tested and while demonstrating an atypical for lactobacilli resistance to tetracycline, erythromycin and clindamycin no plasmid DNA was detected in each of the 3 strains. The results obtained are in conformity to those declared by the Biomed-Lublin, the Lakcid producer.

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ATYPICAL TETRACYCLINE RESISTANCE IN NATURAL STRAINS OF LACTOCOCCUS LACTIS

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Among 500 natural strains of Lactococcus lactis we identified 6 which showed an atypical resistance to tetracycline. The level of resistance determined with the E-test was high reaching in all the 6 strains 96 μg/ml at least. PCR analysis demonstrated presence of genes encoding ribosome protection proteins of two types - tetM and tetS. Next the transmissibility of these atypical tetracycline resistances was tested in both intra- and inter-genus conjugal transfer. These studies showed that some of the tetM and tetS genes can be transferred through conjugation into other L. lactis or Enterococcus faecalis strains. Further experiments showed that in 4 strains TetR can be linked to the presence of Tn916 transposone and 3 of them can transfer TetR feature by conjugation. In another one strain TetR phenotype is not linked to the presence of Tn916 but nevertheless can be transmitted by conjugation and seems to be located on a plasmid. In two strains no conjugative transfer neither to lactococcal nor enterococcal recipients were observed. Therefore, it is concluded that some atypical, tetracycline resistance genes in L. lactis are located on mobile elements, like plasmids or transposones, which can spread through conjugation.

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Lactobacilli isolates from weaned pigs with ability to compete with pathogenic bacteria

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Bacteria with good antimicrobial and adhesive properties are considered to reduce the attachment and development of pathogens in gastrointestinal tract (GIT). Lactobacilli isolated from 3-5 weeks old weaned piglets were tested in vitro for the following properties: antagonistic activity against potentially pathogenic bacteria (S. aureus, B. cereus, E. coli, C. perfringens etc.), antibiotic resistance, production of organic acids and bacteriocins, survival in conditions simulating GIT and adhesion to mucus. Lb. gasseri K7 - human isolate with in vivo established probiotic properties, and Lb. rhamnosus GG were included as reference. From 12 strains with good antimicrobial activity, two produced beside lactic acid considerable amounts of acetic acid, and at least two produced bacteriocins. Although K7 strain was previously found to adhere well to Caco-2 cells, its adhesion to human mucus was poor in comparison to the porcine isolates and GG strain. 4 pig isolates adhered even better (22.7 - 49.4 %) as Lb. rhamnosus GG (17.5 %). Several porcine isolates adhered to human mucus better than GG and K7 strains. Five of the best adhering strains were investigated for their ability to exclude, compete or displace S. aureusl. An isolate identified as Lb. crispatus, reduced adhesion of S. aureus by 19 % in competition assay. This isolate (4/26) was also the most inhibitory against S. aureus, B. cereus and E. coli by agar diffusion test, produced bacteriocins active against Lb. gasseri and S. aureus, and survived well in simulated gastric and intestinal juice. In addition, good growth in enriched whey at constant pH in bioreactor suggests its potential for probiotic application.

PROBIOTICS AND THE INCIDENCE OF COLORECTAL CANCER: WHEN EVIDENCE IS NOT EVIDENT

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Background: Colorectal cancer (CRC) is the second major cause of death from cancer in Europe, with some 17 per year/100.000 men and 11/100.000 women dying of disease, and similar figures in the US. Dietary factors and colonic microflora seem to play an important role in colorectal carcinogenesis, making the potential protective role of probiotics of overwhelming interest.
Methods and Aim: This article analyzes existing data from human (epidemiological and interventional), and animal models studies to highlight areas for which more evidences are necessary.We interrogated Medline, preMedline, Biosis and Cancerlit for studies analysing the rsik of CRC and the use of probiotics. We also searched the Web of Science and the Cochrane Collaboration Controlled Trials Register, and screened the references of identified papers.
Results: As far as regards the effect of probiotics in animal models of CRC, we identified 22 studies. All but 4 studies used rats treated with carcinogens. Only one study used an animal model with spontaneous tumour growth in the background of colitis (IL-10 knock-out mouse). Most studies employed *Lactobacilli* or *Bifidobacteria*. The outcomes were either surrogates, such as evaluation of DNA damage (4 papers), proliferation/apoptosis (1 paper) or evaluation of the incidence of abberant crypt foci (ACF) (4 papers), or of ACF and/or cancer (13 papers). All but 5 of these studies had positive results. Notably, when prebiotics were evaluated too, the combination with probiotics led to an important synergistic effect. Many other papers evaluated the effect of probiotics on the growth/invasive capacity of CRC cell lines either in vitro or after injection in nude mice. However, those studies do not fulfill the criteria of our search, that is focused on a possible role in reducing the incidence of CRC.
There are few human epidemiological studies specifically designed to analyze the effect of probiotics on CRC incidence, with important confounding factors, such as roles of fibers, other dairy prodcucts and vitamin D often present. Overall, cohort studies failed to detect significant effects, while the majority of case-control studies suggest protective effect of fermented milks against CRC. Interventional studies suggest reduction of surrogate markers for CRC risk, such as fecal water genotoxicity after ingestion of fermented milks or probiotics. However, one recent study showed no significant difference in the development of new CRC following administration of either fibers or probiotics in patients previously resected. A single randomised,double blind, placebo controlled pilot interventional trial aimed to evaluate the reduction in cancer risk biomarkers has been performed. It will describe the impact of the consumption of a symbiotic product containing *Lactobacillus GG, B. lactis Bb12* and oligofructose-enriched inulin (SYNCAN project). However a complete final report is still not available.
Conclusions: In our search of the literature few and conflicting epidemiologic data regarding the impact of fermented dairy products consumption in humans have been gathered. There are no positive data from interventional studies so far.
Therefore, even though an ample body of evidence supports the potential anticarcinogenic action of probiotics on the basis of the results obtained in both in vitro and in vivo animal models, further evidence is very much needed. Systematic review and/or meta-analysis of these data do not seem possible, given the high heterogeneity.

“Assessing a multi strain symbiotic dietary supplement”

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To assess the capability of a preparation containing a number of potentially probiotic strains is not an easy task, due to the complexity of the formulation. A preliminary evaluation could be done by means of standard microbiological plate counts, by grouping bacteria to be monitored according to their ability to grow in specific selective media.
The aim of the present study was to investigate the survival of lactic and spore forming microflora in the intestinal tract of human subjects after ingestion of a probiotic preparation named Probinul. 10 subjects (six supplemented with probiotic product and four supplemented with placebo) were enrolled in this single blind study and each subject was instructed to take one bag per day for 14 consecutive days.
The fecal samples of the 10 volunteers were collected three times: at the beginning of the study (T0) after 14 days (T14) and after 19 days (T19).
Fecal analysis was performed by conventional culturing in order to assess the effective increase in lactic and spore forming microflora through the intestinal tract after the intake of the probiotic preparation.
Fecal samples were diluted into saline solution and cultured on MRS to detect lactic microflora and on PCA after heat treatment at 75 Celsius degrees for 30 minutes to detect spore forming microflora.
All subjects completed the study. Six out of the 10 subjects revealed an increase of the bacterial microflora both on MRS and on PCA plates. These results allowed to identify the six human volunteers supplemented with probiotic preparation.

Influence of fermented milk products on the composition of the faecal microbiota: conventional yoghurt vs. commercial probiotic product

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In this study the influence of a conventional yoghurt and of a commercialy available probiotic drink on the composition of the faecal microbiota was examined, next to other parameters (e.g., cellular components of the immune system, cytokine production, oxidant and antioxidant status in human blood plasma).
Study design: After a pre-adjustment phase of 1 week, female volunteers consumend 100g per day of conventional (n=16) and of probiotic (n=17) product during two weeks (t1-t2), and 200g per day for another 2 weeks (t2-t3), followed by an "wash-out" phase (t3-t4) of two weeks. During the adaption and control period, the volunteers avoided the consumption of fermented food.
After each period (t1, t2, t3, t4) the microbiological composition of faecal samples was analysed (total aerobes, total anaerobes, Gram-negative anaerobes, clostridia, enterobacteria, bifidobacteria, lactobacilli, enterococci). Isolates of bifidobacteria and lactobacilli were identified to species- and strain-level using PCR-based methods. Fluorescent In-Situ Hybridisation was applied as an alternative method.
The microbiota of the voluntees underwent major fluctuations among the individuals and depending on the different measuring points. In general, most of the microbiological parameters were not pronouncedly influenced by the consumption of convential or probiotic yoghurt, although significant effects were detected for anaerobes, clostridia and a certain lactobacilli. The probiotic strain of the yoghurt drink was detected in the faeces based on culture methods followed by identity confirmation by Random Polymorphic DNA (RAPD) typing.
Keywords: Gut flora, yoghurt, probiotic, enumeration.

THE PROBIOTIC FORMULATION LACIDOFIL®/ ENTERCINE® PREVENTS STRESS-INDUCED INCREASE IN COLONIC PERMEABILITY AND SENSITIVITY TO DISTENSION IN RATS

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IBS is characterized by visceral hypersensitivity to gut distension. In rats, acute stress increases colonic sensitivity to rectal distension (RD) resulting from increased colonic paracellular permeability (CPP). In previous studies, the Lacidofil®/ Entercine® (Lactobacillus rhamnosus Rosell 11 and Lactobacillus acidophilus Rosell 52) has been effective in preventing and/or alleviating the symptoms of various gastrointestinal disorders. This study aims to evaluate the effect of the probiotic on hyperalgesia to RD and increased CPP induced by acute stress.

Four groups of female Wistar rats received orally during 15 days either saline or probiotic (8.4 109 CFU/day). To measure abdominal cramps reflecting visceral pain, rats were equipped with electrodes implanted in the abdominal striated muscle. Rats were submitted to a partial restraint stress (PRS) during 2 h. Four hours before and 20 min after PRS, a RD was performed using a Fogarty probe inflated from 0 to 1.2 ml. CPP was determined in rats fitted with an intracolonic (IC) catheter. At day 0, 10 and 15 (20 min post PRS), rats received 51Cr-EDTA (250 µL; 0.7 µCi IC) and CPP was assessed by 51Cr-EDTA recovery in urine for 24 h.

In basal conditions, RD increased ($p<0.05$) the number of abdominal cramps from 0.8 ml of RD. A similar nociceptive response was observed in Lacidofil® rats compared with vehicle. PRS significantly increased ($p<0.05$) the number of abdominal cramps for all RD volumes applied reflecting colonic hypersensitivity. The probiotic reduced ($p<0.05$) the increase in number of abdominal cramps induced by PRS, up to basal level, for all RD volumes. In absence of stress, at day 0 and day 10 similar values of CPP were observed in Lacidofil® vehicle rats (0.85 fi 0.19 vs 0.91 fi 0.09; 0.96 fi 0.23 vs 0.87 fi 0.11 %). PRS increased ($p<0.001$) CPP (2.95 fi 0.11 vs 0.91 fi 0.09 %). Lacidofil® abolished the PRS-induced increase of CPP (1.06 fi 0.05 vs 2.95 fi 0.11 %).

A 2-week treatment with Lacidofil® suppressed the stress-induced visceral hypersensitivity and increase of CPP in rats, suggesting a potential beneficial role of this probiotic in IBS treatment.

Tetracycline resistance genes from Bifidobacterium species of human origin

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INTRODUCTION

Bifidobacteria are indigenous members of the gastrointestinal tract (GIT) microbiota, enjoying a time-honoured reputation as health promoters. The study of antibiotic resistance in bifidobacteria will allow defining antibiotic resistance-susceptibility breakpoints in this class of bacteria and distinguishing between intrinsic (non-specific) and acquired (transferable) resistances. It could also be of help to examine types and levels of resistances already spread among this bacterial community and exclude antibiotic resistant strains in probiotic formulations, as resistances could ultimately be transferred to pathogens.

MATERIAL AND METHODS

Among 76 bifidobacteria strains belonging to several species, 24 were shown to present atypical tetracycline resistances (>8 μg ml-1). Total DNA of resistant strains was used as a template in PCR experiments using universal and tetW-specific primers. Sequences of the amplicons were compared to those on databases and the genetic determinants were located by hybridization.

RESULTS

All bifidobacteria resistant strains gave positive amplification with both universal and tetW specific primers. The sequences proved they all were true tetW genes. Genes were located in the bacterial chromosomes by hybridization, but at a different position in distinct isolates. The gene from a Bifidobacterium longum strain was amplified to completion and sequenced. The sequence was shown to be identical to that of tetW gene from Butyrovibrio frivisolvens, except for two nucleotide changes, one of them in the coding region. In our sequence, a similar 14-amino-acid peptide encoding gene to that of B. fibrisolvens was encountered, but neither upstream or downstream direct repeats nor transposon TnB1230-related sequences were identified.

Antagonistic activity of probiotic strains against H. pylori strains

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Background: Helicobacter pylori infection is associated with gastritis and peptic ulcer disease. So far, the most effective management of H. pylori infection is combined antimicrobial therapy. The antagonistic effect of lactic acid bacteria against H. pylori could also make them potentially useful also in the prevention and therapy against H. pylori infection.

Objective: The aim was investigate the antagonistic activity of 5 probiotic Lactobacillus strains against H. pylori reference strain and clinical isolates.

Material and methods: Five probiotic Lactobacillus strains - L. rhamnosus GG, L. fermentum ME-3, L. acidophilus La5, L. plantarum 299v, L. paracasei 8700:2 were evaluated for their antagonistic activity. The selected target bacteria were H. pylori reference strain (NCTC 11637) and 6 clinical isolates. Antagonistic activity was investigated on agar plate using streak line method.

Results: L. rhamnosus GG, L. plantarum 299v and L. paracasei 8700:2 showed similar moderate inhibitory effect while L. fermentum ME-3, L. acidophilus La5 expressed low antagonistic activity against H. pylori reference strain. All tested lactobacilli except L. acidophilus La5 inhibited H. pylori reference strain significantly better than clinical isolates.

Conclusions: Antagonistic activity of probiotic lactobacilli against H. pylori was strain-dependent in which reference strain was more susceptible than clinical isolates.

Transfer of plasmids harbouring tet(M) and erm(B) from Lb. plantarum to E. faecalis in gnotobiotic rats

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The purpose of this study was to investigate the ability of two selected Lactobacillus plantarum strains, isolated from fermented dry sausages, to function as donors of antibiotic resistance determinants in the gastrointestinal tract. Conjugative transfer of the resistance genes was studied by use of a worst-case model consisting of germ-free Sprague-Dawley rats. The rats were initially inoculated with the recipient Enterococcus faecalis (rifR, fusR) that colonized at approximately 5´109 CFU g-1 faeces. After a week repeated inoculation of either of the two donors Lb. plantarum DG 522 (containing a ~ 40 kb plasmid with tetM) or Lb. plantarum DG 507 (containing a ~ 10 kb plasmid with tetM and a ~ 8.5 kb plasmid with ermB) was initiated. The number of donors was lower and more variable than the number of recipients, but remained within the range of 105-107 CFU g-1 faeces. Two days after introduction of the donor, the first transconjugants were detected in feacal samples. The development in the number of tet(M)-transconjugants was comparable between the two donors. In both cases, the number increased to approximately 5´102 CFU g-1 faeces towards the end of the experiment. For erm(B)-transconjugants the number was slightly higher and increased to approximately 103 CFU g-1 faeces. The transfer mechanism of the resistanceplasmids has not been determined so far. We are currently investigating the ability of the transconjugants to function as new donors in in vitro mating experiments (since this can give indications to whether the resistance plasmids are self-transmissible or only mobilisable).

Surface proteins isolated from Lactobacillus acidophilus inhibit adhesions of enterohemorrhagic Escherichia

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Enterohemorrhagic E. coli, serotype O157:H7 causes acute diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. Adherence of the bacterium to host cell surface is an important virulence factor in determining pathogenesis. We have recently shown that probiotics inhibit E. coli O157:H7 binding to epithelial cells. Surface layer proteins (S-proteins) are located on the outer cell wall of some lactobacillus species. The ability of S-proteins to affect the adherence of pathogenic bacteria has not been investigated. The aim of this study was to determine if isolated S-proteins would adhere to host epithelial cells and thereby prevent binding of enteropathogenic bacteria. S-protein was isolated from Lactobacillus acidophilus, strain R0052 and run on a SDS-PAGE gel to confirm the identity of the S-protein and its purity. HEp-2 cells were infected with enterhemorrhagic E. coli (MOI 100:1), treated with S-protein (140ug/ml and 420ug/ml) alone or pre-treated with S-protein prior to infection with E. coli O157:H7. Immunofluorescence microscopy was employed to detect pathogen adhesion and attaching-effacing lesions. Pre-treatment of host epithelial cells with S-protein prior to infection significantly decreased both E. coli O157:H7 adherence and pathogen-induced attaching and effacing (AE) lesions. In contrast, exopolysaccharides (up to 5000ug/ml) had no effect on E. coli O157:H7 binding and AE lesion formation. Functional analysis of the cellular membrane of Lactobacilli should provide insight into the specific mechanisms of action of probiotics.

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Two membrane proteins from Bifidobacterium breve cooperate to form a functional heterodimeric ABC multidrug transporter.

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Multidrug resistance (MDR) transporters are membrane proteins that prevent the entrance of a variety of structurally and functionally dissimilar toxic compounds into the cytoplasm by actively transporting the compounds out of the cell, therefore playing a crucial role in the intrinsic resistance to antibiotics in living cells. By searching for MDR homologous sequences in the B. breve UCC2003 genome database, we have found that two genes, bbmA1 and bbmA2, display high homologies with ATP-binding cassette-type MDR transporters. Both are located in the same operon, suggesting that they are co-transcribed. The bbmA1/bbmA2 genes were cloned and expressed separately and jointly in Lactococcus lactis. Co-expression of both proteins resulted in an increased resistance to nisin. Furthermore, several structurally unrelated drugs, such as ethidium bromide and Hoechst-33342, are extruded from the cell in higher amounts when both proteins are present. Transport of hydrophobic drugs using insideout membrane vesicles also indicates a possible cooperation between BbmA1 and BbmA2 to form an active transporter. This is the first characterization of a MDR transporter in the genus Bifidobacterium.

In vitro effect of commercial probiotic product isolates and reference strains of Bifidobacterium on cytokine production by human peripheral blood mononuclear cells

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The intestinal flora and its host interaction play an important role in maintaining an immunologically balanced intestinal inflammatory response. Imbalance of the intestinal microflora, resulting from a reduction of 'protective' bacteria, will hence result in intestinal inflammation. In this context, the administration of probiotics has been suggested as a new therapeutic approach for the alleviation of symptoms in inflammatory bowel disease (IBD) patients. During this study, 50 Bifidobacterium strains including commercial probiotic product isolates as well as human reference strains were assessed for their effect on the production of IFN-g, TNF-a, IL-12 and IL-10 by peripheral blood mononuclear cells (PBMC) from healthy individuals. Bifidobacterium strains were able to stimulate cytokine production irrespective of their product or human origin, and this effect was largely strain-dependent. Furthermore, bifidobacteria proved to be potent inducers of IL-10 whereas very low, if not undetectable, amounts of IL-12 were found. It has been shown that IBD patients display an abnormal IL-10/IL-12 ratio as a result of low IL-10 and high IL-12 levels, which is indicative of a pro-inflammatory state (O'Mahony et al., 2005). Our in vitro results suggest that administration of certain Bifidobacterium strains may induce a shift in inflammatory response towards an anti-inflammatory state, resulting in an alleviation of IBD symptoms. However, future experiments with an in vivo mouse colitis model should be performed in order to endorse these observations.

Antimicrobial susceptibility of Bifidobacterium strains from humans, animals and probiotic products

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In this study a total number of 100 strains belonging to 11 bifidobacterial species originating from humans, animals and probiotic products were tested for susceptibility to 15 antibiotics by the agar overlay disc diffusion and micro-broth dilution method. The majority of the strains belonged to the species B. adolescentis, B. animalis subsp. lactis, B. bifidum, B. breve, B. longum biotype infantis and longum and B. pseudocatenulatum. All strains tested were susceptible to amoxycillin, chloramphenicol, erythromycin, vancomycin, quinopristin dalfopristin, rifampicin and with some exceptions to clindamycin. Most strains were resistant to gentamicin, polymyxin B and sulphamethoxazole whereas occasional resistances were found against trimethoprim, ciprofloxacin, minocycline and tetracycline. The therapeutic combination trimethoprim/sulphamethoxazole, on the other hand, was highly active against most strains. Tetracycline resistant (TcR) strains were further investigated for the possible presence of Tc resistance genes (tet genes). The tet(W) gene, which encodes a ribosomal protection protein, was identified in 15 TcR strains including 7 strains from probiotic products belonging to B. animalis subsp. lactis and B. bifidum. In the strains investigated, this gene was present in a single copy on the chromosome and did not appear to be associated with the conjugative transposon TnB1230 previously found in tet(W)-containing Butyrivibrio fibrisolvens. Conjugation experiments are ongoing to assess the transfer potential of tet(W). The present study contributes to a better knowledge of the intrinsic and acquired resistance traits that can occur in bifidobacteria and that should be investigated e.g. as part of the selection of new probiotic strains, safe for human use.

Species of bifidobacteria from faeces and mucosa of healthy Spanish people determined by culturing and 16S rDNA sequence analysis

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INTRODUCTION

Bifidobacteria are among the outstanding populations in the human GIT. Their healthy-promoting effects are thought essential for maintaining a healthy state. Bifidobacterial components have been traditionally addressed by culturing, which is being complemented by molecular techniques, including construction and analysis of 16S rDNA libraries. In this communication we report on the bifidobacterial diversity from faeces and mucosa of healthy Spanish individuals examined by culturing and analysis of 16S rDNA bifidobacterial-specific libraries.

MATERIALS AND METHODS

By culturing, 36 faecal samples from 10 donors were analysed, as well as a unique mucosal sample from four donors. Counting was accomplished by plating on MRS agar. Bifidobacterial colonies were classified by amplification, sequencing and analysis of a segment of their 16S rRNA gene using universal primers. Total DNA from two faecal samples and two mucosal samples was isolated and used as a template to amplify a stretch of bacterial of the 16S rDNA with bifidobacterial specific primers. Amplicons were cloned, and sequences compared to those on databases.

RESULTS

The 196 bifidobacterial isolates were classified as Bifidobacterium longum (96), Bifidobacterium pseudocatenulatum (54), Bifidobacterium bifidum (31), Bifidobacterium adolescentis (9), Bifidobacterium catenulatum (3), Bifidobacterium dentium (2), and Bifidobacterium animalis (1). The 113 clones analysed were assigned to the species B. longum (63), Bifidobacterium dentium (22), B. pseudocatenulatum (21), Bifidobacterium psychroaerophilum (4), B. animalis (2), and Bifidobacterium minimum (1). On the poster, differences and similarities obtained by the two methods and the distribution of species between mucosal and faecal samples and among individuals will be presented.

Regular consumption of short-chain fructo-oligosaccharides improves digestive comfort of subjects with minor functional digestive disorders (FDD)

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Objective: to study the effects of regular consumption of short-chain fructo-oligosaccharides (sc-FOS) on digestive comfort of subjects with minor FDD.

Methods: this comparative randomized double-blind study was carried out in 5 occupational medicine centres in hospital and was separated into two steps. In step 1, 2235 subjects filled a questionnaire to assess the incidence and intensity of digestive disorders. In step 2, 105 of theses subjects were diagnosed as suffering from minor FDD and were randomized in 2 groups, receiving 5 g/d of either sc-FOS (ACTILIGHT) or a placebo during 6 weeks. Incidence and intensity of digestive disorders were assessed at the end of the consumption period using the step 1 questionnaire. A quality of life questionnaire (2) was also addressed at the beginning and at the end of the consumption period.

Results: Step 1: 44% of the subjects suffered from FDD, from which 57% from minor FDD. Step 2: At the end of the consumption period, the intensity of digestive disorders decreased of 15.9% in the sc-FOS group whereas it increased of 4.7% in the placebo group ($p= 0.026$). Digestive disorders persisted for 39% of the subjects in the sc-FOS group and 57% of the subjects in the placebo group ($p= 0.006$). Using the quality of life questionnaire, 96% of the subjects in the sc-FOS group felt an improvement of their digestive comfort (vs. 73% for placebo, $p= 0.022$) and 83% felt less discomfort in their daily activities (vs. 57% for placebo, $p= 0.071$).

Conclusion: regular consumption of sc-FOS (5 g/d during 6 weeks) improves digestive comfort in a working and non-medically treated population.

(1) Multicentre trial led in collaboration with the occupational medicine departments of the hospitals of Bobigny (Dr S. Panserrieu), Poitiers (Dr G. Coulombier), Lille (Pr A. Sobaszek), Montpellier (Dr M. Brabet), Nantes (Dr D. Tripodi) and the gastroenterology department of Nantes (CHU, Hôtel-Dieu, Pr JP Galmiche) in France.(2) Chassany et al. (1999). Validation of a specific quality of life questionnaire for functional digestive disorders. Gut 4(44): 527-533.

Different mechanism could be involved in the inhibition of Salmonella infectiveness by breast milk lactobacilli

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Diarrhoeal and infectious diseases continue to be a major cause of morbidity and mortality worldwide. The manipulation of intestinal microbiota with probiotics has been suggested in the prevention or attenuation of diarrhoea disease.

OBJECTIVE: Evaluate the effect of four breast milk Lactobacillus strains (L.salivarius CECT5713, L.gasseri CECT5714, L. gasseri CECT5715 and L.fermentum CECT5716) on the infectiveness of Salmonella cholerasuis (CECT4155) and the possible mechanisms involved in this effect.

METHODS: After 15 days of oral daily administration of different Lactobacillus strains (4x108CFU), Balb/C mice were infected with Salmonella (106CFU/mice) and mortality was recorded during two weeks. The inhibition of Salmonella adhesion to mucins was then measured by exclusion, competition and displacement assays. The effect of these bacteria on mucins expression by HT-29 cells was also evaluated.

RESULTS: Administration of all lactobacilli reduced the mortality of mice infected with Salmonella. Mice receiving L.salivarius and L.fermentum showed the highest survival rate (80 and 60% respectively, $P<0,05$). Antibacterial compounds produced by Lactobacillus strains could be implicated in this effect, since Lactobacillus supernatants decreased the viability of Salmonella up to 20%. The inhibition of Salmonella adhesion to mucins was also demonstrated for these strains. In this case L.salivarius and L.fermentum rised an inhibition range between 40 and 80%. All the lactobacilli strains tested increased mucins expression, with an important effect on MUC-2 induction by L.fermentum.

CONCLUSIONS: Breast milk lactobacilli have a beneficial effect on Salmonella infection in mice. Inhibition of adhesion to mucins, production of antibacterial compounds and increase of mucin expression could be involved.

Proteome of a bile salt resistant strain of Bifidobacterium animalis

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The aim of this study is to gain insight into the physiological mechanisms that allow the adaptation of Bifidobacterium to bile salts. A resistant derivative of the strain Bifidobacterium animalis IPLA 4549 was obtained by exposure to gradually increasing concentrations of bile salts in MRS broth supplemented with L-cysteine. In previous studies we have shown that resistance to bile salts of this Bifidobacterium bile resistant derivative was related to changes in glycolytic activities and in the ability to adhere to human intestinal mucus. It also has an increased tolerance to low pH and several antibiotics, and higher levels of bile salt hydrolase activity.

In the present study, we analyzed two dimensional electrophoresis protein profiles (pI range 4-7) of cytoplasmic extracts from the pair original strain / bile resistant derivative. We determined proteins involved in the adaptation of B. animalis IPLA 4549 to bile salts by the identification of spots whose expression is specific or increased in the resistant derivative. We observed significant differences of the expression levels of several proteins between the two strains. These include, among others, general stress response chaperones, several enzymes of the glycolysis, and a bile salt hydrolase. To the best of our knowledge, this is the first proteomic approach to study bile salt adaptation in Bifidobacterium animalis.

Production of fructooligosaccharide prebiotics with immobilized biocatalysts

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The short chain fructooligosaccharides (FOS) have importance in human and animal nutrition first of all as prebiotics: They improve the general health conditions via their beneficial effects on the intestinal microbial flora. On the other hand, they can be applied as alternative sweeteners, too. FOS are natural constituents of plants and vegetables but their commercial scale production is performed from sucrose by biosynthetic way using fructosyl transferase (FTF) enzyme. The main advantage of use of immobilized enzyme is that the product syrup can be easily separated from the biocatalyst. In the course of our work, two protein mixtures with considerable transfructosylation activity have been examined: commercial Pectinex Ultra SP-L and a new protein fraction (cell lisate) recovered from a recombinant E. coli bacterium. Immobilization has been carried out via novel type combined fixing onto an anionic exchange resin and by means of entrapment into Ca-alginate gel. The immobilization conditions for each solid-phase biocatalyst have been optimized then the functional parameter optima have been determined. After characterizing the biocatalysts, FOS synthesis experiments were carried out in reactors of different type and scale. The synthesis runs with Ca-alginate entrapped protein fraction have given most promising results for further scale-up studies of the process.

“Prebiotics and probiotics: the gut microflora management”

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Because of its resident microbiota, the human colon is one of the most metabolically active organs. Gut bacteria predominantly ferment undigested food materials and the nature of the fermentation may influence in different ways host’s health. For example, the end products of carbohydrates fermentation are often positive for the host, while end products of proteins metabolism may be toxic. The use of diet to influence the microflora composition aiming to fortify those components thought to be positive is object of an increasing attention for functional foods scientists. In this context both prebiotics and probiotics play a significant role. Also synbiotics, which combine the two approaches, are emerging as useful tools to condition gut microbial composition, but they are not going to be discussed here. Many studies are focused on tools to manage microflora composition and the enhancement of molecular tools into gut microbiology enable now to understand more completely gut biodiversity and effective responses to diet.
AAT, founded June 2005, is the first spin-off of the Catholic University and working to conceive the requirements of food and pharmaceuticals companies as well as public and private clinical institutions interested in microbiological analysis, taxonomical identification by molecular tools, bacterial molecular typing, studies of the microbial ecology of human and animal body districts, metabolic engineering, bioinformatic analysis, bibliographic updating in the field of probiotic bacteria, creation and maintaining of bacterial collections, clinical and nutritional research and biosafety.

EVALUATION OF TECHNOLOGICAL AND FUNCTIONAL PROPERTIES OF THE NEW PROBIOTIC LACTOBACILLUS FERMENTUM ME-3

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FAO/WHO guidelines (2002) for probiotics in human food include in vitro studies and clinical trials. The choice of probiotic into food products depends also on the suitability of the strain in manufacturing.

Aim. Viability and stability of functional properties of L. fermentum ME-3, a probiotic with high antioxidativity and antimicrobial activity were detected in fermented milk products and probiotic capsule.

Methods. All products were industrially manufactured. ME-3 inoculation rate in fermented milk products was 5x108 CFU/g. Viable count of ME-3 was analysed throughout shelf life. ME-3 from products was identified by AP-PCR. Reisolates were tested for TAA (linolenic acid test) and antimicrobial activity (streak line procedure in modified MRS). Results. Viability of ME-3 was good in all products (1x107 to 1x109 CFU/g). No interactions with starter cultures. Antagonistic activity of ME-3 isolates from capsule and kefir was significantly higher ($p<0.001$) than the original base value. TAA values from milk products were different: isolates from sour cream (25.0%±13.2) being the best. TAA values of ME-3 incorporated into cheese increased at 8 months from the cheese preparation. TAA value of ME-3 capsule isolates remained high (21±10.6).

Conclusions. ME-3 suited well with technology of fermented milk products. ME-3 lacks the antagonistic activity against starters and survives in various products. High TAA and antimicrobial activity of ME-3 in different carriers prove the stability of its functional properties in food and food additives. Stabile antioxidative property of ME-3 serves as a natural antioxidant in food and antimicrobial activity offers putative protection against food pathogens.

Production and storage stabilization of vaginal probiotics biomasses

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Lactobacilli exert a probiotic function in the vaginal human flora limiting the proliferation of pathogenic species by producing organic acids and H2O2; recent studies evidence also the viralicide effect of some selected strains, leading to an increasing interest towards lactobacilli strains that can be able to adhere to vaginal mucosae, to modulate the composition of urogenital microflora and to prevent infections. The necessity of introducing probiotic biomasses as active component of food and pharmaceutical preparations needs the development of new production and storage technologies to grant a prolonged shelf-life.
The aims of this research are the metabolic characterization and the productivity optimization of selected vaginal lactobacilli strains, in particular L. crispatus. During experiments H2O2, lactic acid and exopolysaccharides in fermentation media were measured. The research was also focused to improve survival of probiotic biomasses in lyophilized forms, also formulating innovative cryopreservative solutions.
Growth experiments were performed to analyze culture conditions and organic acids production, using different carbon and nitrogen sources.
Biomasses from fermentation were treated with different crioprotectant and then freeze-dried to analyze the vitality during the storage.
The crioprotective molecules analyzed were trehalose, dextrin and ectoines in different amounts.
We observed a significative crioprotective effect for these molecules, in terms of reduction of the cellular mortality due to osmotic and thermal shock in the freeze-drying process. Vitality during prolonged storage was also efficiently maintained, in particular in the presence of trehalose and ectoines. Moreover ectoine has a comparable effect to trehalose even if used in five-fold lesser concentrations.

Construction of an oligonucleotide microarray to detect antibiotic resistance genes in lactic acid bacteria (LAB)

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A thematic microarray consisting out of nearly 300 oligonucleotide probes was constructed for the screening of lactic acid bacteria (LAB) strains for the presence of a large variety of antibiotic resistance genes. Included were genes responsible for resistance to the following classes of antibiotics: Aminoglycosides, Extended Spectrum β-lactamases (ESBL), Chloramphenicol, Macrolides Lincosamides Streptogramins (MLS), Sulfonamides, Tetracyclines, Trimethoprim, and Vancomycin. Oligonucleotides were modified with a 5' C6-amine linker and spotted on QMTEpoxy slides (Quantifoil Micro Tools GmbH). The specificity of the oligonucleotides was tested by using a variety of reference strains with known phenotype and genotype. For this DNA was isolated from the reference strains and fluorescently labelled with Cy5 using the BioPrime DNA labeling system (Invitrogen BV). After hybridisation the microarrays were scanned with a confocal laser scanner (ScanArray ExpressHT; Perkin Elmer) and the hybridisation patterns were analysed using the software package ArrayVision (Imaging Research, Ontario, Canada). Although the majority of the developed primers turned out to be specific, the specificity, however, of a substantial set of oligonucleotides could not be verified. Either due to the lack of appropriate reference strains, absence of DNA information or incorrect design of the oligonucleotides.

Modulation of the immune response by the non-bacterial fraction derived from kefir

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Probiotic microorganisms can exert their beneficial properties as viable cells or by modulating immune response via their metabolites. Kefir, a complex fermented milk, modulates the immune response at local (intestinal mucosa) and distant sites, upon recent studies conducted in our lab. The aim of this project was to study the effects of the non-bacterial fraction of kefir on the gut immune response. BALB/c mice received orally kefir supernatant for 2, 5 or 7 days. The number of IgA+, IgG+, IL-4+, IL-10+, IL-6+, IFNgamma+ and TNFalpha+ cells was determined on histological slices of small (SI) and large intestine (LI) and cytokines were also measured in blood serum and intestinal fluids by ELISA. In the SI, the number of IgA+ cells increased for all feeding periods assessed, but not for IgG+ cells, compared to control mice. The cytokines IL-4+, IL-10+ and IL-6+ cells showed an enhancement when compared to IFN+ and TNF+ cells. The same immune response profile was observed in the LI, but to a lesser magnitude. This might be due to the highest absorption of soluble metabolites in the LI. In blood serum, cytokines (mainly IL-10) increased for all feeding periods. In the intestinal fluid we observed that only IL-6 was secreted into the lumen. IL-6 was also produced by intestinal epithelial cells as observed in our previous studies. We concluded that the soluble metabolites of kefir stimulate the gut immune response increasing the IgA+ cells and cytokine release, with an important induction of the regulatory cytokine IL-10.

Molecular methods to identify Lactobacillus and Bifidobacterium species from food, feed and faeces of human and animal origin

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Lactobacillus and Bifidobacterium species play an important role as starters used in fermentation, as probiotic cultures and as health promoters in the human intestine. At present, more than 90 species of the genus Lactobacillus and 31 species of the genus Bifidobacterium are recognised, although the number of species comprised in these genera remains a matter of debate. Due to this great number of species, identification based on physiological and biochemical criteria does not always give unambiguous results, is time-consuming and sometimes unreliable because of adaptive changes of species to growth conditions.In this study, multiplex-PCR, ARDRA and TTGE was used for grouping lactobacilli and bifidobacteria, respectively, followed by species-specific PCR to confirm the identification of the isolates at the species level. These molecular techniques proofed to be rapid and reliable screening methods for the identification of distinct Lactobacillus and Bifidobacterium isolates.

Use of DNA Microarray for the Identification of Antibiotic Resistant Genes in Streptococcus thermophilus

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The increase of Antibiotic Resistant (AR) populations in the food chain community is now well recognized as source of antimicrobial resistance determinants for the gut flora and is generally caused by horizontal gene transfer. In this context the EU-project "Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain" (ACE-ART) is focused on Lactic Acid Bacteria (LAB). These bacteria might not represent clinical risk in themselves, but they can be vehicles of AR genes; the presence of such genes has been reported in several LAB, but never in Streptococcus thermophilus.
Here we report the assessment of a 50 and 60-mer oligonucleotides DNA based microarray for the identification of AR genes in a significant number of S. thermophilus strains.
The AR genes represented by the oligonucleotides on the microarray belong to: Aminoglycoside, Extended Spectrum β-lactamase (ESBL), Chloramphenicol, Macrolide Lincosamides and Streptogramin (MLS) group, Sulfonamide, Tetracycline, Trimethoprim and Vancomycin.
All strains were obtained from the UCSC culture collection except for 11 strains that were isolated from raw milk during the study and all originated from dairy products. To validate the results obtained by microarray analysis all isolates were subjected to PCR and MIC testing on Iso-sensitest medium (with lactose 1% w/v) based on microdilution method.tetS and ermB genes were found in different S. thermophilus isolates. Although for Streptomycin very high MIC values were observed no Sm related genes were found by microarray analysis.

Effect of a symbiotic preparation on the clinical manifestations of irritable bowel syndrome, constipation-variant: results of a multicenter trial

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Introduction. Irritable Bowel Syndrome (IBS) is frequently associated with a modified equilibrium of the different species of intestinal bacteria. A few studies are currently available concerning the efficacy and safety of probiotic administration in IBS; moreover concepts relating to the use of probiotics in patients with constipation variant IBS are scattered and inconclusive. Recently a symbiotic drug has become available in clinical practice; this preparation is constituted by a probiotic, Bifidobacterium longum W11 and by the short chain oligosaccharide prebiotic Fos Actilight.
Aim: to evaluate the efficacy of this symbiotic on clinical symptoms and on bowel habit in patients with IBS.
Sixty-five Centres participated to an open, uncontrolled, multicentric trial; each centre enrolled an average of 10 subjects, with an M/F ratio of 0.65 and age-range of 18-90 years. All patients had a diagnosis of constipation-type IBS, according to the Roma II criteria. A specific questionnaire was administered to all patients at the beginning and at the end of the study, inquiring about symptoms, stool frequency, concomitant treatments and/or diseases. All subjects received the symbiotic at the dose of 3 g (one bag) daily for at least 36 days.
Results: A total of 636 patients were treated (250 males, 386 females). At enrollement, 35.8% of subjects declared not to assume any drugs, 27.7% reported intake of fibres, 12.4% of sedatives, 26.6% of laxatives, 15.1% of other drugs. According to a visual scale, symptom severity was present in the first two grades (no symptom and low grade symptom) from 3.0% to 26.7% and from 9.1 to 44.8 % ($p<0.001$) for meteorism and from 8.4% to 44.1% and from 19.3% to 39% for abdominal pain. On the contrary, in more severe classes (moderate and severe symptom), symbiotic administration induced a significant reduction in symptom frequency, both for meteorism and abdominal pain. A significant increase in stool frequency was observed, from 2.9 ± 1.6 times/week at enrolment to 4.1 ± 1.6 times/week at the end of treatment. Diarrhoea episodes reduced, progressing from 0.4 ± 1.02 before treatment to 0.1 ± 0.4 after treatment ($p<0.0001$)
Conclusions: administration of a symbiotic preparation to patients with IBS can significantly modify not only the clinical picture, by reducing abdominal pain and meteorism in patients with moderate to severe symptoms at enrolment, but also intestinal function, increasing significantly the weekly rate of stool frequency.

Evaluation of the probiotic food supplement Probio-Stick on stress-induced symptoms in patients: a double-blind, placebo-controlled randomized trial

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Stressful life events alter the well-being and produce various disorders such as gastrointestinal, cardiovascular, social and psychological symptoms. Probiotics, when ingested in sufficient amount, have beneficial effects on the health and in particular on the gastrointestinal system. The aim of the present study was to investigate the effects of a probiotic preparation (Probio-Stick) on stress-induced symptoms in patients.
METHODS: A double-blind, placebo-controlled, randomized study was conducted over a three-week period in patients with symptoms of stress (n = 64). All patients received a probiotic (Probio-StickT) containing Lactobacillus acidophilus Rosell-52 and Bifidobacterium longum Rosell-175 (3x 109 cfu/sachet) or a sensorially identical placebo without probiotics. Patients completed a questionnaire on stress-induced symptoms at the beginning and the end of three-week period. Severity of stress-induced symptoms (gastrointestinal, cardiovascular, social, mental, sleep and psychological symptoms) was evaluated by visual analogue scales (VAS). The variations of VAS scores were calculated for the both groups receiving probiotics or placebo.
RESULTS: The consumption of Probio-Stick resulted in a significant improvement in gastrointestinal symptoms (-16.42 vs -7.65 for placebo; p = 0.034). The probiotic treatment tend to improve the cardiovascular symptoms (-9.94 vs -5.35; p = 0.058). In contrast, the probiotic supplementation did not significantly modify the social ,psychological, mental ,sleep symptoms induced by stressful life events. No adverse reactions were reported.
CONCLUSION: The results indicate that ProbiostickT can provide a beneficial effect on gastrointestinal symptoms of patients with chronic stress.

INTESTINAL MUCIN GENE MODULATION IN VIVO USING ORALLY ADMINISTERED PROBIOTIC BACTERIA

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Intestinal cell culture studies demonstrate mucin gene modulation, an innate protective mechanism, can be modulated by probiotic bacteria. Mucins are large glycoproteins that bind enteropathogens thereby limiting access to intestinal mucosa. MUC2 and MUC3 are the major mucins in the large and small intestine, respectively. We investigated whether rats fed probiotic organisms increased mucin expression in a regional and time-specific manner within the intestinal tract. Lactobacillus rhamnosus R0011 or Bifidobacterium bifidum R0071 were added to water of Sprague-Dawley rats daily so either 10E7 or 10E9 CFU/day were ingested. Following 2, 5 and 10 days, animals were sacrificed and intestinal segments were excised for RNA isolation and histology. rMuc2 and rMuuc3 mRNA were analyzed via Taqman RT-PCR, and protein levels confirmed using immunohistochemical analysis using an rMuc3 antibody. Results show with B. bifidum rMuc3 expression in jejeunum was greater after 2 days (204+/-42% control, mean+/-SE) compared to expression at 10 days (72+/-17% control, $p<0.05$). By 10 days, rMuc3 expression was similar to baseline levels ($p>0.05$). Similar results were found in ileal segments. Colon rMuc2 expression was similar between control animals and those administered probiotics (108+/-12% control, $p>0.05$).
Results were similar with L. rhamnosus administration. No concentration dependent alteration in rMuc3 expression was demonstrated. Immunohistochemical staining of jejunum showed direct correlation with mRNA expression following B. bifidum administration. Orally administered probiotics demonstrate site specific and time-dependent effects on mucin expression and may offer an indicible means of protection for the intestinal tract.

Synergistic combinations of prebiotics and probiotics

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The aim of the present study was to identify prebiotics that can be used to promote survival and colonization of probiotic cultures in the gastrointestinal (GI) tract (Lactobacillus acidophilus LAFTIr L10, Bifidobacterium lactis LAFTIr B94 and Lactobacillus casei LAFTIr L26).

Firstly, a range of carbohydrates including soybean oligosaccaride (SOS), oligofuctose (FOS), long chain inulin, raffinose, fructo-oligosaccharide (FOS), beta-glucan hydrolysate and arabinogalactan was screened for their stimulatory effect on growth of probiotics in vitro. Secondly, prebiotics that stimulate growth of probiotics in vitro were further assessed for their effects in vivo, using an animal model. Animals were fed a diet supplemented with the prebiotic carbohydrate or glucose as a control.

The results demonstrate that the probiotic cultures utilize a range of prebiotics. L. acidophilus utilize SOS, FOS and long chain inulin. L. casei utilize FOS, inulin, SOS, beta-glucan hydrolysate while B. lactis utilize the widest range of carbohydrates, including SOS, raffinose, raftilose, beta-glucan hydrolysate, inulin and arabinogalactan.

Subsequent studies in vivo demonstrate that several of these prebiotics increase intestinal population levels of probiotics, resulting in an extended presence of probiotic cultures after terminating feeding with these cultures. Results presented here indicate that several of these prebiotics may be used to improve survival, colonization and the effects of probiotics in the human GI tract.

In vitro Selection of Probiotic Bacterial strains from Mother's Milk(Human)

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Bacterial antagonism has been recognized for over a century but in recent years this phenomenon has received more scientific attention, particularly in the use of various strains of lactic acid bacteria. one important attribute of many lactic acid bacteria is their ability to produce antimicrobial compounds called "Bacteriocins". Bacteriocins are used as natural food preservatives in the production of foods with enhanced shelf life and safety. There is a growing consumer awareness of the link between diet and health. A total of 10 samples of milk were evaluated for the presence of Bacteriocin producing lactic acid bacteria by modified Kirby-Bauer method.From 10 of these samples, 50 strains of lactic acid bacteria were isolated. when evaluated by disc diffusion assay, 10 of these strains having bactericidal effect on Vibrio choloerae, Klebsiella pneumoniae, Salmonella typhi and Salmonella paratyphi. All compounds produced by these lactic acid bacteria fully inactivated by proteolytic enzymes, which indicates the proteinaceous nature of compound. Cultivation parameters for this selected lactic acid bacterial strain were studied in 3 litre Applikon fermenter.

The antimicrobial activity of the bacteriocins produced by the lactic acid bacteria isolated in this work could act as a potential barrier to inhibit growth of pathogens. These microbes can be used as food preservatives especially in baby foods and these probioitcs are expected to dominate the world of medicine.

ANTIOXIDANT COMPOUNDS FROM WHEAT SPROUTS : CITOTOXICITY ON TUMOR AND NORMAL CELL LINES AND PRELIMINARY RESULTS ON THE FIRST STAGES OF HUMAN ATHEROSCLEROSIS

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In the last two decades an increasing interest has been focused on the possible relationship between free radical mediated oxidative damage and cardiovascular diseases, cancer and aging. Accordingly many Authors gave particular attention to diet composition especially with regard to the assumption of fruit and vegetables containing natural products with antioxidant activity. In this context we have reported that wheat sprouts contain a potent cocktail of antioxidant compounds including reducing glycosides and polyphenols (J.Clin. Gastroenterol. 38, S123-S126, 2004; J. Agric. Food Chem. 52, 5201-5206, 2004). We have also demonstrated that the wheat sprout antioxidant activity is able to protect DNA against the antioxidant stress induced by hydroxyl radicals formed via the Fenton reaction (Fe2+/H2O2) (J. Food Sci. 67, 2918-2922, 2002). In this paper we have evaluated the toxicity of the wheat sprout extracts in normal and tumor cell lines (MDCK, HELA). The wheat sprouts extract, at concentrations ranging from 5 to 50 micrograms/ml, causes an inhibition of tumor cells (HeLa) growth of 20-35%, compared to the untreated control. On the contrary the extract doesn't influence the growth of the utilized normal cell line (MDCK).
Following the promising results obtained by oral administration of wheat sprout powder to old dogs affected by cataract (Biogerontology 6 (4), 2005, in press), we studied the effects obtained by supplementing the diet of patients affected by moderate hyperlipemia (LDL > 160 mg/dL e/o triglycerides > 150 mg/dL) (Metabolism 52, 1191-5, 2003) and with a cardiovascular risk less than 10% . The early stages of atherosclerosis are represented by endothelial dysfunction that may be usually evaluated in humans by a noninvasive ultrasound method as brachial artery flow-mediated vasodilatation (FMV). We studied 13 moderately hyperlipemic patients (3 men and 10 women) who were under isocaloric hypolipidic diet. Before and after the treatment with 3-4 g daily of wheat sprout powder, total cholesterol, HDL and LDL cholesterol, triglycerides and FMV were evaluated . After 6 weeks of treatment the values of cholesterol, LDL, HDL e triglycerides were almost stable. Vice versa an increase of FMV after treatment with wheat sprout powder has been observed even if the data are just within the limits of the statistical significance (5.9+/-4.0 vs 8.0+/-3.3 %, p = 0.051). Interestingly the direct analysis of FMV delta values distribution (delta = FMV after treatment -FMV before treatment, registered in each patient) allow us to evaluate the statistical significance of the hypothesis corresponding to positive influence of the treatment with wheat sprouts powder on the brachial artery vasodilatation. Indeed, the opposite hypothesis, denoted by H0 (Hypothesis 0), corresponds to increments in the percentage variation of the artery diameter being independent zero mean (gaussian) random variables. Under H0, sample variance for the increments is estimated to be 11.25, that is a mean square error of 3.35. Then, the sample mean of the increments 1.88 allows to reject H0 with an error of about 28%.
It is worthwhile noting that, if we restrict attention to the cases in which no vasodilatation occurs without treatment, then the increment corresponding to treatment is very high, and leads to reject H0 with an error of about 0.24%. However, because of the very low number (three) of such cases, this finding may only provide a good hint for future work.
In conclusion the dietary supplementation with wheat sprouts powder seems to improve the brachial artery vasodilatation in early stages of hyperlipemia.

Cultivation-independent assessment of maternal sources for bacterial colonization of the neonate gut

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Human milk is an important factor in the initiation, development and composition of the neonatal gut microbiota. Bacteria commonly isolated from this biological fluid, such as staphylococci, streptococci, micrococci, lactobacilli and enterococci can be considered as components of the natural microbiota of the human milk. The aim of this study was to investigate whether the way of delivery could influence the bacterial population in breast milk. Using a culture-independent approach based on 16S rRNA gene diversity we examined samples from breast skin, maternal rectal and vaginal swabs, colostrum, breast milk, amniotic fluid, meconium and feces from the babies obtained from five programmed caesarean sections and five natural deliveries of healthy women. Denaturating gradient gel electrophoresis (DGGE) of 16S rRNA gene amplicons was performed to characterize the diversity of Lactobacillus, Enterococococcus and Bifiobacterium-specific primers. In addition, 16S rRNA clone libraries were constructed to investigate the dominant microbial community within colostrum, breast milk, meconium and feces. Analysis with Bionumeric software did not reveal a clear clustering of caesarea versus natural delivery groups in any of the DGGE profiles, indicating that the bacterial communities found in the baby and breast milk must also be originating from other locations than just the mothers vaginal or faecal microbiota. From most of the samples the 16S rRNA genes in the clone library were related to those of Photoharbdus luminiscens and Serratia spp. In contrast, 16S rRNA genes related to (percentages) Lactococcus lactis, Lactobacillus spp., Staphylococcus spp., Streptococcus spp and Leuconostoc spp. were only found in all breast milk and colostrums samples, suggesting that the bacterial composition of the breast milk is likely to depend on more than one factor alone. Overall, this study suggests that the way of delivery does not play a major role in the succession of the bacterial communities in breast milk and faeces.

Probiotics for disease prevention in experimental Crohn's disease

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Therapeutic potential of probiotics in clinical and experimental ileitis is still controversial. SAMP1/YitFc and TNFdeltaARE mice are unique models of spontaneous ileitis that closely resemble Crohn's disease.
Aim of the present study was to test the efficacy of the multiple probiotic compound VSL#3 for prevention of small bowel disease in these animal models.
Three-week-old SAMP1/YitFc (n=11) and seven-week-old TNFdeltaARE mice (n=8) were supplemented with VSL#3 [50x10(9)CFU/day/mouse] in their diet, and compared to age-matched mice (SAMP=11, TNF=8) that did not receive a supplement. Treatment period was 6 weeks for SAMP and 10 weeks for TNF mice. Histological assessment of ileitis was performed in a blind fashion using a validated scoring system. Feces were collected before and after the treatment period and total DNA isolation was performed and evaluated by real-time PCR with specific primers for VSL#3 bacteria. Mesenteric lymph node (MLN) cells were partly evaluated for markers expression by flow-cytometry, and partly cultured for 48 h under stimulation with anti-CD3 Ab, and measured for cytokines production.
SAMP mice that received VSL#3 supplementation had consistently reduced ileal inflammatory scores compared to control mice (3.2 ± 0.9 vs. 11.6 ± 1.8, $p<0.005$); disease was completely prevented in five mice (45%). Despite a trend for amelioration, the difference between VSL#3-treated TNF mice and controls didn't reach statistical significance. No significant difference was found in MLN cell composition or cytokines secretion between treated and control groups.
VSL#3 administration prevented the development of chronic ileitis in SAMP1/YitFc mice, without a direct effect on MLN lymphocytes.

COMPARISON OF THE FAECAL MICROBIAL POPULATIONS OF PATIENTS SUFFERING FROM IRRITABLE BOWEL SYNDROME WITH SYMPTOM-FREE AGE- AND SEX-MATCHED CONTROLS

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Irritable bowel syndrome (IBS) is a common gastrointestinal (GI) disorder of unknown etiology. In previous studies indications of the GI tract microbiota abnormalities have been obtained but the significance of the GI tract bacteria in IBS remains inadequately studied. The aim of our study was to update the current knowledge concerning the putative role of GI microbiota in the IBS and to apply real-time PCR for comparing the faecal microbiota of IBS patients with controls. For this purpose 27 IBS patients fulfilling the Rome II criteria and 22 control subjects devoid of GI disorders were recruited. The patients were divided into diarrhoea-, constipation- or alternating type - IBS. Each patient and control subject gave three faecal samples at different time points of the investigation (0, 3 and 6 months). For accurate and precise detection and quantification of 20 different bacterial species and groups, real-time PCR with TaqMan or SYBR Green chemistry was used. The real-time PCR assays had coverage of approximately 300 species. As a result variation in the normal microbiota was observed between individuals. When Kruskall-Wallis signed rank analysis was used for group-wise comparison of the subject groups significant differences were observed. As to presence of intestinal pathogens, no indications of the presence of Helicobacter spp. or C. difficile were found in either of the subject groups, but in one case of Campylobacter jejuni was diagnosed. As a conclusion, the faecal microbiota of the IBS subgroups seem to differ from each other, which is not unexpected due to differences of the symptoms experienced by these patients. The comparison of the results from various studies describing alterations of faecal microbiota in IBS is difficult due to different IBS criteria used and the development of bacterial taxonomy. In conclusion, this study supports the earlier suggestions of the changed GI microbiota in IBS.

INTESTINAL MICROBIOTA IN CELIAC DISEASE

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Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of gluten proteins in genetically susceptible individuals. The peptides generated after gluten digestion bind the DQ2 and DQ8 molecules of antigen-presenting cells activating an abnormal immune response mediated by T cells in the small intestine. This generally results in inflammation and tissue damage, leading to flattening of the intestinal villi and reduction of the absorptive capacity. Clinical manifestations of CD are presently known to include the typical malabsorption syndrome (chronic diarrhoea, abdominal distension, etc) and a spectrum of extraintestinal disorders.Currently, CD is the commonest lifelong disorder in Europe and US showing a prevalence 0.7-2%. Despite of that, the only alternative for celiac patients is to adhere to a lifelong strict gluten-free diet. The compliance with this dietary guide is very difficult due to the presence of gluten in the majority of processed foods and patients continue suffering from gastrointestinal symptoms, nutritional deficits and higher health risks (autoimmune diseases, osteoporosis, cancer, etc.). In addition, a proportion of patients (5-30%) fail to improve after a gluten-free diet (refractory CD). The pathogenesis of CD is very complex, involving interactions between genetic, immunologic and environmental factors. The ingestion of gluten is the only environmental factor that has been clearly linked to CD so far, but others such as stress and microbial infections are being considered as possible additional triggering elements. In order to determine the potential role of the intestinal microbiota on the onset and evolution of CD a comparative analysis of the composition of the faecal microbiota of celiac and healthy infants is being carried out by using cultural and molecular techniques. The elucidation of the microbial changes associated to CD could lead to a better understanding of the environmental factors involved in the pathogenesis of this disease. In addition, it could contribute to the development of intestinal intervention strategies based on the use of probiotics aimed at improving the intestinal barrier function and restoring the intestinal homeostais on celiac patients.

Treatment of acute infectious diarrhea in infants and children with a mixture of three Lactobacillus rhamnosus strains. A randomized, double-blind, placebo-controlled trial

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Background: Multiple studies document that probiotics are effective in treating infectious diarrhea (ID) in children. L. rhamnosus GG is the most extensively studied in treatment of diarrhea but effectiveness of other strains has been poorly examined.
Aim: To determine whether L. rhamnosus strains (573L/1; 573L/2; 573L/3) (Lakcid L®) would be effective in shortening acute diarrhea in children.
Methods: Randomized, double-blind, placebo-controlled trial. Subjects: children 2 months - 6 years of age, with acute diarrhea, administered Lakcid L or placebo. Primary outcome measure: duration of diarrhea. Secondary: weight gain in first 24 hours; no. of stools in consecutive days; duration of parenteral rehydration; diarrhea lasting over 7 days; adverse events and GI tract colonization by administered strains.
Results: 87 children analyzed ITT. Mean duration of diarrhea in the treated group: 83.6 ± 55.6 h; in placebo group: 96 ± 71.5 h (p=0,36). In rotavirus infection: 77.5 ± 35.4 h vs 115 ± 66.9 h (p=0,03), respectively. Duration of parenteral rehydration 14.9 ± 13.7 h vs 37.6 ± 32.9 h; (p=0,006).
Conclusions: Administration of L. rhamnosus strains shortens the duration of rotaviral diarrhea in children but has no influence on duration of diarrhea of any etiology. Intervention shortens the time of intravenous rehydration. The administered probiotic strains were able to colonize the gut of treated children.

Selection of Therapeutically Efficacious Lactic Acid Bacteria Cultures for Probiotic Use in Commercial Poultry

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During the last four years, our laboratory has worked toward the identification of probiotic candidates for poultry which can actually displace Salmonella and other enteric pathogens which have colonized the gastrointestinal tract of chicks and poults. Selection of 11 lactic acid bacteria (of the genus Lactobacillus or related) have beene efficacious in the treatment of Salmonella infected chicks and poults. In laboratory challenge studies, 80-90% reductions in Salmonella recovery rates from challenged chicks treated with the candidate probiotic culture were typical. By selecting Salmonella infected flocks pre-slaughter, we have demonstrated that treating such flocks, approximately two weeks prior to slaughter, can markedly reduce environmental Salmonella recovery in commercial turkeys and broilers. Treatment of idiopathic enteritis in commercial poults also compared favorably to selected antibiotic therapy in recent studies. Large scale commercial trials have indicated that appropriate administration of this probiotic mixture to turkeys increased body weight gain at processing by approximately 230 grams with over 120 flocks evaluated, with similar performance gains observed in more limited commercial trials with broilers. Administration of dietary lactose at a very small concentration (0.1%) greatly enhanced the growth rates of probiotic turkeys under commercial conditions and furthered reduced total production costs. These data indicate that selection of therapeutically efficacious probiotic cultures with marked performance benefits in poultry is possible, and that defined cultures can sometimes provide an attractive alternative to conventional antimicrobial therapy. The specific results of these studies will be presented.