September 16 / 18, 2007

4TH PROBIOTICS & PREBIOTICS NEW FOODS

JOINTLY WITH

XXX INTERNATIONAL CONGRESS ON MICROBIAL ECOLOGY AND DISEASE (SOMED)

ROME

Università Urbaniana

THE MEETING IS ORGANIZED BY

OLTRE LA NUTRIZIONE - onlus

SOMED – Society for Microbial Ecology and Disease

Under the patronage of

Azienda Complesso Ospedaliero "San Filippo Neri" - Roma
FIMAD (Federazione Italiana Malattie Apparato Digerente)
FISM (Federazione delle Società Medico-Scientifiche Italiane)
INRAN (Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione)
ISS (Istituto Superiore di Sanità)
Ministero della Salute (requested)
"Sapienza" Università di Roma, II Facoltà di Medicina e Chirurgia

ORGANISING SECRETARIAT

e meeting&consulting
Via F. Denza, 19/A
00197 Roma
Ph +39 06 80693320
Fax +39 06 80692586
probiotici2007@emec-roma.com
www.probiotics-prebiotics-newfood.org
www.emec-roma.com

SUNDAY, SEPTEMBER 16TH

Lecture in honour of:
Elie Metchnikoff: an optimistic philosopher
L. Morelli

POTENTIAL ROLE OF NATURAL ANTIOXIDANTS IN THE PREVENTION OF SOME HUMAN DISEASES

Development of biomarkers of oxidative stress and inflammation to predict the effects of natural antioxidants
P. Patrignani, S. Tacconelli

***Morinda Citrifolia*, fermented papaya, wheat sprouts and white tea are popularly claimed to be source of potent cocktails of antioxidant compounds. But is it really true?**
V. Marsili, I. Calzuola, S. Perni, G.L. Gianfranceschi

Nutrition and cardiovascular disease
G. Lupattelli, A.R. Roscini

FUNCTIONAL FOOD PREPARATIONS: EVIDENCE BASED CLAIMS ON FUNCTIONALITY, TECHNOLOGICAL ISSUES, SAFETY AND EFFICACY

Antibiotic susceptibility of bacteria isolates from probiotic products available in Italy
G. Blandino, D. Fazio, I. Milazzo

Double benefit claims for antimicrobial and antioxidative probiotics
M. Mikelsaar

How bifidobacterial genomics could help in understanding probiotic-prebiotic functionality
M. Ventura

Human intestinal microbiota and nutrition homeostasis
B. Shenderov, V. Aleshkin

Relationship between number of bacteria and their probiotic effects
E. Bertazzoni Minelli, A. Benini

MONDAY, SEPTEMBER 17TH

STATE OF ART

Functional genomics of probiotic bacteria
T.R. Klaenhammer

Interactomics in the human intestine
W.M. de Vos

OMICS OPENING NEW X-BIOTICS MARKETS?

Exploitation of *Lactobacillus rhamnosus* GG genome data
S. Tynkkynen, T. Salusjärvi, W.M. de Vos, R. Korpela, T. Mattila-Sandholm

PROBIOTICS AND CANCER CHEMIOTHERAPY

Cancer gene therapy with anaerobic and non pathogenic bacteria
A. Saggioro

IMMUNE RESPONSE MODULATION PLAYED BY THE NEW CONCEPT PROBIOTICS

Immune response modulation played by new concept probiotics
M. Rescigno, E. Mileti

Lactobacillus paracasei strain B 21060 and human T cell proliferation
F. Caprioli

The role of a synbiotic (FLORTEC, Bracco SpA, Italy) on the plasma levels and peripheral blood mononuclear cell expression of cytokines in patients with ulcerative colitis: a pilot study
A. Federico, C. Tuccillo, E. Grossi, R. Abbiati, N. Garbagna, R. Salerno, C. Mucherino, A. Tiso, M. Romano, C. Del Vecchio Blanco, C. Loguercio

IBD

Solute carriers (SLC) in inflammatory bowel disease, a potential target for probiotics?
M. D'Amato

Understanding why probiotic therapies can be effective in treating IBD
R.N. Fedorak

Saccharomyces Boulardii and IBD
M. Guslandi

Indications and challenges of probiotics in the management of patients with arthralgias and spondylarthropathies in Crohn's disease and ulcerative colitis
O. Karimi, A.S. Peña

ANTIBIOTICS AND ALTERATIONS IN INTESTINAL FLORA

The hypervirulent antibiotic resistant *Clostridium difficile* in Europe
P. Mastrantonio, F. Barbanti, P. Spigaglia

The gnotobiotic mouse model to study the effect of antibiotics on human fecal microbiota
A. Collignon

Antibiotic susceptibility of *Bifidobacterium* strains
M. Saarela, J. Mättö

Transfer of ermB-plasmid from lactobacillus to enterococcus faecalis in the intestine of gnotobiotic rats
L. Feld, M. Danielsen, K. Hammer, A. Wilcks

Identification and molecular analysis of tetracycline and erythromycin resistance genes from lactic acid bacteria isolated from an italian dairy product
C. Devirgiliis, D. Coppola, S. Barile, A. Caravelli, G. Perozzi

PROBIOTIC IN HUMAN DISEASE

Probiotics and Irritable Bowel Syndrome: rationale, mechanisms and efficacy
M. Camilleri

Microbiological characteristics of some italian probiotics
L. Drago

MICROBIAL ECOLOGY AND HUMAN HEALTH

Lecture:
Why not all probiotics are equal
G. Reid

Adhesins selected in the environment and bacterial pathogenecity
L. Vezzulli, B. Repetto, E. Pezzati, G. Giusto, M. Stauder, A. Karbowiak, C. Pruzzo

Apparent antibiotic misuse in environmental ecosystems and food
E. Bezirtzoglou, A. Alexopoulos, C. Voidarou

Colonization of rectal and vaginal epithelium by three different *Lactobacillus* strains administered orally
P.B. Heczko, M. Strus, P. Kochan, Z. Chełmicki, A. Chełmicki, T. Gosiewski, A. Pałucha

CELIAC DISEASE

Microflora associated characteristics in children with celiac disease
E. Norin, B. Tjellström, L. Stenhammar, T. Midtvedt

Antagonist effect of a naturally occurring peptide in celiac disease
M. Silano, F. Maialetti, M.R. Dessì, O. Vincentini, M. Petrassi, M. De Vincenzi

MICROBIAL BIOFILMS AND HUMAN INFECTIONS

Microbial biofilms in the human gastrointestinal tract
S. Macfarlane

Switch from planktonic life to biofilm formation: a major event in pneumococcal pathogenesis
C. Trappetti, F. Iannelli, A. Kadioglu, P. Andrew, G. Pozzi, M.R. Oggioni

The role of multispecies microbial biofilms in the occlusion of biliary stents
E. Guaglianone, R. Cardines, P. Mastrantonio, R. Di Rosa, A. Penni, G. Puggioni, A. Basoli, F. Fiocca, G. Donelli

A new bacterial biofilm model for chronic CF lung infections
S. Molin, J. Haagensen

NEW MOLECULAR TARGET FOR FUNCTIONAL FOODS EFFICACY

Effects of vegetal foods and molecular biomarkers
M. Porrini

Post-prandial glucose and insulin response is improved by bread fortified with germinated wheat seedlings
G. Andersen, P. Köhler, V. Somoza

USE OF NEW FOODS IN EXPERIMENTAL AND HUMAN STUDY

Whole grain health benefit: the human studies
A. Costabile

Role of carnitine in butyrate metabolism
G. D'Argenio

Functional milk as adjuvants therapy of chronic hepatitis C
F. Morisco, B. Russo, G. Maio, V. Lamura, D. Amoruso, P. Vitaglione, V. Fogliano, C.Carella, N. Caporaso

Satiating effect of new fiber-rich foods
P. Vitaglione, L. Scalfi

DIET VERSUS DRUGS IN DYSLIPIDEMIAS

Lipid metabolism and bowel health: an up-date on knowledge about probiotic/prebiotic supplementation and cholesterol metabolism
A.F.G. Cicero

PEDIATRIC DAY

Opening lecture:
The possible role of the intestinal microflora of infants in the subsequent development of atopic responses
A.B. Onderdonk

Round Table
AGE-RELATED NUTRITIONAL APPROACH

Functional compounds in the newborn formula
O. Hernell

Toward the European indications for complementary feeding
C. Agostoni

Round Table
CLINICAL EFFECTS BY FUNCTIONAL FOODS IN INFANT FORMULA

Short-term and long-term effects of long-chain polyunsaturated fatty acids in infant nutrition
T. Decsi

PROBIOTICS AND ALLERGY

Probiotics and allergic rhinitis
G. Ciprandi

Allergen free probiotics
L. Mogna

Probiotics and immune system … beyond the probiotics
A.M. Castellazzi, C. Valsecchi, G.L. Marseglia

Update on research with *Lactobacillus reuteri*
E. Connolly

Probiotic bacteria for treatment of gastroenteritis
J. Amil Dias

TUESDAY, SEPTEMBER 18TH

INTESTINAL ECOLOGY: SUGGESTION FOR CLINICAL PRACTICE

Probiotics and Irritable Bowel Syndrome: rationale and clinical evidence for their use
G. Barbara, V. Stanghellini, C. Cremon, R. De Giorgio, L. Gargano, F. Pallotti, R. Corinaldesi

INTERACTIONS OF THE ORAL AND GUT MICROBIOTA WITH THE HOST AND THEIR IMMUNE SYSTEM

Probiotics protected mice against experimental infections
L. Quercia Vieira, L. Martins dos Santos, E. Neumann, A. Paulo da Silva, L. Nobre Moura, J.R. Nicoli

Mechanisms involved in the intestinal interaction between host and bifidobacteria
P. Brigidi

Use of VSL #3 probiotic mixture for prevention and therapy of allergic diseases: studies in mouse model of allergic sensitization to inhalant and food allergens
G. Di Felice, M. Boirivant

HEALTH PROMOTING EFFECTS OF PROBIOTICS: UNDERSTANDING OF MECHANISMS

Health, probiotics and inflammation
E. Mengheri

Response of resident gut bacteria to the application of probiotics or prebiotics
K.P Scott, J.C. Martin, P. Louis

Effect of the probiotic agent *Clostridium butyricum* M588 strain on enteric pathogens
S. Kamiya, H. Taguchi, T. Osaki, K. Oka , M. Tanaka, M. Takahashi

FACULTY

C. Agostoni (Italy)

J. Amil Dias (Portugal)

G. Barbara (Italy)

E. Bertazzoni Minelli (Italy)

E. Bezirtzoglou (Greece)

G. Blandino (Italy)

P. Brigidi (Italy)

M. Camilleri (USA)

F. Caprioli (Italy)

A.M. Castellazzi (Italy)

A.F.G. Cicero (Italy)

G. Ciprandi (Italy)

A. Collignon (France)

E. Connolly (Sweden)

A. Costabile (U.K.)

M. D'Amato (Italy)

G. D'Argenio (Italy)

M. De Vincenzi (Italy)

W.M. de Vos (The Netherlands)

T. Decsi (Hungary)

C. Devirgiliis (Italy)

G. Di Felice (Italy)

L. Drago (Italy)

R.N. Fedorak (Canada)

L. Feld (Denmark)

G.L. Gianfranceschi (Italy)

E. Guaglianone (Italy)

M. Guslandi (Italy)

P.B. Heczko (Poland)

O. Hernell (Sweden)

S. Kamiya (Japan)

T.R. Klaenhammer (USA)

G. Lupattelli (Italy)

S. Macfarlane (U.K.)

P. Mastrantonio (Italy)

E. Mengheri (Italy)

M. Mikelsaar (Estonia)

L. Mogna (Italy)

S. Molin (Denmark)

L. Morelli (Italy)

F. Morisco (Italy)

E. Norin (Sweden)

M.R. Oggioni (Italy)

A.B. Onderdonk (USA)

P. Patrignani (Italy)

A.S. Peña (The Netherlands)

M. Porrini (Italy)

C. Pruzzo (Italy)

L. Quercia Vieira (Brazil)

G. Reid (Canada)

M. Rescigno (Italy)

M. Romano (Italy)

ELIE METCHNIKOFF: AN OPTIMISTIC PHILOSOPHER

Lorenzo Morelli
Istituto di Microbiologia-Università Cattolica del Sacro Cuore
Via Emilia Parmense 84- 29100 Piacenza-Italy

If the currently used criteria for the evaluation of the scientific activities of scientists could be applied to researcher of the past, I am sure that Elie Metchnikoff could rank very high in the “highly cited scientist” list. The “introduction” sections of papers dealing with probiotics or the ecology of the gut microbiota (more than two papers per day published in the last three years!) have the “compulsory” citation of the E .Metchnikoff book: “The prolongation of life: optimistic studies” published in 1907.

Pasteur was the first to say that life of upper animals would be impaired by the absence of indigenous microorganisms but was his colleagues Metchnikoff who, exactly one century ago, to observe that: " …the different susceptibilities of people to the harmful action of microbes and their products. Some can swallow without any evil result a quantity of microbes which in the case of other individuals would produce a fatal attack of cholera. Everything depends upon the resistance offered to the microbes by the invaded organism." (Metchnikoff, 1907).

He also stated that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1907).

This sentence describes in a clear way the "probiotic concept", the use of health promoting bacteria able to exert a positive role on intestinal flora, even if the word probiotics was coined several dozen years later; then we can assume today we are going to celebrate the first century of life of probiotics.

It could be then interesting to details on the man who is credited to have started a century, and we hope more and more long, history of scientific research and industrial application.

Ilia Metchnikoff was born in “the land of Panassovka, which belonged to the Mtechnikoff family (Life of Elie Metchnikoff, 1845-1916, written by the wife, Olga, published in 1918) in the province of Kharkoff, (Kharkov, Ukraine).

Metchnikoff received his bachelor's degree from the University of Kharkov (1864) and completed his doctoral degree at the University of St. Petersburg (1868).

Fascinated by science in all his aspects (he published his first scientific paper when he was aged 16) his academic education was basically as a zoologist. And served as professor of zoology and comparative anatomy at the University of Odessa (1870–82).

Germany and Italy were the two places outside Russia were he completed his scientific education.

During these periods he came across the books of C. Darwin, which had a decisive influence on the future directions of his researches.

He decided to focus on comparative embryology of animals and this kind of research allowed him to get in touch with the Oxonian P.Chalmers Mitchell, the Secretary of the Zoological Society of London and translator into English of most of future papers and books of Metchnikoff.

In Messina, Italy (1882–86), while studying the origin of digestive organs in starfish larvae, he observed that certain cells, unconnected with digestion, surrounded and engulfed foreign particles that he had introduced into the bodies of the larvae. He called these cells phagocytes and named the process phagocytosis.

Metchnikoff further developed this finding while working at the Bacteriological Institute, Odessa (1886–87), and at the Pasteur Institute, Paris (1888–1916); Metchnikoff had therefore contributed to one of the most important discoveries about the immune response. Perhaps his most notable achievement was his recognition that phagocytosis is the first line of defence against acute infection in most animals, including humans. This work formed the basis of Metchnikoff's cell-mediated theory of immunity (1892), a hypothesis that engendered much opposition, particularly from those scientists who claimed that only soluble substances and not cells could pose a barrier to pathogens.

Later vindicated, Metchnikoff's work on phagocytes won him the Nobel Prize in 1908.

Metchnikoff devoted the last decade of his life to investigating means of increasing human longevity. This interest was a further step in the research on immunity; in his Nobel lecture, held in Stockholm in 1908, Metchnikoff stated the Nobel Prize on Medicine (which was shared with paul Ehrlich) "for work on immunity", also “concerns the resistance of the body the disease”.

This was a clear suggestion that in the last of his life Metchnikoff was enlarging his area of research from the “immunity” to the more broad “resistance” concept; in the first (Nature of man, 1903)of the two books he wrote on this subject he described the “Disharmonies” in al living beings, from insects to humans. This book is a good example of how to use scientific data for philosophical considerations. Let me quote from the last chapter of this book: ” Man, who is descendant of some anthropoid ape has inherited a constitution adapted to an environment very different from that now surrounding him…. The sudden change in his natural conditions has brought about a large series of organic disharmonies …The greatest disharmonies of the constitution is that of the morbid nature of old age …”.

Metchnikoff had then posed the accent on the problem of an old age suffering from illness and then not really a “gold age”.

A few years later, in the second book devoted to this subject, the widely cited “The prolongation of life” Metchnikoff provided , in nine sections, a detailed view of his ideas on ageing and senility.

This book is more a philosophical “essay” than a paper on intestinal or food microbiology and among the 34 chapters of this book only three deal with bacteria and the intestine.

In the first among these three he suggested that the large intestine is the reason of the relatively short life of mammals, compared to other vertebrates. He disregarded it as a digestive organ and proposed a negative role “…it attended with disadvantages that may shorten the actual duration of life. The accumulation of waste

matter, retained in the large intestine for considerable periods, becomes a nidus for microbes which produces fermentation and putrefaction harmful for the organism."

The, in the following chapter he suggested a positive correlation between a long life span and the presence of a colon of reduced dimension also containing a low quantity of bacteria.

As he realise that it is not possible to have an "aseptic" colon , then he suggested to "disinfect the content of the large intestine".

After several but unsuccessful experiments made "*in vivo*" using lactic acid, he turned to the swiss prof. Massol, who provide Metchnikoff with a sample of "Bulgarian yahourt". The team of Metchnikoff (Dr. Cohendy and Michelson) isolated a strain they named "*Bulgarian bacillus"* to be used to ferment milk to be used in subjects exhibiting putrefactive-type fermentations. Results of this administration were a marked decrease in the fecal amount of products of putrefaction. In addition, he found that a predominantly Gram-positive flora was established and that the bacillus persisted in stools 8-12 days after the start of the treatment. Cohendy kindly provided Metchnikoff with the strain (which is still available in ATCC under the number 521, as *L.helveticus*) .

This strain however, "also gives a disagreeable taste of tallow" and Metchnikoff suggested to associate it with another lactic bacterium, the so called "paralactic bacillus" (whose real taxonomic position is unclear) to obtain a more pleasant flavour

Metchnikoff made an extensive use of the strain in both animal and humans experiments, showing the presence of a bacteriocin and also some "probiotic" activities exerted by heat-killed cells of this bacterium.

But the most interesting developments made by Metchnikoff using this strain deal with the evidences he obtained that lactic acid bacteria present in products such as yogurt, leben, kefir etc were not able to survive into the human gut as well as the *Bulgarian bacillus* was.

More, he established a company to produce and commercialize products containing the Bulgarian bacillus as an ingredient of fermented milk but also as a pill!

He was then a real forerunner of the present probiotic era, where beneficial bacteria are present in foods and in nutritional supplements; he was also an enthusiastic consumer of his own product " For more than eight years I took soured milk as first prepared from boiled milk inoculated with the lactic leaven. Since then…(I) have adopted the pure cultures that I have described. I am very pleased with the results"

He died in 1916 at 71 years of age (well above the average life expectancy at the time)

References.

Three books are available about E. Metchnikoff , his life and works:

1.E. Metchnikoff "The nature of man" 1903 (ARNO Press, New York, 1907)

2. O. Metchnikoff "Life of E. Metchnikoff" 1908 (Books for Libraries Press, Freeport, New York, 1972)

3. E. Metchnikoff "The prolongation of life" 1907 (English and Italian text; Mofin Alce- Novara ; 2000)

Development of biomarkers of oxidative stress and inflammation to predict the effects of natural antioxidants

Paola Patrignani and Stefania Tacconelli

Department of Medicine and Center of Excellence on Aging, "G. d'Annunzio" University, Chieti, Italy

Several lines of evidence suggest that reactive oxygen species (ROS) play a role in the development and progression of many diseases. In addition to cause a direct damage to the cell, oxidative stress leads to the induction of inflammation through the activation of proinflammatory transcription factors. This translates into the induction of pro-inflammatory cytokines and products of arachidonic acid mostly through the inducible form of cyclo-oxygenase, i.e. COX-2.

Biomarkers predictive of anti-oxidant and anti-inflammatory effects will be of valuable help to guide the use of natural antioxidant in the treatment of human disease.

Oxidative stress

In vitro studies unequivocally demonstrate that all vascular cells produce ROS and that ROS mediate diverse physiological functions in these cells. ROS are constantly generated *in vivo* and can cause oxidative damage to DNA, lipid, and protein (1,2). The short half-life of these species makes them ideal signalling molecules, but it also confounds their measurement in complex biological systems. In fact, a variety of methods have been proposed and applied for measurement of oxidative DNA, lipid, and protein damage. However *ex vivo* indices of oxidant stress could have questionable veracity in assessing the actual rate of lipid peroxidation *in vivo*. A valid biomarker should be a major product of oxidative modification, it should be a stable product, not susceptible to artifactual induction or loss during storage.

Plasma or urine measurement of F_2-isoprostanes appears to provide a sensitive, specific and non invasive index of non-enzymatic lipid peroxidation and it has been used extensively to assess *in vivo* lipid peroxidation in several human disease states (3,4). Isoprostanes (isoPs) are a family of prostaglandin (PG)-like compounds formed non-enzymatically through free radical catalysed attack

on esterified arachidonate followed by enzymatic release from cellular or lipoprotein phospholipids. Among F_2-isoPs, 8-iso-$PGF_{2\alpha}$ (also referred as IPF_{2a}-III) is the most frequently measured in urine samples (5). Enhanced generation of 8-iso-$PGF_{2\alpha}$ has been reported in a variety of syndromes associated with oxidant stress: coronary ischemia-reperfusion syndromes, acute coronary syndromes, Alzheimer's disease, chronic obstructive pulmonary disease and cystic fibrosis; in association with several cardiovascular risk factors, including hypercholesterolemia, hyperhomocysteinemia, diabetes mellitus, renovascular hypertension and cigarette smoking (4,6). Thus, measurement of 8-iso-$PGF_{2\alpha}$ provides a reliable tool for identifying populations with enhanced rates of lipid peroxidation (6). Measurements of the unmetabolized 8-iso-$PGF_{2\alpha}$ can be performed in both plasma and urine; however, most studies have examined its urinary excretion because of the non-invasiveness of the procedure and lack of *ex vivo* artifactual formation resulting from auto-oxidation of lipids. Mass spectrometry methods have been developed for the measurement of both 8-iso-$PGF_{2\alpha}$ but their use is limited by time-consuming and high costs (7,8). We have developed RIA techniques using- highly specific antisera - for the measurement of 8-iso-$PGF_{2\alpha}$ (9).

Inflammation

Cyclooxygenase-2 (COX-2) is an immediate early gene with many regulatory sites which is induced in response to different proinflammatory stimuli. COX-2-dependent prostaglandin(PG)E_2 is an important mediator of inflammation. We have developed the blood assay for the assessment of COX-2 inhibition (10). It consists in the assessment of PGE_2 levels in LPS-stimulated whole blood. It can be used as a marker to predict drug efficacy in humans (10,11). Moreover, measurement of a major metabolite of PGE_2 (PGEM) in urine could be a useful biomarker of inflammation *in vivo* (11).

References

1. Griendling, K.K., and FitzGerald, GA. (2003) Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation* 108,1912--1916.

2. Griendling, K.K. and FitzGerald, GA. (2003) Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulation* 108,2034--2040.
3. Patrono, C., and FitzGerald, G.A. (1997) Isoprostanes: potential markers of oxidant stress in atherothrombotic disease. *Arterioscler Thromb Vasc Biol* 17,2309--2315.
4. Patrignani, P., and Tacconelli, S. (2005) Isoprostanes and other markers of peroxidation in atherosclerosis. *Biomarkers*. 10, S24--9.
5. Rokach, J., Khanapure, S.P., Hwang, S.W., Adiyaman, M., Lawson, J.A., and FitzGerald, G.A. (1997) Nomenclature of isoprostanes: a proposal. *Prostaglandins* 54,853--873.
6. Davi, G., Falco, A., and Patrono, C. (2004) Determinants of F_2-isoprostane biosynthesis and inhibition in man. *Chemistry & Physics of Lipids* 128,149--163.
7. Pratico, D., Lawson, J.A., and FitzGerald, G.A. (1995) Cyclooxygenase-dependent formation of the isoprostane, 8-epi prostaglandin F2 alpha. *J Biol Chem*.270,9800--9808.
8. Li, H., Lawson, J.A., Reilly, M., Adiyaman, M., Hwang, S.W., Rokach, J., and FitzGerald, G.A. (1999) Quantitative high performance liquid chromatography/tandem mass spectrometric analysis of the four classes of F(2)-isoprostanes in human urine. *Proc Natl Acad Sci U S A*. 96, 13381--13386.
9. Wang, Z., Ciabattoni, G., Creminon, C., Lawson, J., Fitzgerald, G.A., Patrono, C., and Maclouf, J. (1995) Immunological characterization of urinary 8-epi-prostaglandin F2 alpha excretion in man. *JPET* 275,94--100.
10. Patrignani P, Panara MR, Greco A, et al. (1994) Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. *J Pharmacol Exp Ther*. 271, 1705--1712.
11. Capone ML, Tacconelli S, Di Francesco L, Sacchetti A, Sciulli MG, Patrignani P. (2007) Pharmacodynamic of cyclooxygenase inhibitors in humans. Prostaglandins Other Lipid Mediat. 82, 85--94.

MORINDA CITRIFOLIA, FERMENTED PAPAYA, WHEAT SPROUTS AND WHITE TEA ARE POPULARLY CLAIMED TO BE SOURCE OF POTENT COCKTAILS OF ANTIOXIDANT COMPOUNDS. BUT IS IT REALLY TRUE ?

Valeria Marsili, Isabella Calzuola, Stefano Perni and Gian Luigi Gianfranceschi

CEMIN (Centro di Eccellenza per Materiali Innovativi e Nanostrutturati), Università degli Studi di Perugia, Via Elce di Sotto, 06123 Perugia Italy

Abstract

Hydroalcoholic extracts from *Morinda citrifolia*, fermented papaya, wheat sprouts and white tea were tested on the potassium ferricyanide reduction and on the superoxide ion scavenging activity. Moreover the peroxidase activity was measured in the aqueous extracts from the same sources, as marker of redox enzymatic activity. The results show that the extracts from , wheat sprouts and white tea exert, at concentration of 1mg of extract/ ml, strong reducing and radical scavenging activities. The antioxidant activity of the fermented papaya at the same concentration is much lower. Wheat sprouts contain also a strong peroxidase activity (165 UI/g of sprouts powder) that on the contrary is almost completely absent in *Morinda citrifolia*, fermented papaya and white tea. Moreover the wheat sprouts extract appears able to inhibit the NO production in Raw 264.7 cells induced by LPS.

Introduction

In the last decades an increasing interest has been focused on the antioxidant compounds. This because the oxidative stress, due to an unbalanced increase of pro-oxidant compounds and/ or to a decrease of antioxidant molecules, has been soundly related to several pathologies and to aging progress (1). Many studies report the positive effect of natural antioxidants. A systematic screening of total electron-donating antioxidant was carried out in many dietary plants and fruits (2). Single antioxidant molecules (vitamins, thiols, polyphenols) or cocktails of antioxidant compounds have been tested in the treatment and/or prevention of cardiovascular diseases, neurodegenerative diseases, diabetes, cancer and aging related-diseases. In particular some vegetal plants or fruits have been indicated by scientific reports and often claimed by advertising spots as source of potent antioxidant compounds. To check how sound this claim is, we tested hydroalcoholic extracts from Morinda citrifolia (Noni) (2), fermented papaya (3), wheat sprouts (4) and white tea (5) on the potassium ferricyanide reduction and on the superoxide ion scavenging activity. Moreover the peroxidase activity has been measured in the aqueous extracts from the same sources, as marker of redox enzymatic activity.

Materials and Methods

Materials utilized for the study of the antioxidant activity.

Wheat sprout powder (Biogerm) was obtained from Germinal Life S.r.l., Perugia, Italy, *Morinda citrifolia* dry extract (Noni) from Sangalli s.r.l (Treviglio, BG, Italy), fermented papaya powder from the commercial product Immun'Age (produced by OSATO International, Japan) and white tea from a drugstore.

Preparation of the extracts

2 g of the above reported materials were suspended and homogenized (by means of a Waring Blendor) with 40 ml of water-ethanol (30/70, v/v) and centrifuged at 10.000g for 30 min at 4°C. The super, after storage at -20°C for 24 h, was again centrifuged at 10.000g for 30 min at 4°C and the pellet discarded. The ethanol was then removed by evaporation and the aqueous residue lyophilized. All of the dried extracts after lyophilization were resuspended in 2 ml of water. The extraction by 70% ethanol-30% water is useful for a good recovery of partially hydrophobic molecules such as vitamins A and E. polyphenols, reducing glycosides. We checked that more than

90% of the antioxidant molecules are re-extracted by water from the lyophilized samples of the various sources.

NBT (Superoxide Scavenging) Assay

Scavenging activity of the superoxide radical generated "in vitro" by the hypoxanthine-xanthine oxidase system, was measured following the inhibition of NBT reduction, on the basis of the method described by Kirby and Schmidt (7). Details of some arrangements we performed are reported in (5).

Total reducing power

The total reducing power of the extracts was measured by utilizing potassium ferricyanide as reagent, following the method of Yen and Chen (8). Details of some arrangements we performed are reported in (5).

Peroxidase Activity

The peroxidase activity was measured by the absorbance increase at 460 nm of o-dianisidine in the presence of hydrogen peroxide (9).

Results

The results in Table 1 shows that the extracts from *Morinda citrifolia* (Noni), wheat sprouts and white tea exert, at the concentration of 1mg of extract/ml, strong reducing and radical scavenging activities. Surprisingly the antioxidant activity of the fermented papaya, at the same concentration, is much lower. Wheat sprouts contain also a strong peroxidase activity (165 UI/g of sprouts powder) that on the contrary is almost completely absent in Morinda citrifolia, fermented papaya and white tea. TLC and HPLC analysis of the extracts show that the antioxidant compounds contained in each one of those sources substantially differ from the other ones. These data suggest a potential synergic effect by the extracts from the various sources. A positive result was obtained by mixing the extracts from wheat sprouts (1/2) and white tea (1/2) (+24.3%) and from Morinda citrifolia (1/3), wheat sprouts (1/3) and white tea (1/3) (+17.3%). The here reported results show that Morinda citrifolia, wheat sprouts and white tea alone and even more in association may be potentially an effective defence for the human health. In this context researches are in progress to check the potential synergic effect performed by extracts obtained from wheat sprouts (90%) associated with Lactobacillus GG (10%).The low antioxidant activity we observed in fermented papaya extract only apparently disagrees with the previously reported data. In fact the antioxidant activity observed by several Authors was obtained with a high concentration of fermented papaya extract (IC50 at about 10 mg/ml, (4)).

Table 1: Antioxidant activity of extracts from 1g of material

	Reduced potassium ferricyanide (μmoles) (5)	Scavenged superoxide ion (μmoles) (5)
Morinda Citrifolia	10.665 ± 0.36	1.349 ± 0.09
Fermented Papaya	1.052 ± 0.52	0.135 ±0.01
Wheat sprouts	12.911 ± 0.12	1.052 ± 0.11
White Tea	17.057 ± 0.89	1.379 ± 0.12

The values are reported as mean ± standard deviation of five independent samples.

Taking into account the relationship between oxidative stress and inflammation process, the hydroalcoholic extract from wheat sprouts was tested on the production of NO in Raw 264.7 cells induced by LPS. In fact the inhibition of NO synthesis has been reported as test of anti-inflammatory activity. The research was also prompted by the biochemical and functional

peculiarities of wheat sprouts extracts shown in our laboratory "in vitro" (antioxidant activity) and in animal systems (reduction of senile cataract). The NO production was strongly inhibited by the wheat sprouts extract at concentration of 1 and 5 mM (45% and 70% of inhibition, respectively).
This inhibition may be compared with that obtained with a pure antinflammatory drug such as the diclofenac at concentration of 50 and 100 μg /ml.

Discussion

The impetus for studying and treating patients with antioxidant therapy is due to its role in protecting against cellular injury (for example motor-neural injury) in both animal models and in vitro studies (10). However some points should be taken into account. First, the intestinal adsorption is a crucial step that could strongly quench the potential benefic effect of orally administrated natural compounds (11). Second, the potential efficacy of a vegetal product is measured by titrating the antioxidant activity. In effect the antioxidant compounds can be accompanied by other active molecules that could exert a positive or negative synergic action. For example the amphiphilic structure of glycosilated polyphenols and/or phospholipids could promote the internalization of active molecules into cells. Liu et al. reported that complex with phospolipids results in a strong increase of natural polyphenols bioavailability in rat "in vivo" (12).

References

(1) Droge W. Free radicals in the physiological control of cell function. Physiol. Rev. 2002;82:47-95.
(2) Halvorsen BL, Holte K, Myhrstad MCW, Barikmo I, Hvattum E, Remberg SF, Wold AB, Haffner K, Baugerod H, Andersen LF, Moskaug JO, Jacobs DR, Blomhoff Jr. and R. A systematic screening of total antioxidants in dietary plants. J. Nutr. 2002;132:461-471.
(3) Sang S, Cheng X, Zhu N, Stark RE, Badmaev V, Ghai G, Rosen RT, Ho C. Flavonolglycosides and novel iridoid glycoside from the leaves of *Morinda citrifolia*. J Agric. Food Chem. 2001;49:4478-4481.
(4) Imao K, Wang H, Komatsu M, Hiramatsu M. Free radical scavenging activity of fermented papaya preparation and its effect on lipid peroxide level and superoxide dismutase activity in iron-induced epileptic foci of rats. Biochem. Mol. Biol. Int. 1998;45:11-23.
(5) Calzuola I, Marsili V, Gianfranceschi GL. Synthesis of antioxidants in wheat sprouts. J. Agric. Food Chem. 2004;52:5201-5206.
(6) Santana-Rios G, Orner GA, Amantana A, Provost C, Wu SY, Dashwood RH. Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. Mutat. Res. 2001;495:61-74.
(7) Kirby AJ, Schmidt RJ. The antioxidant activity of Chinese herbs for eczema and placebo herbs. Ethnopharmacology 1997;56:103-108.
(8) Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem. 1995;43:27-32.
(9) Lebedeva OV, Ugarova NN, Berezin IV. Kinetic analysis of o-dianisidine oxidation by hydrogen peroxide in the presence of horseradish peroxidase. Biokhimija 1997;42:1372-1379.
(10) Simpson EP. Antioxidant treatment for amyotrophic lateral sclerosis. Lancet Neurol. 2005;4:266.
(11) Arts IC, Sesink AL, Hollman PC. Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. J. Nutr. 2002;132:2823-2824.
(12) Liu A, Lou H, Zhao L, Fan P. Validated LC/MS/MS assay for corcumin and tetrahydrocorcumin in rat plasma and application to pharmacokinetic study of phospolipid complex of corcumin. J. Pharmaceutic. Biomed. Anal. 2006; 40 : 720-727.

Nutrition and cardiovascular disease

Graziana Lupattelli, MD, Anna Rita Roscini MD.
Medicina Interna, Angiologia e Malattie da Arteriosclerosi
Dipartimento di Medicina Clinica e Sperimentale
Università degli Studi di Perugia

Increasing scientific evidence show that diets particularly rich in fruit, vegetables, fish and vegetable oils have protective health effects. The so-called Mediterranean diet is considered protective towards cardiovascular disease: its main components are monounsaturated fats replacing saturated ones, fish intake, low intake of red meat, moderate intake of wine, daily fresh fruit use and moderate intake of cakes and eggs. Although there are different versions of Mediterranean diet, e.g. Italian, Spanish and Greek, in all of them a central position is occupied by the use of extravirgin olive oil. Studies on this kind of alimentation are mainly observational, but it has been hypothesized that this style of diet lowers LDL cholesterol, has a positive impact on glycemia and allows a high intake of "protective" substances, such antioxidants. For instance, the benefits induced by olive oil seem not only due to the substitution of saturated fatty acids with oleic acid but particularly to the presence of antioxidant substances such as polyphenols.

Oxidative stress, and particularly lipid peroxidation, is involved in the pathogenesis of atherosclerosis through endothelial injury. In humans, endothelial impairment may be evaluated by the assessment of arterial capacity to vasodilate, a noninvasive ultrasound method estimating posthyperemic brachial flow mediated vasodilation (FMV). Endothelial dysfunction occurs in the presence of cardiovascular risk factors, represents the first step of atherogenesis and is a sensitive prognostic test of future coronary heart disease. Nevertheless, FMV can be improved by drugs, such statins or ACE inhibitors, and modifications of the style of life, such as nutrition and/or physical activity.

In a pilot study we examined the vascular effects of an "acute" diet change, and particularly the introduction of extravirgin olive oil (EVO) as a unique source of fat in coronary heart disease patients previously using a diet rich in saturated (or other kinds of monoinsaturated) fats, and never been eating olive oil as a fat supplementation. After 8 weeks endothelial reactivity, expressed as flow mediated vasodilation, significantly improved in the group eating EVO comparing to the control group left to the usual alimentation, whereas lipid and glycemic parameters did not show any modification, thus indicating a beneficial effect of EVO independent of plasmatic metabolic values.

Mediterranean diet is characterized also by the intake of fruit and vegetables. *Cynara Scolymus* (artichoke) is an herbaceous perennial plant native of North Africa, Canary isles, and Southern Europe, with countries from this area being the leading producers in the world. Artichoke leaves were used in ancient herbal medicine for several diseases. It is known that leaves of this plant are particularly rich in phenolic compounds, such as mono- and dicaffeoylquinic acids and flavonoids, an heterogeneous group of phytochemicals that can act as potent inhibitors of LDL oxidation via several mechanisms. The administration of artichoke leave extract to hypercholesterolemic patients significantly improved flow mediated vasodilation, indicating a potential beneficial effect of artichoke on vessel capacity to vasodilate, a consequence which does not seem not to be mediated by the slight hypocholesterolemic effect of the plant, but probably by its antioxidant properties.

Also wheat germ *(Triticum Vulgare)* contains antioxidant substances such as vitamin E and polycosanols such as octacosanol (and also calcium, zinc, manganese, magnesium, vitamin B); we tested the diet supplementation of this substance in moderately hypercholesterolemic patients, evaluating lipid parameters and endothelial function by means of flow mediated vasodilation and soluble adhesion molecules, sVCAM (Vascular Cell Adhesion Molecule) and sICAM (Intercellular Adhesion Molecule). Lipid parameters did not show any modification, whereas FMV increased and sICAM decreased, as a biochemical marker of improved endothelial function.

The influence of nutrition on the development of atherosclerotic process and on the progression of cardiovascular disease is still under debate and needs to be fully explored yet; nevertheless foods allowing a high intake of vitamins, antioxidants and monounsaturated fats seem to be the "key elements" of a cardioprotective diet.

REFERENCES

Ancel Keys, Trichopoulou A, Kouris-Blazos A, Wahlqvist M et al: Diet and overall survival in elderly people. Br Med J, 1995; 311: 1457–60

Keys A, Menotti A, Karvonen MJ et al: The diet and 15-year death rate in the Seven Countries Study. Am J Epidemiol, 1986; 124: 903–15

Ruit-Gutierrez V, Muriana FJG, Guerrero A: Plasma lipids, erythrocyte membrane lipids and blood pressure of hypertensive women after ingestion of dietary oleic acid from two different sources. J Hypertens, 1996; 14: 1483–90.

Marchesi, S., Lupattelli, G., Schillaci, G., Pirro, M., Siepi, D., Roscini, A.R., Pasqualini, L., Mannarino, E., 2000. Impaired flow-mediated vasoactivity during post-prandial phase in young healthy men. Atherosclerosis 153, 397–402.

Lupattelli G., Marchesi S, Bombardini R. Roscini AR, Trinca F, Gemelli F, Vaudo G, Mannarino E. Artichoke juice improves endothelial function in hyperlipemia. Life Sciences 76 (2004) 775–782

Antibiotic susceptibility of bacterial isolates from probiotic products available in Italy

Blandino G, Fazio D, Milazzo I.
Department of Microbiological Sciences-University of Catania-Italy

Routine antibiotic susceptibility testing of lactic acid bacteria (LAB) and bifidobacteria may be advisable in a number of instances e.g. for checking the biosafety of potentially probiotic isolates.[1,2] In fact, there is concern over the possible spread of antibiotic resistance determinants from bacteria used in probiotic products. However, there is still a lack of agreement on the MIC interpretative breakpoints mainly for *Lactobacillus* spp.[1-4]

The FEEDAP Committee[5] recently proposed microbiological breakpoints that can be relevant to identify strains with acquired and potentially transferable antibiotic-resistance in microbial feed additives. However, the results provided by different methods cannot be compared because they are influenced by test media, growth conditions and test assay.

Moreover, another aspect of probiotic bacteria is that under certain circumstances, they can cause infections in humans. A new CLSI document, "Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria", was published in 2006 as approved guidelines (M45/A)[6] and includes *Lactobacillus* sp, *Leuconostoc* spp and *Pediococcus* spp. The document suggests for this genera broth microdilution MIC tests, with cation-adjusted Mueller Hinton broth (CAMHB-LHB) supplemented with 2.5%-5% lysed horse blood and MIC interpretative criteria.

The aim of the present study was to compare the minimum inhibitory concentrations (MICs) obtained for antimicrobial agents using different methods and/or test media against 15 strains of *Lactobacillus* spp, 5 *Streptococcus thermophilus*, 1 *Enterococcus faecium*, and 8 *Bifidobacterium* spp isolated from 15 probiotic products.[8] The susceptibility of the 29 isolates was assayed by the E-test method (Abbiodisk) using De Man Rogosa-Sharpe agar (MRS) for *Lactobacillus* and *E. faecium,* MRS agar plus cystein for *Bifidobacterium* spp, and Muller Hinton agar + 5% sheep blood for *S. thermophilus,* with different conditions of incubation.

The MICs obtained by the E-test were compared to the MICs by broth microdilution test using CAMHB-LHB for *Lactobacillus* spp[6] and *Enterococcus* spp[7] , and CAMHB for *Streptococcus* spp[7].

Intrinsic resistance to vancomycin was confirmed for *L. paracasei, L. salivarius* and *L. plantarum.* All the isolates of *Lactobacillus* were susceptible to ampicillin. Species–dependent antibiotic susceptibility was detected for the cephalosporins tested; gentamicin and ciprofloxacin had variable activity. Atypical resistance to erythromycin was detected for one strain of *L. salivarius.* Isolates of *S. salivarius* and *E. faecium* were susceptible to the tested antibiotics (Table 1).

In the antimicrobial susceptibility of *Bifidobacterium* spp (CLSI guidelines are not currently available), we compared the MICs obtained by the E-test on MRS agar and the new test medium LSM (Lactic acid bacteria Susceptibility Test Medium)[9] both supplemented with cysteine (anaerobic incubation at 37 °C for 48h) (Table 2).

The isolates of *Bifidobacterium* spp were susceptible to vancomycin, ampicillin, cefotaxime, and erythromycin. The MICs of gentamicin was ≥ 8 mg/L.

MICs of several antibiotics for the majority of the strains of *Lactobacillus* and *S. thermophilus* were equal with both methods. However, the MICs of ampicillin and the cephalosporins were 1 to 2 MIC $\log_2$ steps lower with the E-test than with microdilution for some strains of *Lactobacillus* spp. Using the E-test method MICs of vancomicina, and β-lactams against some strains of *Bifidobacterium* spp were higher on LSM + cys than on MRS + cys.

On the basis of microbiological[5] and clinical breakpoints,[6] the observed resistances, genus or species dependent, seemed to be intrinsic except for erythromycin in 1 *L. salivarius* strain.

In conclusion, MICs obtained following the CLSI guidelines (2006)[6] for *Lactobacillus* may give new data to better define the cutoff values for separating strains with acquired resistance from susceptible strains.[3,4] Moreover, these results confirm that the E-test on LSM + cys is an applicable technique for susceptibility testing of bifidobacteria and generally for testing individual strains, e.g. by a probiotic culture producer.[9]

References

1) Charteris WP, Kelly PM, Morelli L, Collins JK (1998) Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. J Food Prot 61:1636-1643.

2) Charteris WP, Kelly PM, Morelli L, Collins JK (1998) Antibiotic susceptibility of potentially probiotic *Bifidobacterium* isolates from the human gastrointestinal tract. Lett Appl Microbiol 26:333-337.

3) Florez AB et al.(2005) Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Can J. Microbiol 51:51-58.

4) Florez AB et al.(2006) Susceptibility of *Lactobacillus plantarum* strains of six antibiotics and definition of new susceptibility-resistance cutoff values. Microbial Drug Res. 12, 252-256.

5) European Commission (2005) 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 and veterinary importance. EFSA J. 223: 1-12.

6) Clinical and Laboratory Standards Institute. Methods for antimicrobial and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Approved standard M45-A. Wayne, PA: Clinical and Laboratory Standards Institute, 2006.

7) Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Seventeenth Information Supplement M100-S17 Clinical and Laboratory Standards Institute: Wayne, PA: Clinical and Laboratory Standards Institute, 2007.

8) Milazzo I, Speciale A, Musumeci R, Fazio D, Blandino G (2006) Identification and antibiotic susceptibility of bacterial isolates from probiotic products available in Italy. New Microbiological 29: 281-291.

9) Mältö J.et al (2006) Comparison of three test media for antimicrobial susceptibility testing of bifidobacteria using E-test method. Int. J. Antimicrob. Ag. 28, 42-48.

Table 1. Distribution of MICs (mg/L) of antimicrobial agents for *Lactobacillus* spp, *E. faecium*, *S. thermophilus* by Microdilution broth (M) and the E-test (E).

Antibiotic	Strains		N°	≤ 0.25	0.5	1	2	4	8	16	≥32
Vancomycin	*L. lactis*	*(M)*	1				1				
		(E)					1				
	L.plantarum/L.salivarius	*(M)*	2								2
		(E)									2
	L. acidophilus	*(M)*	4			1	1	2			
		(E)				1	1	2			
	L. paracasei	*(M)*	5								5
		(E)									5
	L.bulgaricus	*(M)*	3			1	1	1			
		(E)				1	1	1			
	S .thermophilus	*(M)*	5	5							
		(E)		5							
	E. faecium	*(M)*	1		1						
		(E)		1							
Ampicillin	*L. lactis*	*(M)*	1	1							
		(E)		1							
	L.plantarum/L.salivarius	*(M)*	2		2						
		(E)		1	1						
	L. acidophilus	*(M)*	4	3	1						
		(E)		2	2						
	L. paracasei	*(M)*	5	5							
		(E)		5							
	L.bulgaricus	*(M)*	3	1		1	1				
		(E)		1	1		1				
	S .thermophilus	*(M)*	5	5							
		(E)		5							
	E. faecium	*(M)*	1		1						
		(E)			1						
Cefaclor	*L. lactis*	*(M)*	1			1					
		(E)				1					
	L.plantarum/L.salivarius	*(M)*	2					1		1	
		(E)						1		1	
	L. acidophilus	*(M)*	4			1		2	1		
		(E)		1				2	1		
	L. paracasei	*(M)*	5					3	2		
		(E)						4	1		
	L.bulgaricus	*(M)*	3							2	1
		(E)								2	1
	S .thermophilus	*(M)*	5	5							
		(E)		5							

Antibiotic	Strains		N°	≤ 0.25	0.5	1	2	4	8	16	≥32
Cefotaxime	*L. lactis*	*(M)*	1								1
		(E)									1
	L.plantarum/L.salivarius	*(M)*	2			1	1				
		(E)			1		1				
	L. acidophilus	*(M)*	4			2	2				
		(E)		1		1	2				
	L. paracasei	*(M)*	5			4		1			
		(E)				4		1			
	L.bulgaricus	*(M)*	3							2	1
		(E)								2	1
	S .thermophilus	*(M)*	5	5							
		(E)		5							
Erythromycin	*L. lactis*	*(M)*	1		1						
		(E)			1						
	L.plantarum/L.salivarius	*(M)*	2	1							1
		(E)		1							1
	L. acidophilus	*(M)*	4	3		1					
		(E)		3		1					
	L. paracasei	*(M)*	5	2	1	1		1			
		(E)		3		1		1			
	L.bulgaricus	*(M)*	3	2			1				
		(E)		2			1				
	S .thermophilus	*(M)*	5		5						
		(E)		5							
	E. faecium	*(M)*	1	1							
		(E)		1							
Ciprofloxacin	*L. lactis*	*(M)*	1		1						
		(E)			1						
	L.plantarum/L.salivarius	*(M)*	2								2
		(E)									2
	L. acidophilus	*(M)*	4						2	1	1
		(E)							2	1	1
	L. paracasei	*(M)*	5			1	1			2	1
		(E)				2				2	1
	L.bulgaricus	*(M)*	3								3
		(E)									3
	S .thermophilus	*(M)*	5			2	1	2			
		(E)				2	1	2			
	E. faecium	*(M)*	1	1							
		(E)		1							
Gentamicin	*L. lactis*	*(M)*	1					1			
		(E)						1			
	L.plantarum/L.salivarius	*(M)*	2					1			1
		(E)						1			1
	L. acidophilus	*(M)*	4			1		2			1
		(E)			1		1	1			1
	L. paracasei	*(M)*	5					1			4
		(E)						1			4
	L.bulgaricus	*(M)*	3					3			
		(E)					1	2			

Table 2. Distribution of MICs (mg/L) of antimicrobial agents for 8 strains of *Bifibacterium* spp by the E-test on 2 media (anaerobic incubation at 37 °C for 48h).

Antibiotic	Media	≤ 0.25	0.5	1	2	4	8	16	≥ 32
Vancomycin	*MRS + cys*	5	1	2					
	LSM + cys		5	1	2				
Ampicillin	*MRS + cys*	8							
	LSM + cys	6	2						
Cefotaxime	*MRS + cys*	2	1	1			4		
	LSM + cys	1	2	1			4		
Gentamicin	*MRS + cys*						2	4	2
	LSM + cys						2	4	2
Erythromycin	*MRS + cys*	7	1						
	LSM + cys	7	1						

Double benefit claims for antimicrobial and antioxidative probiotics

Marika Mikelsaar
Department of Microbiology, University of Tartu

The search for new effective probiotic strains, particularly aimed against atherosclerosis related cardiovascular diseases, is in process. The new EU regulations require evidence of either general balancing and/or enhancing human particular functions or the reduction of risk of certain diseases through the use of probiotic microbes.

Objective: The paper lays out the characteristics of a new probiotic Lactobacillus fermentum strain ME-3 DSM-14241, elaborated according to the new regulations.

Methods and Results. The process of the identification and molecular typing of this probiotic strain of human origin, its deposition in an international culture collection, its safety assessment by laboratory tests and testing on experimental animals and volunteers have been described. Lactobacillus fermentum strain ME-3 possess double functional properties: antimicrobial activity against intestinal pathogens and high total antioxidative activity (TAA) and total antioxidative status (TAS) of intact cells and lysates. The functional efficacy of the antimicrobial and antioxidative probiotic has been proved by eradication of pathogenic microbes and reduction of liver and spleen granulomas in Salmonella Typhimurium infected mice treated by the combination of ofloxacin and Lactobacillus fermentum strain ME-3. In several clinical trials carried out on volunteers and ill persons this probiotic has increased the anti-oxidative activity of blood sera and improved the composition of LDL particles, thus demonstrating a remarkable anti-atherogenic effect. A special industrial technology has been developed to incorporate the new probiotic into food products and supplements and to control the probiotic content in these products. The ME-3 probiotic-containing products are available in Estonia and Finland
Conclusion. The evidence based double claims for enhancing human immunological and antioxidative functions to reduce the risk of infections and atherosclerosis through the use of probiotic microbes have been elaborated.

How bifidobacterial genomics could help in understanding probiotic-prebiotic functionality

Marco Ventura

Department of Genetics, Biology of Microorganisms, Anthropology, Evolution, University of Parma, Italy

Bacterial-genome nucleotide sequencing has revolutionized the genetic, biochemical and molecular biology research on bacteria and, indeed, many higher organisms. Over the past decade, the sequences of more than 500 bacterial genomes have become available in the public domain. Considerable emphasis was initially placed on sequence determination of the genomes of pathogenic bacteria, including food-borne pathogens (NCBI source). However, in recent years, genome sequencing of Gastro Intestinal Tract (GIT) commensals and symbionts, as well as food-grade bacteria has become more established among sequencing projects, currently represented by genome sequences of more than 40 lactic acid bacteria (LAB) and bifidobacteria. Members of the latter group include GIT commensals with probiotic properties. Bifidobacterial species are found in the GIT of mammals with the notable exception of species isolated from the human oral cavity (*Bifidobacterium dentium*), sewage, or the insect gut. Bifidobacteria are representatives of the high G+C Gram positive group of bacteria, belonging to the *Actinobacteria* phylum, within which they constitute a separate order, "*Bifidobacteriales*". This order comprises a single family *Bifidobacteriaceae*, which in turn consists of four genera, *Bifidobacterium*, *Gardnerella, Scardovia* and *Parascardovia*. Except for the *Bifidobacterium* genus, which contains 29 species, the other genera each contain only a single species. Bifidobacterial species are organized in six phylogenetic groups including *B. longum*, *B. boum*, *B. adolescentis*, *B. pullorum*, *B. pseudolongum* and *B. asteroides*.

Bifidobacterial genomics

Of the currently recognized 29 *Bifidobacterium* species, three strains belonging to the *B. longum* and *B. adolescentis* phylogenetic groups have been sequenced to completion, of which only two have been published in full, i.e. *Bifidobacterium longum* biotype longum NCC2705 (Schell et al., 2002) and *Bifidobacterium adolescentis* ATCC 15703, while others, e.g. *B. dentium* Bd1, are at various stages of completion and detailed sequence information for some of these genomes is expected to become publicly available in the near future. Furthermore, genome sequencing of *B. breve* M-16V, *B. breve* Yakult, *B. animalis* subsp. *lactis*, *B. longum* biotype longum, and *B. longum* biotype infantis is underway. These genomes range in size from 1.9-2.9 Mb, and generally display architectural features of a typical bacterial chromosome, such as the co-orientation of gene transcription and DNA replication, a G-rich, C-poor bias in the nucleotide composition of the leading DNA strand, and a typical presumptive origin of replication region, including a gene-constellation near to the origin comprising *rpmH*, *dnaA*, *dnaN* and *recF*, a particular GC nucleotide skew [(G-C)/(G+C)], the presence of multiple DnaA-boxes and AT-rich sequences immediately upstream of the *dnaA* gene.

The number of rRNA operons in bifidobacteria varies between one and five, perhaps reflecting different ecological strategies. The number of transfer RNA (tRNA) genes in the so far sequenced bifidobacterial genomes is relatively stable, i.e. 54 and 56 in *B. breve* UCC2003 and *B. longum* biotype longum NCC2705, respectively. These are representative of all twenty amino acids, with redundant tRNAs for all amino acids except for cysteine, histidine, isoleucine, phenylalanine and tryptophan.

The availability of whole genome sequences is useful to identify new metabolic pathways or pathways that are incomplete. However, so far, the only available genome-sequences data relating to biosynthetic abilities of bifidobacteria is limited to *B. longum* biotype longum NCC2705 strain (Schell et al., 2002). This strain possesses the genes for the synthesis of at least 19 amino acids from ammonium and major synthesis precursors (phosphoenolpyruvate, oxaloacetate, oxoglutarate and fumarate). Notably, genes homologous to those encoding asparagine synthetases and asparaginyl-tRNA synthetase are absent suggesting that *B. longum* biotype longum NCC2705 uses the *gat*ABC/asparaginyl-tRNA pathway to produce asparagines from aspartate. Also cysteine biosynthesis and sulfur assimilation is accomplished by an unusual pathway in this microorganism, which involve homologs of cystathionine γ-synthetase, cystathionine, β-synthetase and cystathionine γ-lyase using succinylhomoserine and reduced sulphur compound that could be provided through metabolic activity of other GIT commensal bacteria. Moreover, this bifidobacterial strain possesses all the enzymes needed for the biosynthesis of pyrimidine and purine nucleotides for glutamine as well as those necessary for the synthesis of folic acid, thiamine and nicotinate (Schell, 2002). Conversely, the metabolic pathways for the production of riboflavin, biotin, cobalamin, pantothenate, lipoate and pyridoxine appear to be absent. All the above mentioned metabolic capacities indicate that this bacterium seems to be well adapted to growth in an environment which is pour for some metabolic substrates (e.g. aminoacids, vitamins and nucleotides).

Comparative bifidobacterial genome analysis

Dotplot comparisons (nucleotide level) of the fully sequenced bifidobacterial genomes revealed a high degree of conservation and synteny across the entire genomes., i.e. *B. longum* biotype longum NCC2705, *B. longum* biotype longum DJO10A, *B. breve* UCC2003 and *B. adolescentis* ACC15703. Preliminary analysis against the draft genome sequences of *B. dentium* Bd1 confirmed and extended this result. Recently, a *B. longum* biotype longum NCC2705-based spotted DNA microarray was employed to compare the genomes of ten bifidobacterial strains, including other *B. longum* biotype longum strains as well as the closely related *B. longum* biotype infantis and *B. longum* biotype suis taxa. Results revealed seven large genome regions of variability, the majority of which encompass DNA with a deviating G+C content. These regions correspond to a prophage remnant, a cluster of genes for enzymes involved in sugar metabolism, such as an α-mannosidase, and a capsular polysaccharide biosynthesis gene cluster, which could play a role in host-bacterium interactions (Rezzonico et al., 2003).

Bifidobacterial genomics and biological lifestyle

Genome sequencing provides the gold standard for analysing the genetic content of an organism, and provides insights about genetic determinants facing the environment where the organism lives. In this context, food components such as complex carbohydrates that escape digestion by mammal's enzymes (and so reach intact the distal part of the colon) have shaped the genomes of the microbiota living in this GIT compartment (e.g. bifidobacteria). Notably, an enormous potential for degradation and utilization of complex carbohydrates can be predicted from the analysis of bifidobacterial genomes. In fact, over 8% of the annotated genes of bifidobacterial genomes have been predicted to encode enzymes involved in sugar metabolism of glucose, galactose, fructose, arabinose, mannose and xylose, as well as prebiotic compounds like fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), gluco-oligosaccharides, xylo-oligosaccharides, lactulose and raffinose. Furthermore, GIT mucus provides a great reservoir of glycans that can be degraded by bifidobacteria, thereby representing a considerable synergistic relationship.

Another example of how the genome content of bifidobacteria reflects its ecological adaptation to the GIT is characterized by genes coding for proteins involved in stress responses. It is, in fact, worth to mention that in their more or less isothermal niche, bifidobacteria possess one of the smallest set of genes coding for heat-shock proteins.

The interaction of bifidobacteria with the humanGIT

Human gut commensals like bifidobacteria are expected to interact with the host through direct contacts between bacteria and host's epithelial cells. Notably, bifidobacteria are predicted to encode cell envelope-associated structures such as exopolysaccharides that are considered to be important extracellular structures for intestinal commensal bacteria to establish themselves within the host. Genome analysis of *B. longum* biotype longum NCC2705 revealed the presence of two regions related to polysaccharide biosynthesis that are flanked by IS elements and depict a considerable divergence in G+C content relative to the remainder of the genome, both evidences would suggest that they have been acquired by horizontal gene transfer (HGT). Interestingly, genes predicted to encode glycoprotein-binding fimbria-like structures, which have been identified in the genome sequences of both *B. longum* biotype longum NCC2705 and *B. longum* biotype longum DJO10A genomes may mediate another way of interaction with the host. In addition, bifidobacterial genomes encode a serpin-like protease inhibitor that has been shown to contribute to host interaction in the GIT. The *B. longum* biotype longum NCC2705 serpin is an inhibitor of human neutrophil and pancreatic elastases, whose release by activated neutrophils at the sites of intestinal inflammation represents an intriguing mechanism of innate immunity (Ivanov et al., 2006).

Bifidobacterial genomics and evolution

In the last years prokaryotic taxonomy has gained from the great advantages obtained by genomic projects. The availability of large genome databases has offered the possibility to investigate phylogenetic relationship between bacteria using alternative molecular markers to 16S rRNA gene. Moreover, phylogenetic investigations based on the concatenation of multiple molecular markers has generated a most reliable image of evolutionary relationships between bacteria. In this context, a phylogenetic analysis of the genus *Bifidobacterium* using a multigene concatenation approach revealed that the projected ancestor of all the genus was most closely related to the current *Bifidobacterium asteroides* species (Ventura et al., 2006).

References

Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, B. Berger, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, R. D. Pridmore, and F. Arigoni. 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A **99:**14422-7.

Ivanov, D., C. Emonet, F. Foata, M. Affolter, M. Delley, M. Fisseha, S. Blum-Sperisen, S. Kochhar, and F. Arigoni. 2006. A serpin from the gut bacterium *Bifidobacterium longum* inhibits eukaryotic elastase-like serine proteases. J Biol Chem **281:**17246-52.

Ventura, M., C. Canchaya, A. Del Casale, F. Dellaglio, E. Neviani, G. F. Fitzgerald, and D. van Sinderen. 2006. Analysis of bifidobacterial evolution using a multilocus approach. Int J Syst Evol Microbiol **56:**2783-92.

Human Intestinal Microbiota and Nutrition Homeostasis

Shenderov Boris, Aleshkin Vladimir

Research Institute of Epidem. Microbiol. after G.N.Gabrichevsky

According to modern data human being needs hundreds and thousands of different physiologically active functional nutrients for normal growth and development. There is traditional opinion that the main source of all this ingredients is the foods. The impact of host intestinal microbiota on human nutritional status is still only vaguely understood.

The review presented gives grant attention to contribution of symbiotic microbiota to anatomy and physiology of human being intestinal tract (epithelial cell growth and development), to the food processing, to` the support of water, ion, energy homeostasis. It will be discussed the role of intestinal microbiota in the protein, carbohydrate, lipid metabolism, in the production of different micronutrients, signal molecules, regulators of host-bacteria interactions (neurotransmitters, peptides, lectins, amines, hormones, vitamins, fat acids, steroids, defenzines, nitrogen oxide, activated phytoestrogens and many others) from exogenous and endogenous origin, in the liver-intestinal macromolecule re-circulation and so on. The analysis of these data allows the authors to come to conclusion that provision of human being with required nutritional ingredients depends on both how well the host is provided with balanced foods and what state of intestinal microecology host has. Searching and identification for symbiotic bacteria participating in provision of humans with different macro- and micronutrients will make it possible to work out the new types of probiotics (metabiotics) with specific predicted metabolic characteristics.

Relationship between number of bacteria and their probiotic effects

Elisa Bertazzoni Minelli, Anna Benini

Dpt. of Medicine and Public Health, Pharmacology Section, University of Verona, Verona, Italy

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host as defined by FAO/WHO. Probiotics encompass live bacteria belonging to natural bacterial flora without pathogenicity which are thought to exert healthy benefits beyond inherent basic nutrition. They consist of different strains of bacteria (Lactic Acid Bacteria-LAB-, Bifidobacteria, Bacilli, *E. coli,* Clostridia, Propionibacteria) and yeasts (*Saccharomyces*) (2, 9). Their utilisation in antibiotics diarrhoea, diarrhoea caused by virus or bacteria demonstrated positive results at standard doses (10^7-10^8 CFU/day), e.g. reduction of stool frequency, mean duration of diarrhoea in adults and children (1).

Traveller' Diarrhoea. The literature on the topic of the efficacy of probiotics in the prevention of traveller' diarrhoea gave conflicting findings.

Protective effects have been shown with probiotic preparations of *L. rhamnosus GG* and *Saccharomyces boulardii*, while a mixture of LA and *L. bulgaricus*, LA or *L. fermentum* preparations have not been shown to be effective.

Differences in the population involved in the studies, the probiotic strains used (and their viability), and methodological and statistical problems - such as subgroup analysis or similar- could explain the discrepancies. Preparations of non-viable *L. acidophilus* showed no efficacy in travellers' diarrhoea prevention. Additional trials may still worth considering with probiotics that have demonstrated a protective effect for the prevention and the treatment of acute infectious diarrhoea in children (1).

A body of data stressed the differences among bacterial species and strains about resistance to intestinal conditions, their survival and colonisation as well as their different probiotic effects. Lactic acid bacteria seem exert better effects when administered in combination.

A number of clinical studies on the effects of probiotics on *H .pylori* infection indicate a suppressed growth of *H. pylori* without eradication, although there are differences in the effectiveness between strains.

Viability. The survival capacities of various strains of *L. acidophilus*, *L. plantarum*, *L. salivarius*, *L. casei* and *L. johnsonii* in acid conditions are higher than that of *L. bulgaricus*. Approximately 1-10% of *L. acidophilus* ingested in fermented product were found to survive until the ileum in several human studies using intestinal intubation techniques (9).

L. plantarum NCIB 8826, *L. salivarius* 433118 and some *Bifidobacterium* spp. (commercial milk product) showed a very high survival capacity. Their concentration in the ileum reached 10^8 and 10^7 CFU/ml respectively after a single-dose; they passed through the ileum at a concentration above 10^5 CFU/ml for more than 5 hours. No small bowel colonization was observed.

Similarly, some *Bifidobacterium* spp. from fermented dairy products and *L. plantarum* NCIB 8826 exhibited a high survival in the whole gastrointestinal tract; 25-30% of the ingested bacteria being recovered from faeces. Faecal concentrations reached 10^8 CFU/g, and these bacteria did non colonised the gut (13).

Other studies in healthy volunteers with different probiotic preparations showed that the faecal concentrations of ingested *L. acidophilus*, *L. reuteri*, *L. salivarius* UCC118 and *L. rhamnosus* strain GG reached around 10^6 CFU/g (9).

Despite the amount up to 10^{11} CFU/day of *Lactobacillus paracasei* subsp. *paracasei* (CRL-431) viable CRL-431 bacteria could not be isolated from the fresh faecal samples from either 2 weeks of treatment and two weeks of wash-out. In contrast, recovery of *Bifidobacteriun animalis* ssp *lactis* (BB-12) exhibited a dose-response relationship with 10^{10} CFU/day being the lowest dose giving a statistically significant chance of recovering viable BB-12 from the faeces (3)

The concentration of probiotics needed to obtain a clinical effect is often quoted as $\geq 10^6$ CFU/ml in the small bowel and $\geq 10^8$ CFU/g in the colon (12).
The faecal recovery of Bifidobacteria and Lactobacilli in healthy subjects exhibited a dose-response relationship with 10^{10} CFU/day (administered) being the lowest dose giving a statistically significant change of recovering viable bacteria from the faeces; a 10-fold increase of ingested bacteria caused the average number of recovered viable BB12 to increase by a factor 20 (10^{13}). It seems evident that the higher the ingested dose, the greater the number of subjects positive for viable bacteria (10^{11} CFU/day) in young healthy adults (2).
The pharmacokinetics of three strains of LAB were studied in the human gastrointestinal tract.
L. plantarum NCIMB 8826 in the ileum reached 10^8 CFU/ml after a single dose (10^8 CFU/ml) in fermented milk. *L. fermentum* KLD and *Lactococcus lactis* MG 1363 showed lower and shorter ileal survival (8).
L. plantarum NCIMB 8826 was present at high concentrations (10^8 CFU/g) in the faeces on the day 7 of the 1-week ingestion period. It was undetectable in the faeces 2 weeks after the end of the ingestion period (9).
In healthy human subject receiving 1g/day (about 3 x 10^{10} viable cells) of lyophilized *S. boulardii* fecal levels were reported to be 1.4 x 10^7/g (4).
A linear relationship was obtained ranging from no protection when a preparation containing 3 x 10^8 viable yeast/mL was the source of drinking water to 85% survival when a preparation containing 3.3 x 10^{10}/ml was employed. The transient presence of high levels of living *S. boulardii* in the gastrointestinal tract of gnotobiotic mice seems to be necessary to exert efficacy in an animal model for human pseudo-membranous colitis (4).
Effects on the immune system. The effects on health or physiology may be either direct or indirect through modifications of the endogenous ecosystem or the immune response, suggesting that a single mechanism of action for all probiotics and all effects is unlikely.
In addition to a direct impact on epithelial cells and cytokine responses, probiotics may also influence the development and activity of regulatory T-cells (9).
The study the immune modulating effects in healthy adults is problematic, because it can not be concluded that the tested bacteria exert no healthy-promoting effects. To evaluate the efficacy of probiotics it may be essential to identify specific target groups of individuals with more specific higher susceptibilities to the potential effects of probiotics, e.g. low bifidobacteria or lactobacilli count, microflora imbalance or intestinal immunological alterations (inflammatory bowel disease, irritable bowel syndrome, etc).
Different preparations of LAB stimulate intestinal lymphoid foci and their accessory cells in different ways lending further support to the notion that live forms of LAB can stimulate specific compartments of the immune system differentially to killed forms.
L. rhamnosus HN001, delivered orally as a viable probiotic supplement in a milk-based substrate is able to enhance phagocytic capacity in mice (3). In the case of immune enhancements a dose of 10^7 *L. rhamnosus* daily for 14 days was enough to enhance the phagocytic capacity of blood leucocytes in mice but a dose of 10^9 was found necessary to enhance the phagocytic capacity of peritoneal cells. Heat-killed *L. rhamnosus* HN001 was effective as live cells in enhancing innate cellular immune function, while only live forms enhanced specific gut mucosal antibody responses to orally administered cholera toxin vaccine (3).
No significant statistically differences were observed for phagocytic activity in blood lymphocytes, IgA faecal concentrations or production of interferon γ and IL -10 in blood cells. The IFN- γ and IL-10 production in blood cells resulted significantly reduced when evaluated according to number of viable faecal bacteria (2)
Cell debris of *L. delbrueckii* subsp. *bulgaricus* MB453 and *L. plantarum* MB 452 stimulates PBMNC when used at concentration higher than 10^4 CFU/mL, while both *L. azidophilus* MB443 and *L. casei* MB451 strains only require concentrations higher than 10^6 and 10^5 CFU/ml. *L. casei* subsp. *rhamnosus* (L. GG) had a very low stimulation capacity compared to other strains (6).

Bifidobacteria stimulates pro- and anti-inflammatory cytokines more significantly than lactobacilli, but the stimulation pattern is different. The highest concentration of bifidobacteria (10^7 CFU/mL) induces PBMNC to produce less pro- and anti-inflammatory cytokines than the lower concentration of the strains (10^3 CFU/mL).

E. coli Nissle, which has been shown to be effective in maintaining remission of ulcerative colitis, has a high stimulating capacity for IL-10 and IL-1β, compared to other strains, but a low capacity for TNF-α (6).

Inflammatory Bowel Disease. The intestinal microflora has been suggested to be involved in the pathogenesis of inflammatory bowel diseases in genetically predisposed subjects with immunological alterations, triggering an overly aggressive cell-mediated immune response (11).

The intestinal microbiota plays a critical role in the pathophysiology of pouchitis, a major complication after ileal pouch and anastomosis in patients with ulcerative colitis (7).

Recent studies have shown that probiotic treatment with VSL#3, mixture of eight different probiotic bacterial strains at high dose (300 billions viable lyophilized bacteria) is effective in maintaining recession in pouchitis. Patients received VSL#3 twice daily dose (3+3g) for nine months or until relapse: 17 of 20 patients remained in remission while all on placebo relapsed. The same preparation administered as prophylaxis once daily (VSL#3, 6 g) maintained antibiotic induced remission for at least a year in patients with recurrent or refractory pouchitis, ameliorating their quality of life (10).

Results in Crohn's disease and in irritable bowel disease are variable, but several probiotics are promising. We need adequate clinical trials, selection of microbial strains with specific characteristics and definition of patients and probiotic appropriate use (number of bacteria, doses, desired effects).

The timing of probiotic administration, the dose and the duration of treatment increased the positive effects in selected patients (8, 10).

Stability. To be effective probiotic cultures must be able to withstanding processing conditions, retain their probiotic properties after processing and survive in sufficient numbers in the product during shelf life storage. The stability of a probiotic is linked to various factors, including genus, species, strain biotype and, above all, the formulation storage conditions.

Conclusions. At the present, we cannot define the optimal amount of bacteria for probiotic effects. We need further investigations to define the effective dose of each genus and strain, and their appropriate utilisation for different clinical situations.

References

1. Allen SJ., Okoko B., et al. Probiotics for treating infectious diarrhoea. Cochrane Database Syst Rev 2004; CD003048
2. Christensen H., Larsen C., et al.. FEMS Immunol. Med. Microbiol. 47: 380-390, 2006
3. Donnet-Hughes A., Rochat F., et al.. J. Dairy Sci 82: 863- 869, 1999
4. Elmer G.W. and Corthier G. Can J. Microbiol 37: 315-317, 1991
5. Gill H.S., Rutherfurd K.J. Br J Nutr 86: 286-0289, 2001
6. Helwig U., Lammers KM., et al. World J Gastroenterol 12: 5978- 5986, 2006
7. Kühbacher T., Ott SJ., et al.. Gut 55: 833- 841, 2006
8. Oozeer R., Leplingard A., et al. Appl. Environm. Microbiol. 72: 5615- 5617, 2006
9. Marteau P. et al. Best Pract Res Clin Gastroenterol 17: 725-40, 2003
10. Mimura T, Rizzello F. et al. Gut 53: 108-14, 2004
11. Sartor BR. . Gastroenterology 126. 1620-1633, 2004
12. Sanders M. E.. Nutrition Reviews 61, n.3: 91-99, 2003
13. Vesa T., Pochart P., et al. Aliment. Pharmacol Ther 14: 823-828, 2000

Functional Genomics of Probiotic Bacteria

Todd R. Klaenhammer.
Department of Food Science, Box 7624, North Carolina State University, Raleigh, NC 27695

Todd R. Klaenhammer*, A. Azcarate-Peril, B.L. Buck, R. Barrangou, and E. Altermann

Department of Food Science, Box 7624, North Carolina State University, Raleigh, NC 27695

Lactic acid bacteria (LAB) have been used in fermentation processes for millennia. More recent applications such as the use of living cultures as probiotics have significantly increased industrial interest. Related bacterial strains can differ significantly in their genotype and phenotype and features from one bacterial strain or species cannot necessarily be applied to a related one. These strain or family specific differences often represent unique and applicable traits. Since 2002, the complete genomes of 13 probiotic lactic acid bacteria have been published. The presentation will discuss these genomes and highlight discoverys of probiotic traits predicted, or functionally linked to genetic content. We have conducted a comparative genomic analysis of four completely sequenced *Lactobacillus* strains versus 25 lactic acid bacterial genomes present in the public database at the time of analysis. Using Differential Blast Analysis (DBA) each genome is compared to the respective remaining three other *Lactobacillus* and 25 other LAB genomes. DBA highlighted strain-specific genes that were not represented in any other LAB used in this analysis and also identified group-specific genes shared within lactobacilli. *Lactobacillus*-specific genes include mucus binding proteins involved in cell-adhesion and several transport systems for carbohydrates and amino-acids. Comparative genomic analysis has identified gene targets in *Lactobacillus acidophilus* for functional analysis of important traits, including adhesion to mucin and intestinal epithelial cells, acid tolerance, bile tolerance, and quorum sensing. Whole genome transcriptional profiling of *L. acidophilus*, and isogenic mutants thereof, has revealed the impact of varying conditions (pH, bile, carbohydrates) and food matrices on the expression of genes and regulatory elements important to probiotic-linked mechanisms and activities.

Key Words: Lactobacillus acidophilus, johnsonii, plantarum, gasseri, genomics, probiotic cultures

Interactomics in the Human Intestine

Willem M. de Vos
Wageningen and Helsinki University, NL and FI

The microbial world within us includes an astonishing array of intestinal microbial communities, consisting of more than 1000 species and dominated by gram-positive bacteria. The dynamics of this microbial diversity in time and space has attracted considerable attention and we have contributed to this by developing and applying a phylogenetic microarray, termed the Human Intestinal Tract (HIT) Chip, to analyze colonization succession, community stability and diet-related community changes. The results indicate that the human host is colonized by a core group of microbes that shows significant stability in time and are likely to have specific interactions with the human host and other microbes. These may include microbial interactions at the substrate and communication level but also adhesion and other physical interactions between microbial and host cells. While global approaches such as stable isotope probing and functional microbiomics are used to delineate these interactions, in many cases relevant microbes are used, such as specific *Lactobacillus* spp. Notably, lactobacilli that are marketed as probiotics have been developed into relevant model systems as their presence can be modulated by consumption. Moreover, a variety of *Lactobacillus* genomes have been characterized allowing for advanced approaches focusing on the response of both the microbe and the host at the global transcriptional or proteome level. This presentation will highlight salient features of these interactions while also providing perspectives for understanding the molecular details of the intestinal interactome.

Exploitation of *Lactobacillus rhamnosus* GG genome data

Soile Tynkkynen[1], Tuomas Salusjärvi[1], Willem M. de Vos[2], Riitta Korpela[1], Tiina Mattila-Sandholm[1]

[1]Valio Ltd, R&D, Helsinki, Finland
[2]Department of Basic Veterinary Science, University of Helsinki, Finland and Laboratory of Microbiology, Wageningen University, The Netherlands

Lactobacillus rhamnosus GG (ATCC 53103) is the most clinically studied probiotic and widely used in fermented milk products around the world. It increases the colonization resistance of the intestine and balances the gut microbiota. Moreover, it improves natural defence by strengthening the mucosal barrier in the intestine and enhances the immune response.

To improve the understanding of the probiotic mechanisms and function of *L. rhamnosus* GG, its complete genome sequence of more than 3 Mb was determined using a variety of sequencing approaches. The coding capacity of *L. rhamnosus* GG genome was predicted to be much larger than that of other widely used probiotic strains, such as *L. casei*, *L. acidophilus*, or *L. johnsonii*. The full genome annotation of *L. rhamnosus* GG and its comparison to the dozen other reported genomes of lactic acid bacteria, provided a variety of testable hypothesis about its function that are now being addressed in a discovery pipeline. Three areas of interest will be discussed in this panel discussion, including medium and growth optimization, probiotic function discovery, and safety assessment.

The growth medium and conditions used in industrial probiotic processes as well the matrix of the final product contribute to probiotic stability, viability and function. Using genomic microarrays of *L. rhamnosus* GG, specific questions related to production processes and optimization of probiotic functionality are studied at the transcriptome level. These studies are complemented by a proteomics and secretomics approach that is applied to the same experimental design, delivering a complete picture of the global cellular response, including post-translational modifications.

L. rhamnosus GG adheres well to mucus and Caco-2 cells *in vitro* and is known to colonize the human intestine for some time. It is expected, that the secretome and other cell surface structures, including polysaccharides and techoic acids, are involved in these processes.

Several secreted proteins of *L. rhamnosus* GG have recently been implied in epithelial cell signalling. Using a variety of approaches based on the *L.rhamnosus* genome, the interactome is being identified in order to characterize its human and microbial components. As efficient genetic tools for *L. rhamnosus* GG have been developed, further functional studies based on its genomic exploration are feasible.

L. rhamnosus GG has a long history of safe use and is the only probiotic that has been thoroughly monitored in a 10-year post-marketing evaluation demonstrating its safety. Hence, its genome analysis will add the current knowledge base of safety traits and may be useful to support further developments such as that of QPS.

Although post-genomics approaches are complex, costly and, in many cases, still have to deliver industrial benefits, we expect, that the genomic analysis of *L. rhamnosus* GG will provide the basis for future improvements.

. .

..

Cancer gene therapy with anaerobic and non pathogenic bacteria

A. Saggioro
Dept. Digestive Diseased & Hepatology and Clinical Nutrition
SS. Giovanni e Paolo and Umberto I Hospitals – Venice Italy

Upon systemic administration, various types of non-pathogenic obligate anaerobes and facultative anaerobes have been shown to infiltrate and selectively replicate within solid tumors. The tumor specificity is based upon the unique physiology of solid tumors, which is often characterized by regions of hypoxia and necrosis. Prokaryotic vectors can be safely administered and their potential to deliver therapeutic proteins has been demonstrated in a variety of preclinical models. Although the amount of clinical experience with bacterial vectors is limited to date, the available data clearly demonstrated the feasibility of bacterial-mediated therapy in humans. A fundamental obstacle in systemic therapy for cancer patients is the specific targeting of therapy directly to solid tumors. The tumour-Clostridium phenomenon describes the specific affinity of spore forming anaerobes to tumour growth. The discovery of strictly intratumoral tetanus toxic infections in tumour-bearing mice after intravenous spore administration gave the impulse to search for non-toxic clostridial isolates with tumour-selective properties for clostridial biotherapy, i.e. oncolysis, as well as serologic tumour diagnostics without any toxic side-effects. Systematic studies of the oncolytic process and its variables on diverse experimental tumours and laboratory animals revealed that tumour liquefaction, converting necrotic tumour parts to putrid abscesses filled with masses of clostridial forms, stops sharply at the viable rim of the blood-supplied tumour tissue. Similar results were observed in clinical trials, particular of gliomas. A strain of the domestic bacterium *Bifidobacterium longum*, which is non-pathogenic and anaerobic, showed selective localization to and proliferation within solid tumors after systemic application. It is proposed a novel approach to cancer gene therapy in which anaerobic and non-pathogenic bacteria of the genus *B. longum* are used to achieve tumor-specific gene delivery and enzyme-prodrug therapy. It has been demonstrated antitumor efficacy in rat bearing autochthonous mammary tumors injected with the transfected *B. longum* directly or intravenously. This method was confirmed to be effective for enzyme-prodrug therapy not only by intratumoral injection but also by systemic administration. Thus, it may be proposed that anaerobic bacteria of the genus *B. longum* were an attractive and safe tumor-targeting vector and transfected *B. longum* was a potential anticancer agent that could effectively and specifically treat solid tumors. There are several issues however that are still unknown and remain major challenges. In this review they will be discussed the major advantages, challenges and shortcomings of bacterial systems for tumor-specific therapy. In addition, we will highlight the requirements needed to advance the approach into clinical trials.

Immune response modulation played by new concept probiotics

Maria Rescigno and Erika Mileti
Department of Experimental Oncology European Institute of Oncology, Milan, Italy

The role of the intestinal microflora in the development and correct functionality of the immune system is becoming increasingly evident (Macpherson and Harris, 2004). A perturbation of the gastrointestinal microflora or unwanted immune responses to this flora have been demonstrated to play a critical role in the pathogenesis of inflammatory bowel disease (IBD) in experimental animal models (Rath et al., 1996; Strober et al., 2002) but recently also in tumorigenesis (Yang and Pei, 2006). For these reasons it has been proposed to modify the intestinal microflora via the administration of probiotics in IBD patients (Fedorak and Madsen, 2004; Jonkers and Stockbrugger, 2003; Sartor, 2005). The beneficial effect of probiotics can be summarized as a reinforcement of the mucosal barrier against deleterious agents (Fioramonti et al., 2003). Probiotics can increase the resistance of the epithelial barrier, compete for intestine colonization, reduce mucous degradation and modulate the immune response. These characteristics are probiotic- and strain-specific indicating that every probiotic could have a different therapeutic usage.
In our studies we decided to compare the ability of different probiotics to act at several levels at mucosal surfaces: on epithelial cells, on dendritic cells and on both. The rational being that since epithelial cells are the first line of defense towards luminal microorganisms, their function could be modulated by the presence of probiotics. Further, we have recently shown that bacteria can gain access across mucosal surfaces through dendritic cells (DCs). DCs can extend dendrities across epithelial cells and sample bacteria directly from the intestinal lumen (Rescigno et al., 2001). However, even though mucosal DCs are exposed to activating Toll-like receptor ligands, still the inflammatory response is kept at bay. How the mucosal immune system can limit the initiation of inflammatory reactions is unknown. We show that, at steady state, ECs condition anti-inflammatory DCs through the constitutive release of Thymic stromal lymphopoietin (TSLP) (Rimoldi et al., 2005). EC-conditioned DCs even though phenotypically activated by bacteria lose the ability to produce interleukin-12. EC-conditoned DCs release interleukin-6, interleukin-10 but not IL-12 and polarize T cells towards a mucosal non-inflammatory T helper-2 phenotype or T regulatory cells even after a strong Th1 inducer as Salmonella Typhimurium. This control is lost in Crohn's disease (CD) patients.
From our first analysis, we compared *S. typhimurium (*SL1344), *L. Plantarum* (NCIMB882) and *L. Paracasei* (B 21060) for their ability to activate human monocyte-derived dendritic cells (hMo-DCs). We found that whereas all of the bacteria were equally capable to induce the phenotypic activation of hMo-DCs in terms of CD83 uporegulation, they induced three different functional activation states. S. typhimurium induced the release of both IL-12 and IL-10, whereas both Lactobacillus strains induced primarily IL-10 and much less IL-12 both at 6 and 24 h after incubation. This had great consequences in the ability of bacteria-activated DCs to polarized naïve T cells. hMo-DCs were incubated with the different bacterial strains for 1 h in medium without antibiotics. The cells were then extensively washed to eliminate extracellular bacteria and genatmycin was added (100 ug/ml) to kill any remaining extracellular and intracellular bacteria. The cells were then incubated with peripheral blood allogeneic purified CD45RA+CD4+ naïve T cells. 5 Days later

intracellular staining for IL-4 and IFN-g was performed on DC-activated T cells to distinguish between Th2 and Th1 T cell polarization, respectively.
As shown in figure 1, the percentage of IFN-g producing T cells was reduced after activation with DCs incubated with both *Lactobacillus* strains suggesting that the latter have reduced ability to induce Th1-polarizing DCs. In addition, *L. Paracasei* (B 21060) favored the development of non-inflammatory Th2 T cells (compare 8.4% versus 4.5% in *L. paracasei* versus *L. plantarum* activated cells). This suggests that whereas Lactobacillus species have reduced ability to drive Th1 T cells (most likely due to lower levels of IL-12 induction by DCs), *L. Paracasei* (B 21060) is even less inflammatory as it drives the preferential development of non-inflammatory Th2 T cells.
Altogether these results suggest that among different probiotics it is possible to select strains that have non-inflammatory poperties and that could be more rationally employed to treat inflammatory bowel disease. Future studies on the activity of probiotics on intestinal epithelial cells and dendritic cells in co-culture will be instrumental to further select non-inflammatory probiotics.

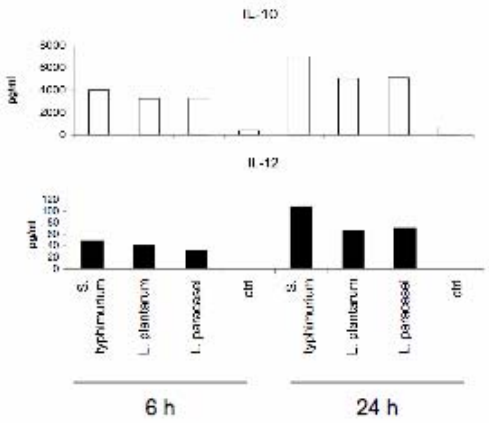
References:

Fedorak, R. N., and Madsen, K. L. (2004). Probiotics and prebiotics in gastrointestinal disorders. Curr Opin Gastroenterol *20*, 146-155.
Fioramonti, J., Theodorou, V., and Bueno, L. (2003). Probiotics: what are they? What are their effects on gut physiology? Best Pract Res Clin Gastroenterol *17*, 711-724.
Jonkers, D., and Stockbrugger, R. (2003). Probiotics and inflammatory bowel disease. J R Soc Med *96*, 167-171.
Macpherson, A. J., and Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol *4*, 478-485.
Rath, H. C., Herfarth, H. H., Ikeda, J. S., Grenther, W. B., Hamm, T. E., Jr., Balish, E., Taurog, J. D., Hammer, R. E., Wilson, K. H., and Sartor, R. B. (1996). Normal luminal bacteria, especially Bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. J Clin Invest *98*, 945-953.
Rescigno, M., Urbano, M., Valzasina, B., Francolini, M., Rotta, G., Bonasio, R., Granucci, F., Kraehenbuhl, J. P., and Ricciardi-Castagnoli, P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol *2*, 361-367.
Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G. M., Nespoli, A., Viale, G., Allavena, P., and Rescigno, M. (2005). Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat Immunol *6*, 507-514.
Sartor, R. B. (2005). Probiotic therapy of intestinal inflammation and infections. Curr Opin Gastroenterol *21*, 44-50.
Strober, W., Fuss, I. J., and Blumberg, R. S. (2002). The immunology of mucosal models of inflammation. Annu Rev Immunol *20*, 495-549.
Yang, L., and Pei, Z. (2006). Bacteria, inflammation, and colon cancer. World J Gastroenterol *12*, 6741-6746.

A) Phenotypic

Treatment	None	*S. typhimurium* (SL1344)	*L. Plantarum* (NCIMB882)	*L. Paracasei* (B 21060)
% CD83+ cells	31,43	60,54	54,84	54,29

B) Functional: cytokines



C) Functional: T cell polarization

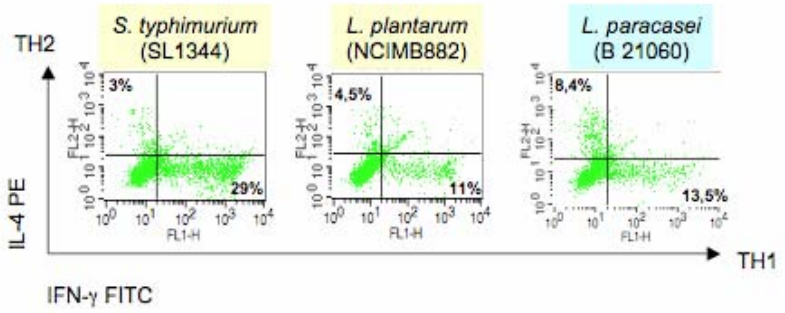


Fig. 1 hMo-DC activation in response to bacteria and T cell polarization.
Human Monocyte-derived DCs were incubated with the different bacterial strains at a ratio of 1:10 (DC:bacteria) for 1 h in medium without antibiotics. Cells were washed and the medium was changed with one containing gentamycin to kill remaining bacteria. The supernatant was collected 6 and 24h later and IL-10 and IL12 were measured by ELISA (B). Cells were detached and analyzed by FACS for CD83 expression (A) or incubated with naïve T cells (C). Intracellular staining for IL-4 and IFN-g was evaluated by FACS after PMA and ionomycin activation.

Lactobacillus paracasei strain B 21060 and Human T Cell Proliferation

Dott. Flavio Caprioli
Department of Internal Medicine – University Tor Vergata of Rome

The intestinal microflora plays several essential roles in the maintenance of the homeostasis and well-being of the human host, from the defence against pathogenic bacteria to the maintenance of the integrity of the intestinal epithelial barrier, to the modulation and instruction of the mucosal immune system. On the other side, intestinal flora may be a target for abnormal immune responses leading to inflammatory reactions and organ damage. In this context, it has been recently suggested that inflammatory bowel diseases (IBD), namely ulcerative colitis (UC) and Crohn's disease (CD) may originate from a dysregulated T-cell mediated immune response against components of the normal microflora, and that modulation of the gut microbiota through probiotics and prebiotics could be beneficial in the treatment of IBD patients; indeed, probiotics have proven effective in the prevention and treatment of intestinal inflammation in IBD patients and in animal models of IBD. The exact mechanism of action of probiotics in modulating intestinal inflammation is currently unknown, even if several theories have been formulated, from the preservation of mucosal integrity, to the production of antimicrobial compounds. A recent line of research has proposed that probiotics can act by modulating immune responses and T lymphocytes functions, both through an antigen presenting cells (APC)-mediated and a direct mechanism; in this context, it is known that T cells express on their surface Toll-like receptors (TLRs), membrane receptors which recognize highly conserved bacterial molecules, raising the possibility that bacteria can act directly on lymphocytes and modulate their functions. Indeed, it has been recently reported that engagement of TLR2 by peptidoglycan (a structural component of the bacterial membrane) enhances T regulatory cells growth. In our work, we aimed to elucidate whether probiotics directly affect T cell activation and growth; specifically, we provided insights on the role of Lactobacillus paracasei on activated peripheral blood and intestinal CD4+ T cell cycling and apoptosis, cytokine production and Foxp3 expression.

Results.
We demonstrate that L.paracasei subsp. paracasei B21060 (10^6/ml), but not L. paracasei subsp. paracasei F19 or L. casei subsp. casei DG, significantly inhibits activated peripheral blood CD4+ T-cell proliferation (Figure 1), while not affecting cellular death (Figure 2). A significantly reduced cell proliferation was observed when intestinal lamina propria CD4+ lymphocytes from both healty subjects and Crohn's disease patients were activated in presence of L.paracasei subsp. paracasei B21060 (not shown).

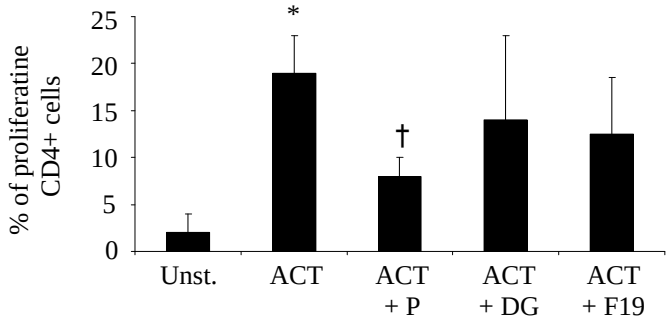


Figure 1. L. paracasei ssp. paracasei B21060 suppresses the growth of activated CD4+ cells. CFSE-labelled CD4+ T cells were either left unstimulated or stimulated with anti-CD3/CD2/CD28 (ACT) in presence or absence of L. paracasei ssp. paracasei B21060 (P), L. paracasei ssp. casei DG (DG), or L. paracasei ssp. paracasei F19 (F19).

Cellular proliferation was assessed by flow-cytometry. Percentage of proliferating CD4+ T cells are presented as the mean ± SD of five separate experiments. *: p<0.001 vs. unst.; †: p<0.01 vs. Act.

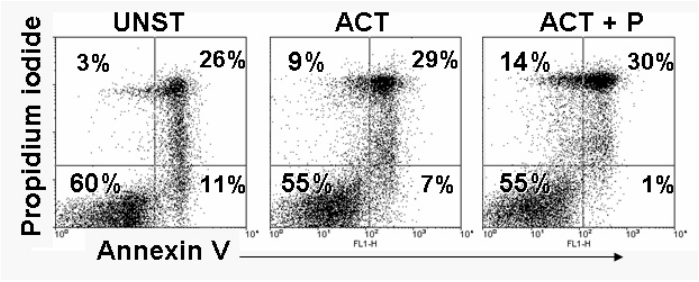


Figure 2. L. paracasei ssp. paracasei B21060 does not affect apoptosis of activated CD4+ T cells. CD4+ cells were either left unstimulated or stimulated with anti-CD3/CD2/CD28 in presence or absence of L. paracasei ssp. paracasei B21060 (ACT and ACT+P, respectively). The percentage of cell death was assessed by FACS analysis of AnnexinV- and/or Propidium Iodide-positive cells. A dot plot representative of four separate experiments is presented in the figure.

Based on these findings, we next assessed the effect of L.paracasei subsp. paracasei B21060 exposure on cytokine secretion by activated CD4+ T cells by means of Enzyme-Linked Immunosorbent Assay. As shown in figure 3A-D, we did not found significant differences in the levels of IFN-γ or IL-10 in the culture supernatants of peripheral blood and gut lamina propria CD4+ lymphocytes extracted from healthy subjects activated in presence or absence of L. paracasei. Additionally, no differences were found between the two groups in the expression of the transcription factor Foxp3 (not shown).

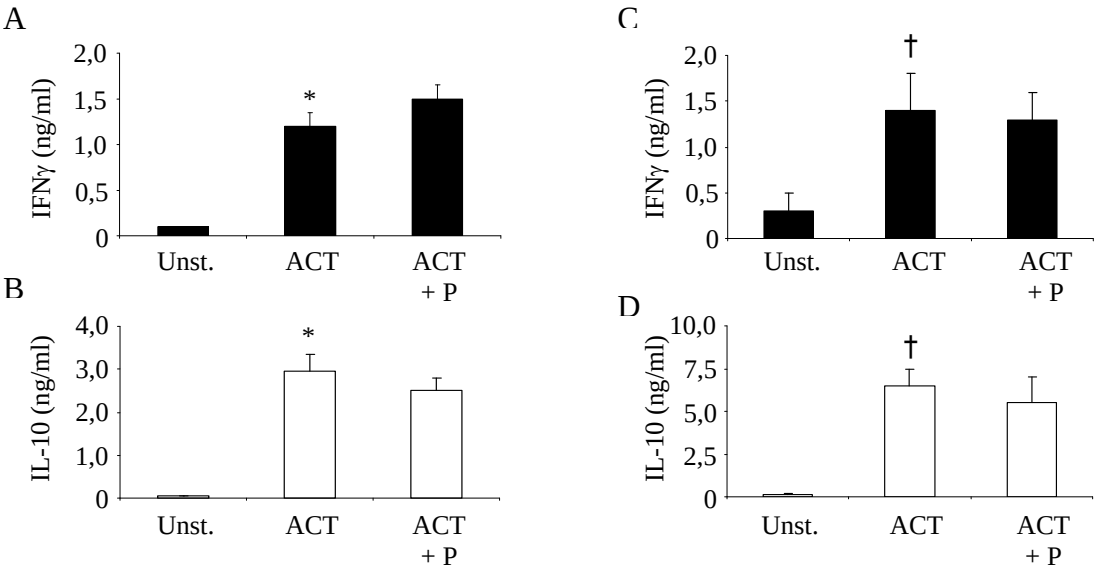


Figure 3. L. paracasei ssp. paracasei B21060 does not affect IFN-γ and IL-10 secretion by peripheral blood and intestinal CD4+ T cells. Peripheral blood (A,B) or healthy subjects lamina propria (C,D) CD4+ T lymphocytes were either left unstimulated or stimulated with anti-CD3/CD2/CD28 for three days in presence or absence of L. paracasei ssp. paracasei B21060 (10^6/ml). After 3 days, the supernatants were collected and analyzed for the IFN-γ (A,C) and

IL-10 (B,D) contents by ELISA. The data are expressed as ng/ml and indicate the means ± SD of five separate experiments. *: $p<0.001$ vs. unst. †: $p<0.05$ vs. unst.

To elucidate the reason of the reduced T lymphocytes proliferation in presence of L.paracasei subsp. paracasei B21060, we focused on MCT-1, a membrane protein of the MCT family, which is involved in the transmembrane transport of lactate, pyruvate, and other monocarboxylates. It has been demonstrated in previous studies that inhibition of MCT-1 in activated T cells blocks the proliferation but not cytokine production, an effect similar to what we observed in CD4+ cells treated with L. paracasei. As shown in Figure 4A, activation of CD4+ cells led to an upregulation of MCT-1 at 24 and 48 hours, an effect inhibited by L. paracasei. This was associated by a significant decrease in intracellular pH (Figure 4B).

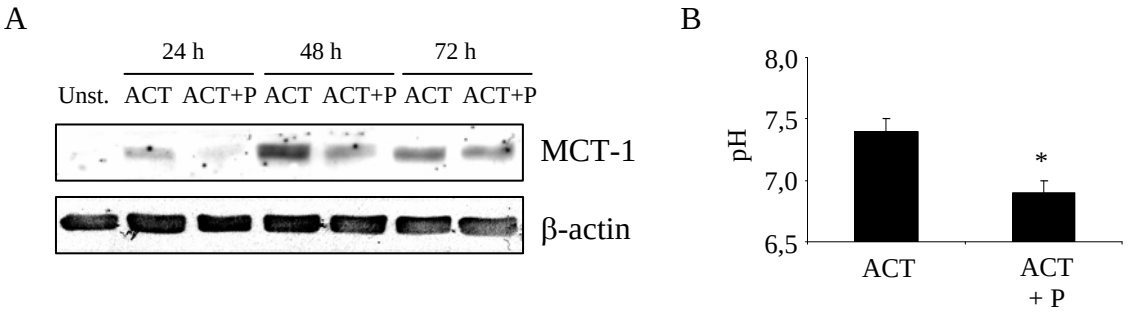


Figure 4. L. paracasei ssp. paracasei B21060 reduces MCT-1 expression in activated CD4+ T cells. A. Representative Western Blot showing MCT-1 expression in CD4+ T cells either left unstimulated (Unst.) or activated with anti-CD3/CD2/CD28 in presence or absence of L paracasei ssp. Paracasei B21060 (10^6/ml) for the indicated time. Data presented are representative of three separate experiments. B. Cells were cultured as indicated for panel A, and the intracellular pH was assessed by flow cytometry. The data indicate mean ± SD of three different experiments. *: $p<0.05$

In conclusion, our study shows that *L. paracasei* subsp. *paracasei* B21060 directly blocks the proliferation of peripheral blood and intestinal T lymphocytes, and that this effect is associated with a downregulation of the transporter MCT-1 on the cellular membrane. However, additional in vivo studies will be necessary to ascertain whether results from these in vitro experiments can be translated to the clinical practice and examine whether *L. paracasei* subsp. *paracasei* B21060 can interfere with T-cell-driven immune responses.

The role of a synbiotic (FLORTEC, Bracco SpA, Italy) on the plasma levels and peripheral blood mononuclear cell expression of cytokines in patients with ulcerative colitis: a pilot study.

Alessandro Federico, Concetta Tuccillo, Enzo Grossi*, Roberto Abbiati*, Nicoletta Garbagna*, Raffaele Salerno, Caterina Mucherino, Angelo Tiso, Marco Romano, Camillo Del Vecchio Blanco, Carmela Loguercio
Gastroenterology Unit, 2nd University of Naples, Naples and *Medical Department, Bracco SpA, Milan, Italy

Introduction

Ulcerative colitis (UC) is a chronic inflammatory and frequently relapsing disease of the gut that ultimately lead to destruction of the intestinal tissue. The pathogenesis of UC likely involves multifactorial interactions among genetic and immunological factors, as well as of environmental triggers (1). A pathologic activation of the mucosal immune system in response to antigens is a key factor in the pathogenesis of UC (2). In patients with UC, the pattern of cytokine expression includes an imbalance between pro-inflammatory and anti-inflammatory reactivity: lymphocytes, monocytes/macrophages and granulocytes are recruited from the blood and they represent the major contributors to tissue perpetuation of inflammation via their production of chemokines and pro-inflammatory cytokines. Over the last decade, abnormal cytokine production have been described in patients with UC (3-5), as plasma and tissue levels: pro-inflammatory cytokines and chemokines are significantly increased and anti-inflammatory cytokines are reduced.

There are no data regarding the effect of Lactobacillus paracasei in patients with ulcerative colitis. Aim of our study was to evaluate the effect of 10 billion CFU/day of a probiotic Lactobacillus paracasei, versus placebo, on serum levels of IL-6, TNFα, IL-8, IL-1β and IL-10 and on mRNA lymphomonocyte expression of TNFα, IL-8, IL-1β in patients with ulcerative colitis.

Patients and Methods

Eighteen volunteer patients (12 males and 6 females, median age 46 years) were enrolled in this randomized study. Patients were affected by a mild-to-moderate UC, confirmed within 6 months by endoscopic evaluation and assessed by Mayo score (6). The diagnosis of UC was made from clinical, endoscopic, and histological data. Patients were already in treatment with 5-ASA products (Mesalazine, median and range: 3200 mg/day, 2400-3200).

Patients were excluded if they had terminated any corticosteroid treatment at least 6 weeks before recruitment, if they had used topical therapies or enemas within the last two weeks, had received antibiotic treatment within the last two weeks, had taken immunosuppressive drugs within the last three months, or had been treated with any investigational drug or device.

According to the study protocol, 9 subjects were treated with a symbiotic preparation containing a prebiotic and a probiotic strain of Lactobacillus paracasei (B21060 strain, deposited at the Collection Nationale de Cultures de Microorganismes, Institut Pasteur, Paris; FLORTEC Bracco S.P.A., Milan, Italy) and 9 were treated with an identical placebo (starch).

The symbiotic preparation was provided in single bags, containing 6 g of lyophilised powder with 5×10^9 CFUs of Lactobacillus paracasei strain B21060. Each subject was instructed to take one bag two times a day (before breakfast and dinner) for 8 consecutive weeks. Bags had to be dissolved in 50 ml of fresh water before oral intake.

At basal level and at 8 weeks serum sample was collected to perform IL-6, TNFα, IL-8, IL-1β and IL-10 determination. Twenty mL of venous blood was collected to perform lymphomonocyte extraction.

Results

Two patients (treated with FLORTEC) dropped out for diarrhea during the first week (event not definitely related to the treatment). Other 16 patients completed the study, the preparation was well-tolerated and very well accepted, no adverse events were observed. In basal condition the patients showed serum alteration of IL-6, TNFα and IL-8; anyone of IL-10 and IL-1β. The treatment with synbiotic, but not with placebo, significantly decreased serum levels of IL-6 and IL-8 (see Table 1).

In lymphocytes, all the cytokines studied were expressed. We found comparable levels of expression of TNFα, IL-1β and IL-8 in placebo and synbiotic group at basal level (Figure 1, 2 and 3). On the other hand, the treatment with synbiotic slightly reduced the expression of TNFα and IL-1β (Figure 1 and 2), while that of IL-8 was significantly lower in respect to that of basal values (Figure 3) ($p<0.01$).

No modifications were assessed in clinical signs during and at the end of treatment: general well-being, rectal bleeding, number of bowel movements/week.

Discussion

Our data show that, in patients with UC treated with mesalazine, there is an imbalance of plasma levels of some proinflammatory cytokines, and that a treatment with FLORTEC is able to restore plasma levels of IL-6 and IL-8. This treatment also significantly affects the expression of IL-8 in lymphocytes from UC patients. These data are in keeping with more than one literature observations.

No studies has been performed with Lactobacillus paracasei in UC.

In our study, a treatment with FLORTEC was able to decrease serum levels of IL-6 and IL-8, but not of TNFα; in addition, FLORTEC slightly affected mRNA lymphomonocyte expression of TNFα and IL-1β, but significantly decreased that of IL-8.

Prebiotic and probiotic therapies are new strategies being used to treat gastrointestinal diseases. These data extend the spectrum of effects of such bacteria on intestinal epithelial function and may justify their use in inflammatory disorders. Recent evidence suggests that the administration of select prebiotics and probiotics, alone or in combination (the latter called "synbiotic" therapy) may improve the clinical outcome of patients with ulcerative colitis: increased length of remission, resolution of symptoms, and improved quality of life following the administration of synbiotic therapy. The literature supports the use of prebiotic, probiotic, and synbiotic therapies in adult ulcerative colitis, but larger and better-designed studies are necessary, including comparative and dose-ranging trials.

References

1. Sandborn WJ, Targan SR. Biologic therapy of inflammatory bowel disease. Gastroenterologt 2002; 122:1592-1608

2. Strober W, Fuss IJ, Blumberg RS. Immunology of mucosal models of inflammation. Ann Rev Immun 2002; 20:495-549

3. Gross V, Andus T, Caesar I, et al. (1992) Evidence for continous stimulation of interleukin-6 production in Crohn's disease. Gastroenterology 102: 514-519

4. Ishiguro Y (1999) Mucosal proinflammatory cytokine production correlates guide endoscopic activity of ulcerative colitis. J Gastroenterol 34: 66-74

5. Reimund JM, Wittersheim C, Dumond S, et al. (1996) Mucosal inflammatory cytokine production by intestinal biopsies in patients with ulcerative colitis and Crohn's disease. J Clin Immunol 16: 144-150.

6. Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. N Engl J Med 1987 Dec 24;317(26):1625-9.

Table 1. Serum levels of IL-6, TNFα, IL-8, IL-1β and IL-10 (M±SD) before and after therapy.

Parameters	Normal values (pg/mL)	Placebo		FLORTEC	
		Basal	8 weeks	Basal	8 weeks
IL-6	3.12-12.5	22.3±3.8	20.8±4.2	21.5±4.1	9.1±2.3*
TNFα	<31.2	58.9±10.4	53.7±8.4	59.7±9.1	54.6±8.4
IL-8	<31.2	149±41	152±37	160±43	50±12**
IL-1β	<10	2.5±1.1	2.7±1.4	2.8±1.2	3.0±1.4
IL-10	5.7-45.7	28.1±8.9	31.4±7.4	32.1±10.3	28.7±9.2

*$p<0.05$ and **$p<0.01$ versus Basal

Figure Legend

Figure 1: mRNA expression of TNFα in lymphocytes from placebo- or synbiotic-treated UC patients. Upper panel shows a representative RT-PCR co-amplification of TNFα/GAPDH transcripts in one placebo-treated patient and in one symbiotic-treated patient. Lower panel shows densitometric scan values of TNFα mRNA levels in 9 placebo-treated and 9 synbiotic-treated UC patients.

Figure 2: mRNA expression of IL-1β in lymphocytes from placebo-or symbiotic-.treated UC patients. Upper panel shows a representative RT-PCR co-amplification of IL-1β/GAPDH transcripts in one placebo-treated patient and in one symbiotic-treated patient. Lower panel shows densitometric scan values of IL-1β mRNA levels in 9 placebo-treated and 9 synbiotic-treated UC patients.

Figure 3: mRNA expression of IL-8 in lymphocytes from placebo- or symbiotic-treated UC patients. Upper panel shows a representative RT-PCR co-amplification of IL-8/GAPDH transcripts in one placebo-treated patient and in one symbiotic-treated patient. Lower panel shows densitometric scan values of IL-8 mRNA levels in 9 placebo-treated and 9 synbiotic-treated UC patients.

Figure 1

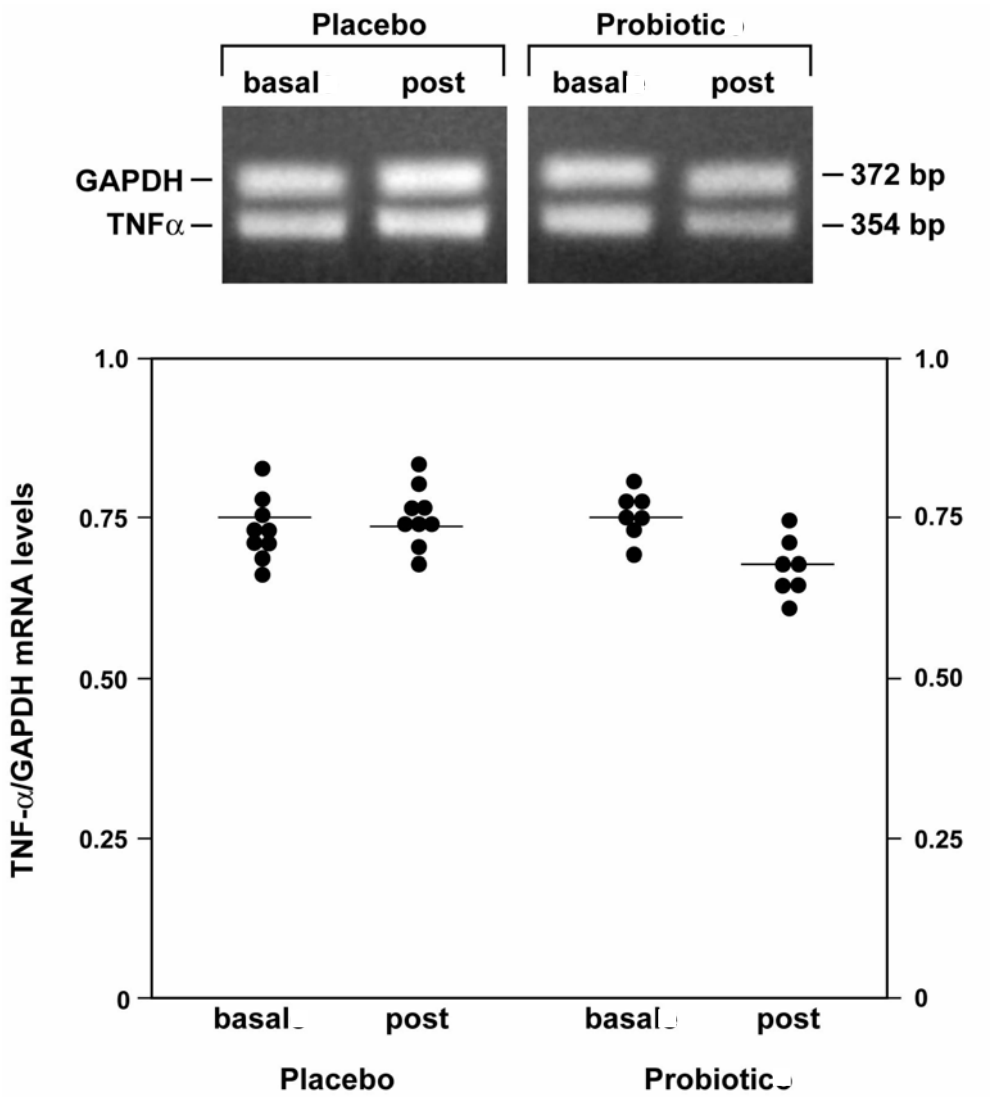

Placebo
Probiotic
basal
post
basal
post
GAPDH
TNFα
372 bp
354 bp
1.0
0.75
0.50
0.25
0
TNF-α/GAPDH mRNA levels
basal
post
basal
post
Placebo
Probiotic

Figure 2

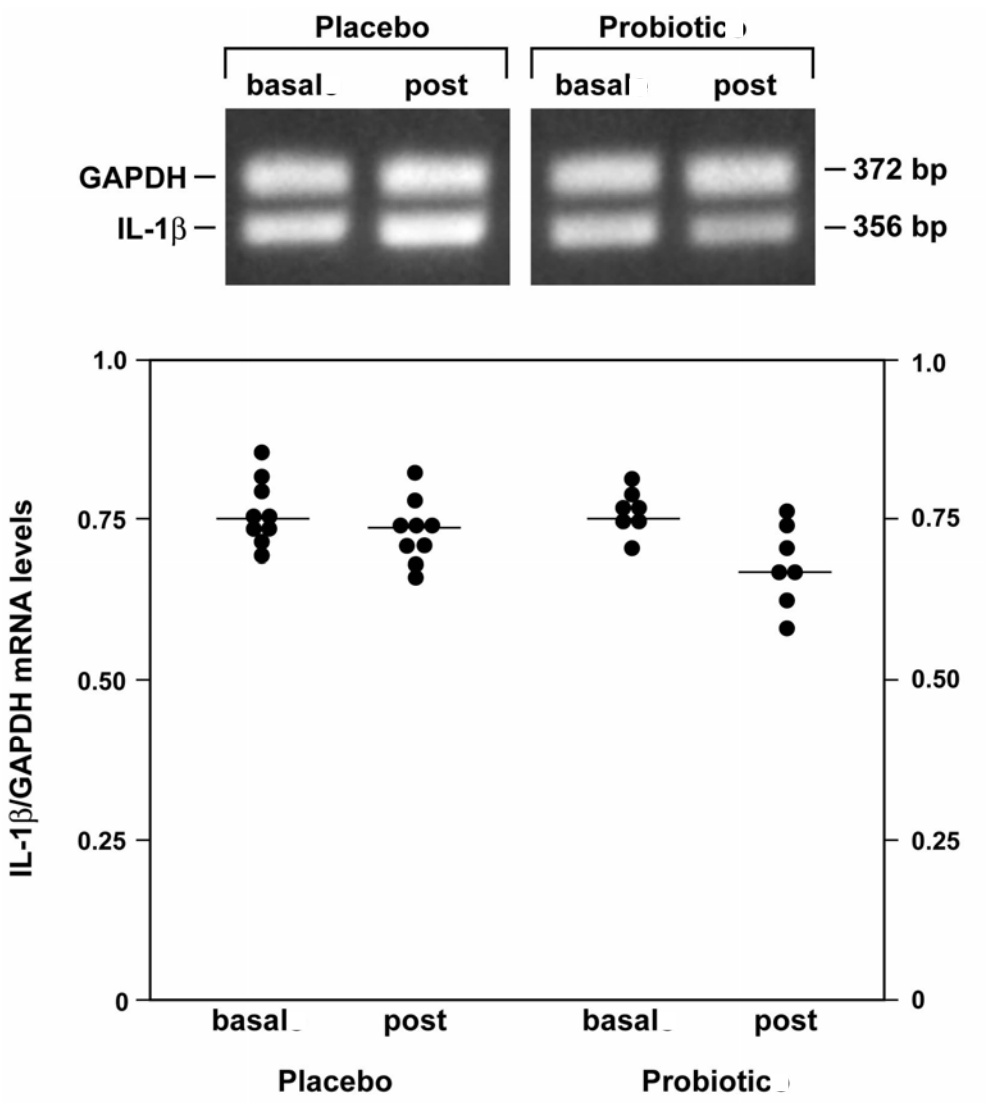
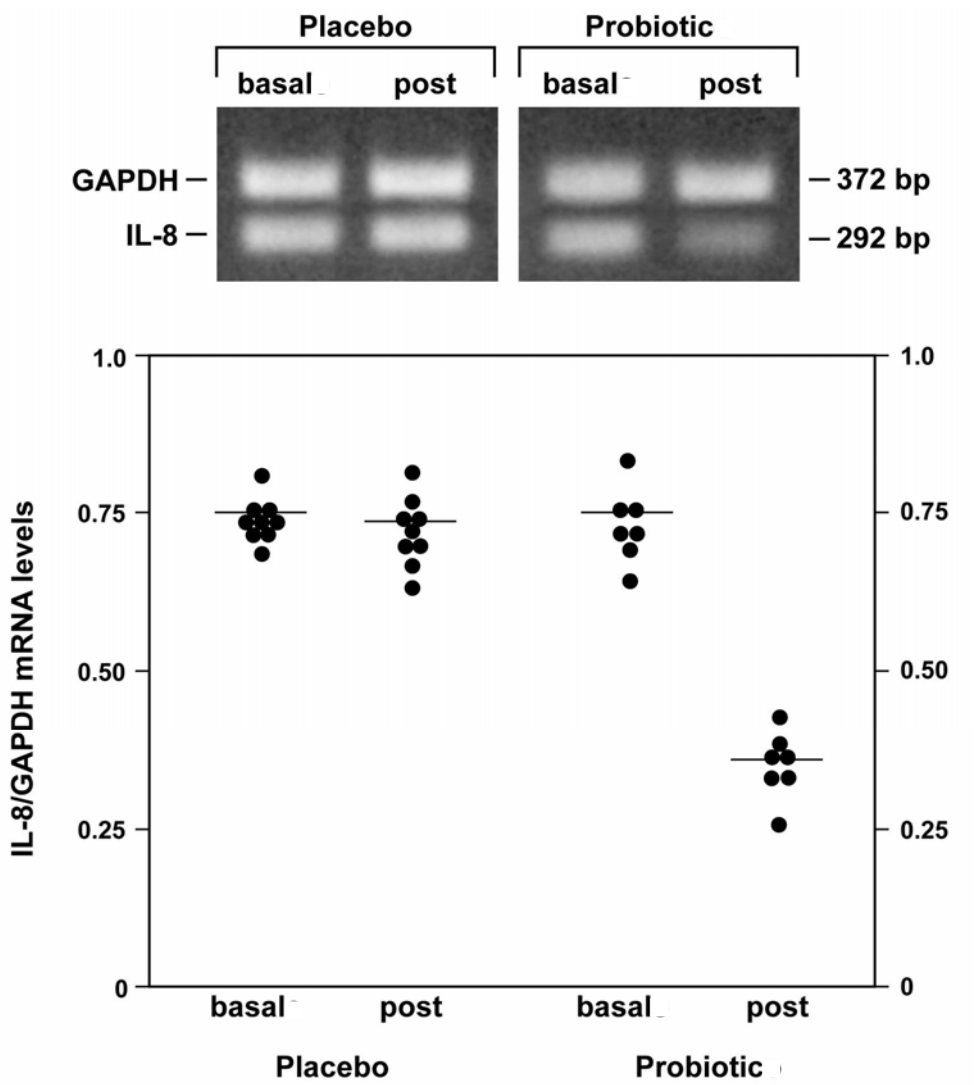

Placebo
Probiotic
basal
post
basal
post
GAPDH
IL-1β
372 bp
356 bp
IL-1β/GAPDH mRNA levels
1.0
0.75
0.50
0.25
0
basal
post
basal
post
Placebo
Probiotic

Figure 3


Placebo
Probiotic
basal
post
basal
post
GAPDH
IL-8
372 bp
292 bp
IL-8/GAPDH mRNA levels
1.0
0.75
0.50
0.25
0
basal
post
basal
post
Placebo
Probiotic

Solute carriers (SLC) in inflammatory bowel disease, a potential target for probiotics?

Mimmi Kotka[1], Agne Lieden[1], Sven Pettersson[1], Vito Trinchieri[2], Luciana Mosca[3] and Mauro D'Amato[1,4]

[1] Dept Microbiology Tumor and Cell Biology and [4] Dept Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden; [2] Dept Infectious Diseases and [3] Dept Biochemical Sciences, "Sapienza" University, Rome, Italy.

Transporter proteins of the solute carriers (SLCs) family have been shown to play a role in the control of intestinal permeability and gut barrier function, and polymorphisms in SLC genes are associated to the susceptibility to inflammatory bowel disease (IBD). Moreover, many SLCs are both targets and mediators of the efficacy of pharmaceutical compounds, and the modulation of such transport systems to increase drug bioavailability is therefore of great interest.

We have undertaken a large-scale project to evaluate whether bacteria can modultate the expression of SLCs in the intestine. In particular, here we report the effect of probiotics on the expression of SLC families 4, 21 and 22, (OAT and OCT transporters, 38 genes in total), which affect the bioavailability of several pharmaceutical compounds. The underlying idea is that a probiotic effect on these transporters would strengthen the potential for combinatorial therapies.

Experiments were carried out in conventional SPF (specific pathogen free) mice, and two groups of treated (2 mg of VSL#3 in 100 ul of PBS daily per os) and untreated (100 ul of PBS only) animals were studied for SLC expression in the intestine by Real-Time PCR at 1 and 20 days post treatment. An additional group of mice which received VSL#3 for 20 days was also analyzed 7 days after the interruption of the treatment.

Probiotics appeared to exert some effect on the expression of the studied genes, with 10% (4/38) of the solute carriers being either up- or down-regulated by VSL#3 administration. However, this effect reached statistical significance (p 0.01) only for the SLC4A11, which showed a 5 times lower expression in VSL#3 than in control mice 1 day post treatment. SLC4A11 (also known as BTR1) is a poorly characterized sodium-borate cotransporter, whose mutations cause the recessive disease corneal hereditary endothelial dystrophy (CHED2).

While VSL#3-driven changes in the expression levels of HCO_3^-/CO_3^- transporters (family SLC4) might impact on gut fluid accumulation and thus have anti-diarrhea effects, the elucidation of SLC11A4 function in the intestine will be key to fully evaluate the relevance of specific findings.

Understanding Why Probiotic Therapies Can Be Effective In Treating IBD

Richard N. Fedorak MD. FRCPC
Director, Centre of Excellence for Gastrointestinal Inflammation and Immunity Research (CEGIIR)
Director, Northern Alberta Clinical Trials and Research Centre (NACTRC)

Zeidler Family Gastrointestinal Health and Research Centre Zeidler Ledcor Building

BACKGROUND

The demonstration that immune and epithelial cells can discriminate between different microbial species has extended the known mechanism(s) of action of probiotics beyond simple barrier and antimicrobial effects. It has also confirmed that probiotic bacteria modulate mucosal and systemic immune activity, and epithelial function. The progressive unravelling of these mechanisms of action has led to new credence for the use of probitotics and prebiotics in clinical medicine. Level I evidence now exists for the therapeutic use of probitics in infectious diarrhea in children, recurrent *Clostridium difficile*-induced infections and postoperative pouchitis. Level II evidence is emerging for the use of probiotics in other gastrointestinal infections, prevention of postoperative bacterial translocation, irritable bowel syndrome, and in both ulcerative colitis and Crohn's disease. Nevertheless, one consistent feature has emerged: not all probiotic bacteria have similar therapeutic effects. Future clinical trials will need to incorporate this fact into trial planning and design.

The use of probiotics and prebiotics as therapeutic agents for gastrointestinal disorders is rapidly moving into "mainstream". Mechanisms of action explain the therapeutic effects and randomized, controlled trials provide the necessary evidence for their incorporation into the therapeutic armamentarium.

The mammalian intestinal tract contains a complex and diverse society of both pathogenic and non-pathogenic bacteria. The majority of research to date has focused on the mechanism by which pathogenic bacteria achieve their detrimental effects. However, more recent research has unveiled a glimpse into the mechanisms of action and potential therapeutic role of indigenous non-pathogenic microorganisms (probiotics). This evolution has been facilitated by our ever-increasing understanding of the mechanism of action of these agents, and by the development of molecular methods for analyzing and identifying complex bacterial communities within the mammalian intestine. A series of review articles have been published outlining the efficacy of probiotics in human health (1-13).

MECHANISMS OF ACTION OF PROBIOTIC BACTERIA

Previous research into the protective mechanisms associated with probiotic bacteria focused on the bacteriology of the gut, and concentrated on intestinal colonization and probiotic-induced suppression of pathogen growth and/or invasion (14). Indeed, the concept of a microbiological balance existing in the intestine, involving competition between probiotic and pathogenic bacteria for specific binding sites on intestinal epithelial cells, has been well established in the literature. However, recent research has turned towards understanding the

role of probiotic bacteria, and their secreted products in enhancing and modulating innate and adaptive immune responses in the host by other mechanisms. With the demonstration that immune and epithelial cells can discriminate between different microbial species through activation of toll-like receptors (15), the idea that probiotics may exert some of their protective functions through modulation of immune activity and epithelial function in both the large and small intestine has arisen.

Effects on Barrier Function

Two papers have demonstrated that certain strains of probiotic bacteria can enhance barrier function through distinctly different mechanisms. Resta-Lenert and Barrett (16) showed that live *Streptococcus thermophilus* and *Lactobacillus acidophilus* could inhibit the adhesion and invasion of enteroinvasive *Escherichia coli* (*E. coli*) into human intestinal cell lines. Epithelial cells exposed to these probiotic bacteria demonstrated enhanced phosphorylation of actinin and occludin in the tight junction region (16). A second novel mechanism of maintaining barrier function was identified by Yan and Polk (17). These authors showed that *Lactobacillus rhamnosus* GG was able to prevent cytokine-induced apoptosis in intestinal epithelial cell models through the inhibition of a tumor necrosis factor (TNF)-induced activation of the pro-apoptotic p38/mitogen-activated protein kinase.

Effects on Immune Function

It has been well documented that probiotic bacteria can interact with epithelial and immune cells, and alter signal transduction pathways in the presence or absence of pathogenic bacteria and cytokines. Epithelial cells respond to whole bacteria and bacterial components in a differential manner, releasing interleukin (IL)-8 in response to pathogenic bacteria such as *E. coli*, but not to probiotic strains (18). Bacterial deoxyriboneucleic acid (DNA) is also recognized in a differential manner by epithelial cells; with pathogenic strains evoking a phosphorylation of the extracellular signal-regulated kinase (ERK) pathway and activation of activator protein (AP)-1 (19), and probiotic strains modulating the nuclear factor kappa B (NF-κB) pathway in response to TNF-alpha (α) (20). However, bacterial DNA has been shown to be both beneficial (21) and detrimental (22) in the treatment of murine colitis. An interesting paper by Ibnou-Zekri et al (23) highlights the idea that activity of probiotic strains *in vitro* may not translate into similar *in vivo* behaviour. Two strains of Lactobacillus which exhibited very similar *in vitro* properties demonstrated distinct differences in colonization patterns and resultant host immune response at both the mucosal and systemic levels (23).

Effects on Systemic Immunity

Oral administration of probiotics has been shown to result in altered immunity at distant mucosal sites, including the female genital tract, the respiratory tract, the skin and the nasal passages. Specific strains of Bifidobacteria and Lactobacillus appear to be promising in the treatment and/or prevention of eczema/dermatitis in infants (24, 25) and children (26). It is interesting to note that in these studies, supplementation with the probiotic did not appear to alter bacterial numbers in the colon. This suggests that these results are due to altered immunity rather than altered colonization.

Probiotic Colonization

Whether or not colonization is critical for probiotics to have their effect remains unresolved. Agarwal et al. (27) studied the ability of *Lactobacillus* GG to colonize the neonatal gut.

Colonization with *Lactobacillus* GG occurred in 21% of infants who weighed less than 1500 g versus 47% of larger infants. Colonization was limited to infants who were not on antibiotics within 7 days of treatment of *Lactobacillus* GG. Thus, the neonatal response to probiotic preparations is dependent upon gestational and postnatal age, and prior antibiotic exposure.

CONCLUSION

The microflora of the gut is a tremendously complex organ and we are only beginning to appreciate the myriad effects that these bacteria have on intestinal function and homeostasis. Progress is being made in identifying molecular interactions between microbes and epithelia, and it is clear that the majority of interchanges are beneficial both to the host, and the microbial population. Deciphering the complex relationship between microbes and epithelia will assist in understanding the etiology of inflammatory bowel disease, and developing therapeutic agents for its prevention or treatment.

REFERENCES

1. Tuohy KM, Probert HM, Smejkal CW, et al. Using probiotics and prebiotics to improve gut health. Drug Discov Today 2003;8(15):692-700.
2. Bengmark S. Use of some pre-, pro- and synbiotics in critically ill patients. Best Pract Res Clin Gastroenterol 2003;17(5):833-48.
3. Steidler L. Genetically engineered probiotics. Best Pract Res Clin Gastroenterol 2003;17(5):861-76.
4. Cummings JH, Macfarlane GT. Gastrointestinal effects of prebiotics. Br J Nutr 2002;87 Suppl 2:S145
5. Kolida S, Tuohy K, Gibson GR. Prebiotic effects of inulin and oligofructose. Br J Nutr 2002;87 Suppl 2:S193-7.
6. Schley PD, Field CJ. The immune-enhancing effects of dietary fibres and prebiotics. Br J Nutr 2002;87 Suppl 2:S221-30.
7. Marteau P, Seksik P, Jian R. Probiotics and intestinal health effects: a clinical perspective. Br J Nutr 2002;88 Suppl 1:S51-7.
8. Saavedra JM, Tschernia A. Human studies with probiotics and prebiotics: clinical implications. Br J Nutr 2002;87 Suppl 2:S241-6.
9. Marteau P, Boutron-Ruault MC. Nutritional advantages of probiotics and prebiotics. Br J Nutr 2002;87 Suppl 2:S153-7.
10. McCartney AL. Application of molecular biological methods for studying probiotics and the gut flora. Br J Nutr 2002;88 Suppl 1:S29-37.
11. Fooks LJ, Gibson GR. Probiotics as modulators of the gut flora. Br J Nutr 2002;88 Suppl 1:S39-49.
12. Teitelbaum JE, Walker WA. Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. Annu Rev Nutr 2002;22:107-38.
13. Marteau PR. Probiotics in clinical conditions. Clin Rev Allergy Immunol 2002;22(3):255-73.
14. Clancy R. Immunobiotics and the probiotic evolution. FEMS Immunol Med Microbiol 2003;38(1):9.
15. Kadowaki N, Ho S, Antonenko S, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med 2001;194(6):863-9.
16. Resta-Lenert S, Barrett KE. Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC). Gut 2003;52(7):988-97.
17. Yan F, Polk DB. Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem 2002;277(52):50959-65.
18. Lammers KM, Helwig U, Swennen E, et al. Effect of probiotic strains on interleukin 8 production by HT29/19A cells. Am J Gastroenterol 2002;97(5):1182-6.

19. Akhtar M, Watson JL, Nazli A, et al. Bacterial DNA evokes epithelial IL-8 production by a MAPK-dependent, NF-kappaB-independent pathway. Faseb J 2003;17(10):1319-21.
20. Madsen K, Jijon H, Yeung H, et al. DNA from Probiotic Bacteria Exerts Anti-Inflammatory Actions on Intestinal Epithelial Cells by Inhibition of NF-kB. Gastroenterology 2002;122:A546.
21. Rachmilewitz D, Karmeli F, Takabayashi K, et al. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. Gastroenterology 2002;122(5):1428-41.
22. Obermeier F, Dunger N, Deml L, et al. CpG motifs of bacterial DNA exacerbate colitis of dextran sulfate sodium-treated mice. European Journal of Immunology 2002;32(7):2084-92.
23. Ibnou-Zekri N, Blum S, Schiffrin EJ, et al. Divergent patterns of colonization and immune response elicited from two intestinal Lactobacillus strains that display similar properties in vitro. Infect Immun 2003;71(1):428-36.
24. Kalliomaki M, Salminen S, Poussa T, et al. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. Lancet 2003;361(9372):1869-71.
25. Kirjavainen PV, Salminen SJ, Isolauri E. Probiotic bacteria in the management of atopic disease: underscoring the importance of viability. J Pediatr Gastroenterol Nutr 2003;36(2):223-7.
26. Rosenfeldt V, Benfeldt E, Nielsen SD, et al. Effect of probiotic Lactobacillus strains in children with atopic dermatitis. J Allergy Clin Immunol 2003;111(2):389-95.
27. Agarwal R, Sharma N, Chaudhry R, et al. Effects of oral Lactobacillus GG on enteric microflora in low-birth-weight neonates. J Pediatr Gastroenterol Nutr 2003;36(3):397-402.

Saccharomyces boulardii and IBD

Mario Guslandi

Gastroenterology Unit, S.Raffaele University Hospital

INTRODUCTION

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

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

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

Quite recently it has shown that S. boulardii has the unique ability to trap T-cells in mesenteric lymph nodes, thus limiting infiltration of inflamed colon and production of pro-inflammatory cytokines (8)

Clinical studies with *Saccharomyces boulardii* in both children and adults have shown that the probiotic is effective in the prevention and/or treatment of various types of diarrhea (antibiotic-associated, enteric feeding-induced, traveler's diarrhea) (2). In addition, due to its protective effect against the specific enterotoxins , *Saccharomyces boulardii* significantly reduces the number of relapses of *Clostridium difficile* infection.(9)

In view of the possible pathogenetic role of bacteria in inflammatory bowel disease (IBD) ,the rationale for employing a probiotic in this condition is to restore the unbalanced indigenous microflora, to inhibit the adverse effects of enteric pathogens and to counteract the inflammatory process by enhancing degradation of enteral antigens and reducing secretion of inflammatory mediators (10). In this respect *Saccharomyces boulardii* is, on theorethical grounds, an excellent candidate for a potential therapeutic role in IBD. We tested the efficacy of that probiotic agent in patients with active and inactive IBD (11,12)

PATIENTS AND METHODS

In the first study (11) 32 patients of both sexes, aged 23-49, with established diagnosis of Crohn's disease of ileum and/or colon in phase of remission (CDAI <150) for at least three months were randomly allocated to a six-month maintenance therapy either with mesalazine alone (500mg of sustained-release microgranules t.i.d.) or with the same mesalazine preparation 500mg b.i.d. plus *Saccharomyces boulardii* 500 mg o.d.. A clinical relapse was defined as CDAI>150 with an increase of 100 points over the baseline values for more than two weeks.

In the second, open-label study (12) 25 patients, aged 19-47, with a clinical flare-up of left-sided ulcerative colitis of mild to moderate degree, while on maintenance with mesalazine since at least three months, and with a history of poor tolerance to corticosteroids, were enrolled. *Saccharomyces boulardii* 250 mg t.i.d. was added for four weeks to the ongoing mesalazine treatment. Clinical evaluation was performed before and after by means of Rachmilewitz 's Activity Index ,calculated on the basis of stool frequency, blood in the stools, general conditions, fever, abdominal pain, sedimentation rate and hemoglobin values. Only patients with a basal score of 9 of higher were

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

RESULTS

In the first study, at the end of the six-month maintenance period, clinical relapses was observed in 37.5% of patients receiving mesalazine alone and in only 6.25% of subjects in the group treated with mesalazine plus *Saccharomyces boulardii* ($p = 0.04$ by Fisher's exact test).
In the second , pilot trial, the theraputic success - endoscopically confirmed - was achieved in 17 patients (68% of cases on an intention-to-treat basis)

DISCUSSION

The rationale for employing probiotics in IBD treatment relies upon the postulated pathogenetic role of intestinal microflora. The mechanisms of action of *Saccharomyces boulardii*, a probiotic with a different taxonomic profile, being a yeast and not a bacterium, may explain the beneficial effects observed in the course of our studies. The recently recognized trapping effect of T cells in mesenteric lymph nodes adds a further rationale for a possible, effective use of *S. boulardii* in the treatment of inflammatory bowel disease.
Our clinical findings are in keeping with the mechanism of action of that probiotic. When combined with mesalazine the it appears to significantly reduce relapses during maintenance treatment of inactive Crohn's disease. Whether *Saccharomyces boulardii* can be effective in this setting also if employed alone is still to be determined. As for its possible therapeutic role in selected cases of mild-to-moderate ulcerative colitis, our preliminary data are encouraging, and further,controlled studies are warranted.

REFERENCES

1. McFarland LV, Bernasconi P. *Saccharomyces boulardii*: a review of an innnovative biotherapeutic agent. Microb Ecol Health Dis 1993; 6:157-71
2. Vandenplas Y. Bacteria and yests in the treatment of acute and chronic infectious diarrhea. Part II: yeasts. Clin Microbiol Infect 1999; 5: 389-95
3. Castagliuolo I, Lamont JT, Nikulasson ST, Pothoulakis C. *Saccharomyces boulardii* protease inhibits *Clostridium difficile* Toxin A effects in the rat ileum. Infect Immun 1996; 64: 5225-32.
4. 4. Buts JP, De Keyser N, De Raedemaeker L. *Saccharomyces boulardii* enhances rat intestinal enzyme expression by endoluminal release of polyamines. Pediatr Res 1994; 36: 522-27
5. Zaouche A, Loukil C, De Lagausie P, Peuchmaur M, Nacry J, Fitoussi F, Bernasconi P, Bingen E, Cezard JP. Effects of oral *Saccharomyces boulardii* on bacterial overgrowth, translocation and intestinal adaptation after small-bowel resection in rats. Scand J Gastroenterol 2000; 35: 160-5
6. Buts JP, Bernasconi P, Vaerman JP, Dive C. Stimulation of secretory IgA and secretory components of immunoglobulins in small intestine of rats treated with *Saccharomyces boulardii*. Dig Dis Sci 1990; 35: 251-5
7. Girard P, Pansart Y, Lorette I, Gillardin JM. Dose-response relationship and mechanism of action of *Saccharomyces boulardii* in castor oil-induced diarrhea in rats. Dig Dis Sci 2003; 48: 770-4
8. Dalmasso G, Cottrez F, Imbert V et al. *Saccharomyces boulardii* inhibits inflammatory bowel disease by trapping T cells in mesenteric lymph nodes. Gastroenterology 2006; 131: 1812-25
9. McFarland LV, Surawicz CM, Greenberg RN. A randomized,placebo-controlled trial of *Saccharomyces boulardii* in combination with standard antibiotics for *Clostridium difficile* disease. JAMA 1994; 271: 1913-8
10. Guslandi M. Probiotics for chronic intestinal disorders. Am J Gastroenterol 2003; 98:520-1

11. Guslandi M; Mezzi G, Testoni PA. *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. Dig Dis Sci 2000; 45: 1462-4

12. Guslandi M, Giollo P, Testoni PA. A pilot trial of *Saccharomyces boulardii* in ulcerative colitis. Eur J Gastroenterol Hepatol 2003; 15: 697- 8

Indications and challenges of probiotics in the management of patients with arthralgias and spondylarthropathies in Crohn's disease and ulcerative colitis

O. Karimi MSc and Prof. A. S. Peña MD, FRCP, AGAF

Department of Pathology and Gastroenterology, Immunogenetics, VU University Medical Centre, Amsterdam, The Netherlands

Summary

Arthralgia and spondyloarthropathy (SpA) of the peripheral and the axial joints are common in patients with inflammatory bowel diseases (IBD). Evidence for this association has been provided by clinical, epidemiological and immunological studies. They have shown the presence of shared inflammatory pathways in gut and joint. Bacterial gut infections such as *Salmonella typhimurium, Yersinia enterocolitica, Shigella, Campylobacter jejuni* may induce reactive peripheral arthritis and 20% of these patients may develop chronic spondylo-arthropathy.

It is not certain that arthralgias in IBD are more frequent than in the general population but clinical articular manifestations compatible with SpA are present in 10-40% of patients with IBD. These enteropathic peripheral arthropathies without axial involvement are subdivided into a pauciarticular of large joints and a bilateral symmetrical polyarthropathy.

The rationale and the challenges of using pre- pro- and synbiotics in the management of patients with IBD with arthralgias and SpA is briefly reviewed. The rationale is based on the modulation of the ubiquitous intestinal flora by bacteria and its products that have been proven to be safe. The challenge is to find the "window of opportunity" to treat the evolutionary stage of joint inflammation. It seems to us that the major aim is not to treat patients who have a self-limited inflammatory joint disorder but those patients with persistent arthralgia in an early phase of the disease. Seronegative and seropositive patients with early arthritis, before damage may occur could be managed by this approach to improve the quality of life and to positively influence the natural course of the disease.

Introduction

There is increasing genetic and immunological evidence supporting a clinical and histological overlap between gut inflammation in SpA and CD. Subclinical gut inflammation was described in several SpA patients [1]. Gastrointestinal infections associated with SpA, usually involve the terminal ileum and sometimes also the colon, in most cases without joint symptoms [2].

Seronegative spondyloarthritides, a subgroup of SpA, is characterized by the consistent absence of the rheumatoid factor, involvement of the sacro-iliac joints and by involvement of peripheral inflammatory arthritis. This subgroup of SpA is also known by the name of non-erosive and non-deforming arthropathies [3]. This group is clinically distinguishable from the group of patients with seropositive rheumatoid arthritis. Clinical articular manifestations compatible with SpA are present in up to 39% of patients with IBD [4]. The prevalence of SpA was shown to be higher in Crohn's disease of the colon (CD) than in ulcerative colitis (UC) [5]. Immunopathological studies have revealed an increased E-cadherin/catenin complex expression in clinical overt IBD and in subclinically inflamed bowel mucosa from SpA patients [6]. SpA is associated with the histocompatibility antigen HLA-B27. This marker and other genetic and environmental factors explain the often observed familial aggregation of SpA and IBD [7]. Involvement of the gastrointestinal tract is a feature of SpA [8], and found in 25-75% of SpA patients [9]. Enteropathic peripheral arthropathy without axial involvement has

been subdivided into a pauciarticular large joint arthropathy and a bilateral symmetrical polyarthropathy. Both sub-groups can be distinguished by the different distribution of the joint involvement and the natural history of the disease. Patients with recorded joint swelling or effusion were classified as type 1 (pauciarticular) when less than five joints were involved and classified as type 2 (polyarticular) when five or more joints were swollen. Patients with joint pain but without evidence of swelling in the joints were classified as suffering from arthralgia [10]. Different HLA associations may define phenotypical distinct sub-groups. In IBD and SpA, there is a polygenic predisposition and a high prevalence of increased intestinal permeability [11].

Pre- pro- and synbiotics in patients with IBD and arthralgia and SPA

Recently, focus has been placed on probiotic, prebiotic and synbiotic therapies, which aim to restore balance to the gastrointestinal microbiota, and reduce intestinal inflammation. However, a greater understanding of the mechanisms behind their action on the gastrointestinal microbiota is required in order to determine which prebiotic, probiotic or synbiotic is the most beneficial [12]. Probiotics have been assessed extensively in animal models, with a number of clinical trials also demonstrating potential therapeutic benefits. *Lactobacillus* GG has been shown to be effective in the prevention and treatment of T-cell dependent experimental arthritis in two animal models [13].
Prebiotics have been studied less extensively, however, they may become an ideal treatment or co-treatment option due to their capacity to increase endogenous *lactobacillus* and *bifidobacteria*. Prebiotics are dietary substances, mostly non-digested carbohydrates, which promote the growth and metabolic activity of beneficial enteric bacteria. These agents attenuate experimental colitis but have not been well studied in CD. The chronic intestinal and extraintestinal inflammation associated with CD is due to an overly aggressive T cell response to discrete antigens from a subset of luminal bacteria most likely of commensal origin. This susceptibility is determined by genes that encode immune responses, barrier function or bacterial clearance. The onset of inflammation or reactivation of quiescent disease is triggered by environmental stimuli. In this complicated theory, inflammation is dependent on the interaction between genetic, microbial, environmental, and immunologic factors [14].
We have undertaken a proof of concept study for the safety and efficacy of probiotics in patients with quiescent IBD who suffered from arthralgia for more than two weeks. The results suggest that VSL#3 may be an alternative treatment for arthralgia in patients with IBD [15, 16].
Combined probiotics and prebiotics (synbiotics) can restore a predominance of beneficial *Lactobacillus* and *Bifidobacterium* species however as far as we know no studies have been performed in this group of patients.

Mechanism of action of pre- pro- and synbiotics in arthralgia and SpA in IBD

Probiotics benefit the host by improving its intestinal microbial balance and probably complement the normal nutrition [17]. Although the full mechanisms of action are still unclear, proven beneficial activities are: 1) inhibition of pathogenic bacterial growth or epithelial attachment preventing invasion, 2) improvement of the epithelial barrier function, 3) promoting homeostatic immunoregulation by the induction of IL-10, TGF-β and by the inhibition of TNFα and IL-12 synthesis [14] and 4) inhibition of the p38 mitogen-activated protein kinase (MAPK) pathway [18]. The “p38 MAPK” is a mediator of endotoxin-induced production of COX2 in enterocytes [19].

In acute and chronic active bowel inflammation of SpA patients E-cadherin mRNA is upregulated [20]. Since E-cadherin is also a ligand for the αEβ7 integrin on intra-epithelial T-cells it may play a role in the homing of specific T-lymphocyte populations to the gut mucosa. In fact, phenotypic characterization of IL-2 expanded T-cell lines (CD3 and CD8) from mucosal biopsies show upregulated αEβ7 expression in CD [21]. Other immune alterations found in the synovium and colonic mucosa of SpA patients are an increased number of the macrophage scavenger receptor CD163 [22]. These findings demonstrate that the innate immune system rather than the acquired immunity may be involved in the inflammatory process of early arthritis. Prebiotics have several proposed mechanisms of action, including the enhancement of luminal concentrations of endogenous *Lactobacilli* spp. and *Bifidobacterium* spp. Pre-, pro- and synbiotics stimulate the production of short chain fatty acids, particularly butyrate enhancing the water-holding capacity of the stool [14].

The future

Future research in this field needs to focus on determining which probiotics are the most efficacious in patients with arthritis, SpA and IBD. How the genetic and bacterial profiles of the patient will influence treatment responsiveness. In this direction an interesting study has been published. A glass-based microarray, based on the identification of 2625 expressed sequence tags that are differentially expressed in the colon of patients with CD or SpA was used to analyze colon biopsy specimens from 15 patients with SpA, 11 patients with CD and 10 controls. The genes expressed from patients with SpA with subclinical chronic gut inflammation, cluster together and are more related to those with CD than the controls. This result suggests that some of these genes can act as an early genetic marker for evolution to CD in SpA patients [23].

Conclusion

Based on the results of experimental animal models, the concepts described in this review and our preliminary clinical observations, we believe that the use of probiotics may be effective in the management of patients with IBD suffering from arthralgia and/or SpA. Controlled randomized clinical trials to investigate the as yet unresolved issues with regard to efficacy, dose and duration of use and/or single or multi-strain formulation are necessary to prove this beneficial effect. A major challenge at present is to find markers to be able to identify those patients in the early phase of the disease.

References

1. Schatteman L, Mielants H, Veys EM, Cuvelier C, De Vos M, Gyselbrecht L, Elewaut D, Goemaere S. Gut inflammation in psoriatic arthritis: a prospective ileocolonoscopic study. J Rheumatol 1995;22:680-3.
2. Smale S, Natt RS, Orchard TR, Russell AS, Bjarnason I. Inflammatory bowel disease and spondylarthropathy. Arthritis Rheum 2001;44:2728-36.
3. Gravallese EM, Kantrowitz FG. Arthritic manifestations of inflammatory bowel disease. Am J Gastroenterol 1988;83:703-9.
4. de Vlam K, Mielants H, Cuvelier C, De Keyser F, Veys EM, De Vos M. Spondyloarthropathy is underestimated in inflammatory bowel disease: prevalence and HLA association. J Rheumatol 2000;27:2860-5.
5. Veloso FT, Carvalho J, Magro F. Immune-related systemic manifestations of inflammatory bowel disease. A prospective study of 792 patients. J Clin Gastroenterol 1996;23:29-34.

6. Demetter P, De Vos M, Van Damme N, Baeten D, Elewaut D, Vermeulen S, Mareel M, Bullock G, Mielants H, Verbruggen G, De Keyser F, Veys EM, Cuvelier CA. Focal up-regulation of E-cadherin-catenin complex in inflamed bowel mucosa but reduced expression in ulcer-associated cell lineage. Am J Clin Pathol 2000;114:364-70.
7. Gonzalez S, Garcia-Fernandez S, Martinez-Borra J, Blanco-Gelaz MA, Rodrigo L, Sanchez del Rio J, Lopez-Vazquez A, Torre-Alonso JC, Lopez-Larrea C. High variability of HLA-B27 alleles in ankylosing spondylitis and related spondyloarthropathies in the population of northern Spain. Hum Immunol 2002;63:673-6.
8. De Keyser F, Elewaut D, De Vos M, De Vlam K, Cuvelier C, Mielants H, Veys EM. Bowel inflammation and the spondyloarthropathies. Rheum Dis Clin North Am 1998;24:785-813, ix-x.
9. De Vos M, Mielants H, Cuvelier C, Elewaut A, Veys E. Long-term evolution of gut inflammation in patients with spondyloarthropathy. Gastroenterology 1996;110:1696-703.
10. Orchard TR, Wordsworth BP, Jewell DP. Peripheral arthropathies in inflammatory bowel disease: their articular distribution and natural history. Gut 1998;42:387-91.
11. Holden W, Orchard T, Wordsworth P. Enteropathic arthritis. Rheum Dis Clin North Am 2003;29:513-30, viii.
12. Geier MS, Butler RN, Howarth GS. Inflammatory bowel disease: current insights into pathogenesis and new therapeutic options; probiotics, prebiotics and synbiotics. Int J Food Microbiol 2007;115:1-11.
13. Baharav E, Mor F, Halpern M, Weinberger A. Lactobacillus GG bacteria ameliorate arthritis in Lewis rats. J Nutr 2004;134:1964-9.
14. Balfour Sartor R. Bacteria in Crohn's disease: mechanisms of inflammation and therapeutic implications. J Clin Gastroenterol 2007;41:S37-43.
15. Karimi O, Pena AS. Probiotics in arthralgia and spondyloarthropathies in patients with inflammatory bowel disease. Prospective randomized trials are necessary. Rev Esp Enferm Dig 2005;97:570-4.
16. Karimi O, Pena AS, van Bodegraven AA. Probiotics (VSL#3) in arthralgia in patients with ulcerative colitis and Crohn's disease: a pilot study. Drugs Today (Barc) 2005;41:453-9.
17. Berg RD. Probiotics, prebiotics or 'conbiotics'? Trends Microbiol 1998;6:89-92.
18. Yan F, Polk DB. Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem 2002;277:50959-65.
19. Grishin A, Wang J, Hackam D, Qureshi F, Upperman J, Zamora R, Ford HR. p38 MAP kinase mediates endotoxin-induced expression of cyclooxygenase-2 in enterocytes. Surgery 2004;136:329-35.
20. Demetter P, Baeten D, De Keyser F, De Vos M, Van Damme N, Verbruggen G, Vermeulen S, Mareel M, Elewaut D, Mielants H, Veys EM, Cuvelier CA. Subclinical gut inflammation in spondyloarthropathy patients is associated with upregulation of the E-cadherin/catenin complex. Ann Rheum Dis 2000;59:211-6.
21. Elewaut D, De Keyser F, Van Den Bosch F, Lazarovits AI, De Vos M, Cuvelier C, Verbruggen G, Mielants H, Veys EM. Enrichment of T cells carrying beta7 integrins in inflamed synovial tissue from patients with early spondyloarthropathy, compared to rheumatoid arthritis. J Rheumatol 1998;25:1932-7.
22. Baeten D, Demetter P, Cuvelier CA, Kruithof E, Van Damme N, De Vos M, Veys EM, De Keyser F. Macrophages expressing the scavenger receptor CD163: a link between immune alterations of the gut and synovial inflammation in spondyloarthropathy. J Pathol 2002;196:343-50.
23. Laukens D, Peeters H, Cruyssen BV, Boonefaes T, Elewaut D, De Keyser F, Mielants H, Cuvelier C, Veys EM, Knecht K, Van Hummelen P, Remaut E, Steidler L, De Vos M, Rottiers P. Altered gut transcriptome in spondyloarthropathy. Ann Rheum Dis 2006;65:1293-300.

The hypervirulent antibiotic resistant *Clostridium difficile* in Europe.

Paola Mastrantonio, Fabrizio Barbanti and Patrizia Spigaglia on behalf of ESGCD
Department of Infectious,Parasitic and Immune-mediated Diseases
Istituto Superiore di Sanità
Rome,Italy

Infection with toxigenic *C.difficile* results in a spectrum of disease ranging from mild self-limiting diarrhoea to fulminant colitis (1-3)and asymptomatic toxin-positive carriers have also been identified (4). The prevalence of *C.difficile* associated diarrhoea (CDAD) may vary depending on the institution and patient population studied (5). The microorganism is the leading cause of nosocomial diarrhoea in adults from industrialized countries (6) and many hospital outbreaks throughout the world have been described (7-9).

Primary virulence factors are two large toxins:toxin A(TcdA) and toxinB (TcdB) which disrupt the actin cytoskeleton of intestinal epithelial cells by the UDP-glucose dependent glucosylation of proteins of Rho and Ras subfamilies (10). A third toxin,named binary toxin, (actin-specific ADP-ribosyltransferase) has been described from *C.difficile* strain CD196 in 1998 (11). It is encoded by two genes (*cdt*A and *cdt*B). Prevalence of binary toxin genes in humans varies from 1.6% to 20.8% and its pathogenic role still remain unclear (12-14).

Standard treatment includes withdrawal of the inducing antibiotic and use of oral metronidazole or vancomuycin in seriously ill patients. The major complications of treatment are failure to respond and relapses or reinfections after treatment discountinued.

Since 2003,increasing rates of CDAD with more severe courses have been reported in Quebec (Canada) and United States (15-17). Investigators from Quebec,noted an increase in the frequency and severity of *C.difficile*-associated disease in the 2000. Furthermore, the disease seemed to be more serious and refractory to therapy wih an increased mortality rate.

During the same years, the Centers for Disease Control and Prevention in US reported an increased frequency and severity of the CDAD. Complications included toxic megacolon,septic shock,requirement for colectomy and death (18).

This trend was due to the rapid spread of a specific clone belonging to PCR-ribotype 027 or pulsotype NAP1 (19). This strain is toxinA/toxinB positive,contains the genes for binary toxin and has an 18-bp deletion and a frameshift mutation in the gene *tcdC* hypothesized to result in a deregulated expression of toxins A and B.These strains are high producers of toxins in vitro compared to other toxinotypes. Moreover, these strains show a high level of resistance to fluoroquinolones.(20).

A two-month prospective study was conducted in 38 hospitals from 14 different European

countries,coordinated by the European Study Group on *Clostridium difficile*, in order to get an overview on the phenotypic and genotypic features of C. *difficile* isolates in 2005. Cultures were performed in each laboratory of Microbiology according to local standard techniques.Then,strains were sent to three central laboratories for toxinotyping, PCR-ribotyping and antimicrobial susceptibility tests (MIC determination), respectively.
Four hundred and eleven *C.difficile* were isolated from diarrheic patients with suspected CDAD and characterised (21). The great majority (75.7%) were toxinotype O and 24.3% were toxin variant strains including toxinotypes I,III,IV,V,VI,VIII,IX and XXIV. All strains toxin A negative-toxinB positive were identified as toxinotype VIII.
All *C.difficile* strains were susceptible to vancomycin and metronidazole.Resistance rates to erythromycin,clindamycin,tetracycline and moxifloxacin varied widely from one country to another. 46% of strains resulted resistant to fluoroquinolones.
Sixty six different PCR ribotypes were identified.The six major ribotypes were 001,002,014,017,020,027.
The prevalence of the 027 epidemic strain was 6.2%. All 027 strains were positive for binary toxin genes, had an 18-bp deletion in *tcd*C gene and were resistant to erythromycin and moxifloxacin. Patients infected with a 027 strain were likely to have a more severe disease (OR=2.52, 95% CI 0.92-6.85, p=0.04) and to have been more specifically treated by metronidazole or vancomycin (OR=7.23, 0.99-149, p=0.02).
On-going epidemiological surveillance of CDAD cases with periodic characterization of the strains is needed to detect clustering of cases in time and space and to monitor the emergence of a specific hypervirulent clone.

References

1. Fekety R. Antibiotic-associated diarrhoea and colitis.*Current Opin.Infect.Dis*.1995,8:391-397

2. Tedesco F.J.Barton R.W.,Alpers D.H. Clindamycin-associated colitis. A prospective study. *Ann.Intern.Med*.1974,81:429-433

3. Gerding D.N.,Olsen M.M.,Peterson L.R.,et al. *Clostridium difficile* associated diarrhoea and colitis in adults: a prospective case-controlled epidemiological study. *Arch.Intern.Med*.1986,146:95-100

4. Simor A.E.,Yake S.I.,Tsimidis K. Infection due to *Clostridium difficile* among elderly residents of a long-term care facility. *Clin.Infect.Dis*.1993,17:672-678

5. Gerding D.N.,Johnson S.,Peterson I.,Mulligan M.,Silva J. *Clostridium difficile* associated diarrohea and colitis *Infect.Contr.Hosp.Epidemiol*.1995,16:459-476.

6. McFarland L.V.,Mulligan M.E.,KwoK R.Y.,Stamm W.E. Nosocomial acquisition of *Clostridium difficile* infection. *N.Engl.J.Med*.1989,320:204-210

7. Barbut F.,Mario N.,Meyohas M.C.,Binet D.,Frottier J.,Petit J.C. Investigation of a nosocomial outbreak of *Clostridium difficile*–associated diarrhoea among AIDS patients by random amplified polymorphic DNA assay. *J.Hosp.Infect*. 1994,26:181-189

8. Cartmill T.D.,Panigrahi H.,Worsley M.a.,McCann D.C.,Nice C.N.,Keith E. Management and control of a large outbreak of diarrhoea due to *Clostridium difficile* . *J. Hosp.Infect*. 1996,27:1-15

9. Muto C.A.,Pokrywka M.,Shutt K.,et al. A large outbreak of *Clostridium difficile*-associated disease with an unexpected proportion of death and colectomies at a teaching hospital following increased fluoroquinolone use. *Infect.Control Hosp.Epidemiol*.2005,26:273-280

10. Voth D.E.,Ballard J.D. *Clostridium difficile* toxins:mechanism of action and role in disease. *Clin Microbiol.Rev*. 2005,18:247-263

11. Popoff M.R.,Rubin E.J.,Gill D.M., Bouquet P. Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain. *Infect.Immun*.1998,56:2299-2306

12. Goncalves C.,Decre D.,Barbut F.,Burghoffer B.,Petit J.C. Prevalence and characterization of a binary toxin from *Clostridium difficile. J.Clin Microbiol*.2004,42:1933-1939

13. Pituch H.,Rupnik M.,Obuch-Woszczatynski P.,Grubesic A.,Meisel-Mikolajczyk F.,Luczak M.Detection of binary toxin genes (*cdt*A and *cdt*B) among *Clostridium difficile* strains isolated from patients with *C.difficile* associated diarrhoea (CDAD) in Poland. *J.Med.Microbiol.* 2005,54:143-147

14. Rupnik M.,Kato N.,Grabnar M.,Kato H. New types of toxin A negative ,toxin B positive strains among *Clostridium difficile* isolates from Asia. *J.Clin.Microbiol*. 2003,41:1118-1125

15. Loo VG, Poirier L, Miller MA, et al. A Predominantly Clonal Multi-Institutional Outbreak of *Clostridium difficile*-Associated Diarrhea with High Morbidity and Mortality. *N Engl J Med* 2005.

16. McDonald LC, Killgore GE, Thompson A, et al. An Epidemic, Toxin Gene-Variant Strain of *Clostridium difficile*. *N Engl J Med* 2005;353:2433-41.

17. Pepin J, Valiquette L, Alary ME, et al. *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity.*Can.Med.Ass.J* 2004;171(5):466-72.

18. Pepin J, Valiquette L, Cossette B. Mortality attributable to nosocomial *Clostridium difficile*-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *Can.Med.Ass.J*. 2005;173(9):1037-42.

19. Warny M.,Pepin J.,Fang A. et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe.*Lancet* 2005, 366:1079-1084

20. Pepin J.,Saheb N.,Coulombe M.A.,Alary M.E.,Corriveau M.P.,et al. Emergence of fluoroquinolones as predominant risk factor for *Clostridium difficile*-associated diarrhoea: a cohort study during an epidemin in Quebec. *Clin.Infect:Dis*. 2005,41:1254-1260

21.Barbut F.,Mastrantonio P.,Delmeè M.,Brazier J.,Ackermann G.,Bouza E.,et al. for the European Study Group on *Clostridium difficile* (ESGCD). Prospective Study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterization of the isolates. *Clin.Microbiol.Infect*. 2007 (in press)

The gnotobiotic mouse model to study the effect of antibiotics on human faecal microbiota

Anne Collignon, EA 4043, USC INRA "Ecosystème Microbien Digestif et Santé", faculté de Pharmacie, Université Paris Sud-XI et Hôpital Jean Verdier, AP-HP, France

The intestinal tract of humans is a complex bacterial ecosystem which plays an important role in human health and disease concerning nutrition and immunological balance of the host. The intestinal microbiota is responsible for colonization resistance and immune response to pathogens. This microbiota is subjected to variations according to diet, environment and treatments particularly antibiotic treatment which disrupt its balance and barrier effect. Antibiotic-associated diarrhoea and *Clostridium difficile* diseases are a common complications of most antibiotic treatments. Amoxicillin-clavulanic acid is widely used and is well-known for disturbance and disruption of intestinal microbiota. The use of probiotics such as *Saccharomyces boulardii*, a non-pathogenic yeast has shown efficacy in the prevention and in treatment of these disorders (7).

The human microbiota-associated mouse model is a valuable tool for studying the ecosystem and modifications of human microbiota (2, 4, 9). Using a gnotoxenic mouse model associated with an intestinal human microflora allows to eliminate ethic problems concerning clinical trials with human volunteers.

The complexity of the digestive ecosystem consisting mainly of obligatory anaerobic bacteria makes traditional plate culture and identification of faecal anaerobic bacteria difficult. The development of molecular techniques based on sequence variability of ribosomal RNA genes has led to the identification of many anaerobic species that have not previously been cultured (6).

We analyzed the composition of the faecal microbiota in a human flora-associated mouse model and evaluated the effect of antibiotherapy (amoxicillin-clavulanic acid) and of the yeast *S. boulardii* before, during and after the antibiotic treatment. The molecular analysis of the microbiota was performed with Fluorescence In Situ Hybridization associated to flow cytometry using specific 16S rRNA targeted probes.

Materials and methods

The human microbiota-associated mouse model was obtained from germ-free mice (C3H mice, INRA , Jouy en Josas, France) feded a suspension of human faecal microbiota as previously described (1).

In a first set of experiment, one group of 6 mice were orally intubated with 0.4ml of an amoxicillin-clavulanic acid solution (150 mg/kg) during 7 days and one group of 6 mice were similarly treated with saline and served as control.

In a second set of experiment, six human microbiota-associated mice were gastrically intubated with 0.5 ml of a suspension of *S. boulardii* (10^9 CFU/ml) daily for 14 days and six mice were gastrically intubated in the same way with sterile water (control mice). A second group of 12 human microbiota-associated mice were orally intubated with 0.4 ml of an amoxicillin-clavulanic acid solution (150 mg/kg) for 7 days (day 1 to 7). Six mice were orally intubated with 0.5 ml of a suspension of *S. boulardii* (10^9 CFU/ml) simultaneously for 14 days (day 1 to 14) and six mice were given sterile water (control mice).

The faeces of each mouse were collected for the molecular analysis and the enumeration of the total anaerobic bacteria and the *Enterobacteriaceae*.

For hybridization, the probes were designed for the *Eubacteria*, *Bacteroides-Porphyromonas-Prevotella*, *Clostridium coccoides-Eubacterium rectale*, *Faecalibacterium prausnitzii, Clostridium histolyticum*, *Lactobacillus-Enterococcus* and *Enterobacteriaceae* groups and the *Bifidobacterium* genus (1, 5).

The statistical analysis was assessed with two tests: the Wilcoxon test and the Student test.

Results and discussion

In the first set of experiment, we evaluated the effect of amoxicillin-clavulanic acid on the composition of the microbiota. For the total microbiota, the difference between the treated and control groups with both techniques was not significant: the enumeration in the control group was 2.3 ± 2.3 10^{10} CFU/g and 4.7 ± 1.7 10^{10}CFU/g in the treated group; with the EUB 338 FITC probe the total hybridized bacteria in the control group was 67.3 ± 2.6 % of 100 000 events and for the treated mice 69.1 ± 2.9 %. The antibiotic treatment did not quantitatively modify the level of the total flora assessed by culture or FISH.

By FISH, the most abundant groups were detected with the *Clostridium coccoides-Eubacterium rectale* and *Bacteroides* group probes. Similarly, in healthy volunteers, the most abundant group are the *C. coccoides - E. rectale* group (20% to 36%) and the *Bacteroides* group around 20% (5). The *C. coccoides* group was stable in the control group (from 40.7 ± 1.6% to 45.6 ± 2.8%) but decreased dramatically in the treated group during the antibiotic treatment (3.9 ± 0.8%). After antibiotic stop, the level increased to reach at day 14 the initial level. The *Bacteroides* group in the control group had a steady level of 35.9 ± 4.3% whereas in the treated group it increased during antibiotic treatment to reach the level 58.5 ± 4.5% at day 6. After antibiotic stop, the level decreased to 38.6 ± 5.7% at the end of the experiment to reach the same level as in the control group.

In the second set of experiments with *S. boulardii,* the level of the yeast was stable in the faeces during the experiment. The yeast achieves steady-state concentration within 3 days and is cleared from the stools rapidly, within 2-5 days after discontinuation showing that the bacterial populations exert a barrier effect. *S. boulardii* did not quantitatively alter the total anaerobic microbiota nor the different groups of the normal dominant microbiota : there was no significant difference between the mice treated with *S. boulardii* and the control mice for neither of the two groups.

With amoxicillin clavulanic acid treatment, the role of *S. boulardii* on faecal microbiota was assessed. The total microbiota studied by culture or FISH was stable during the experiment in the control mice as well in the mice treated with the yeast. The yeast treatment associated or not with amoxicillin clavulanic acid did not quantitatively alter the total microbiota. Before antibiotic treatment, the level of the *C. coccoides-E. rectale* group accounted for 52% of bacterial cells. After one day of the antibiotic treatment, the level in the two groups decreased dramatically to reach 9% in the control group and 11% in the yeast treated group until the antibiotic administration was stopped at day 7. Then, the level increased regularly and at days 17, 22 and 24 in the group treated with *S. boulardii* it increased faster ($P < 0.05$) than in the control mice to reach the initial level at day 29. The *Bacteroides* group in the two groups of mice increased during the antibiotic treatment and decreased at the antibiotic stop to reach the level at the beginning . The decrease rate was faster for mice treated with the yeast than for the control mice, with a significant difference ($P < 0.05$) at days 17 and 22.

The present study showed that *S. boulardii* treatment more rapidly restores the balance of the dominant anaerobic microbiota in human microbiota associated-mice treated with amoxicillin-clavulanic acid. These results may explain, at least in part, the beneficial effects of *S. boulardii* in preventing AAD in humans.

In conclusion the human faecal microbiota associated mouse is a model to study the ecology of the human intestinal tract. Similar results have been obtained in humans treated by antibiotics or healthy humans during administration of probiotics (8, 10). It offers a possible standardization of the faecal microbiota. In addition, this model has been used to follow antibiotic gene transfers in the digestive tract and the role of mobile elements in these transfers. We have demonstrated in a model of gnotobiotic mice that a Tn*1549*-like element is able to transfer by conjugation from *Clostridium symbiosum* MLG101 to *Enterococcus faecium* 64/3 and to *Enterococcus faecalis* JH2-2 (3). Intra and inter-generic transfer of vancomycin resistance genes occur and could be responsible for the spread of resistance between commensal and pathogenic bacteria.

References

1- Barc, M.C., Bourlioux, F., Rigottier-Gois, L., Charrin-Sarnel, C., Janoir, C., Boureau, H., Doré, J. & Collignon, A. (2004). Effect of amoxicillin clavulanic acid on human fecal flora in a gnotobiotic mouse model assessed with fluorescence hybridization using group specific 16S rRNA probes in combination with flow cytometry. *Antimicrob Agents Chemother* **48,** 1365-68

2- Imaoka A., Setoyama, H., Takagi, A., Matsumoto S. & Umesaki, Y. (2004). Improvement of human faecal flora-associated mouse model for evaluation of the functional foods. *J Appl Microbiol* **96,** 656-663.

3- Launay A, Ballard SA, Johnson PD, Grayson ML, Lambert T. (2006). Transfer of vancomycin resistance transposon Tn1549 from Clostridium symbiosum to Enterococcus spp. in the gut of gnotobiotic mice. Antimicrob Agents Chemother. 50(3):1054-62.

4- Raibaud, P., Ducluzeau, R., Dubos, F., Hudault, S., Bewa, H. & Muller, MC. (1980). Implantation of bacteria from the digestive tract of man and various animals into gnotobiotic mice. *Am J Clin Nutr* 33, 2440-2447.

5- Rigottier-Gois, L., Le Bourhis, A.G., Gramet, G., Rochet, V. & Doré, J. (2003). Fluorescent hybridization combined with flow cytometry and hybridization of total RNA to analyse the composition of microbial communities in human feces using 16S rRNA probes. *FEMS Microbiol Ecol* 1456, 1-9.

6- Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P. & Doré, J. (2000). Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl Environ Microbiol* 66, 2263-2266.

7- Surawicz, C.M., Mc Farland, L.V., Greenberg, R.N., Rubin, M., Fekety, R., Mulligan, M.E., Garcia, R.J., Brandmarker, S., Bowen, K., Borjal, D. & Elmer, G. (2000). The search for a better treatment for recurrent *Clostridium difficile* disease: use a high-dose vancomycin combined with *Saccharomyces boulardii*. *Clin Infect Dis* 31, 1012-1017.

8- Vanhoutte, T., V., De Preter, E., De Brandt, K., Verbeke, J., Swings, & G., Huys. (2006). Molecular monitoring of the fecal microbiota of healthy human subjects during administration of lactulose and Saccharomyces boulardii. *Appl Environ Microbiol* 72, 5990-5997.

9- Wong, WC., Hentges, D.J. & Dougherty, S.H. (1996). Adequacy of the human fecal microbiota associated mouse as a model for studying the ecology of the human intestinal tract. *Microbial Ecol Health Dis* 9, 187-198.

10- Young, VB. & Schmidt, TM. (2004). Antibiotic-associated diarrhea accompanied by large-scale alterations on the composition of the fecal microbiota. *J Clin Microbiol* 42, 1203-1206.

Antibiotic susceptibility of *Bifidobacterium* strains

Maria Saarela & Jaana Mättö, VTT Technical Research Centre of Finland, Espoo, Finland.

Bifidobacteria are common inhabitants in the human and animal intestines, and are generally associated with good intestinal health. They are increasingly used as probiotics and one target for their probiotic use is attenuation of ecological disturbances in the microbiota caused by antibiotic therapy. Bifidobacteria are typically susceptible to majority of clinically relevant antibiotics such as penicillins, cephalosporins and macrolides, and bifidobacterial population is therefore vulnerable to changes during antibiotic administration. However, their susceptibility to tetracycline is variable (Moubareck et al. 2005, Delgado et al. 2005, Mättö et al. 2007).

Tetracycline group antibiotics are widely used for therapeutics and prophylaxis of clinical infections in humans and animals and in some countries they are additionally used as growth promoters in animals. Tetracycline resistance is increasingly common in bacteria living in a variety of ecological niches and several transferable determinants have been linked to the resistance (Roberts 2005). Ribosomal protection protein type resistance genes, especially *tet*(W), have been detected in several *Bifidobacterium* species (Moubareck et al. 2005, Aires 2007). However, the data on the species distribution of bifidobacterial population and changes in antibiotic susceptibility patterns caused by tetracycline administration is scarce.

The aim of the present study was to investigate the influence of oral doxymycin therapy on the susceptibility of bifidobacteria to antimicrobial agents with the focus on tetracycline-group antibiotics. Faecal samples were collected from 10 subjects receiving doxymycin therapy together with probiotics (*Lactobacillus acidophilus* LaCH-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12) and 10 control subjects (consuming probiotics only). Bifidobacterial population was analysed by culturing followed by species- and strain-level identification. Antibiotic susceptibility patterns of the bifidobacterial isolates were assessed by Etest on LSM-cys test medium.

Although doxycycline consumption did not have a large impact on the GI-survival of the probiotics, it had a detrimental effect on the bifidobacterial population (Fig.1).

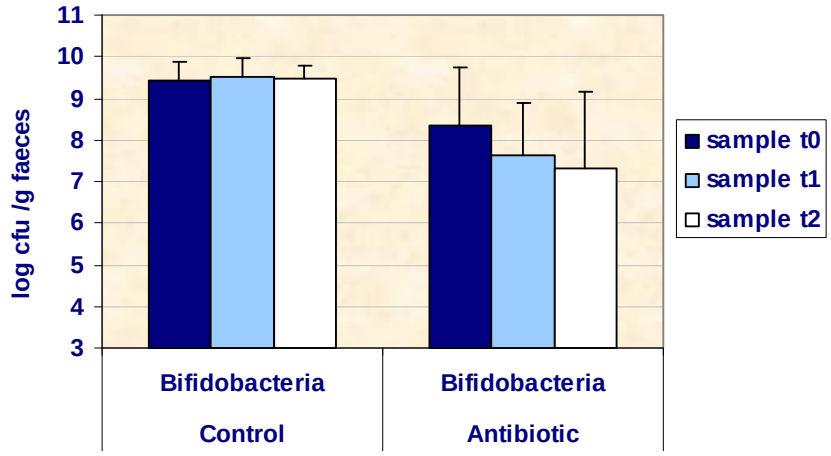


Fig. 1. Numbers of bifidobacteria in the control and antibiotic group. Note that subjects consuming antibiotics were not asked to postpone their treatment and thus some of their t0 samples were obtained after the treatment was started resulting in lower initial bifidobacterial levels.

The proportion of a tetracycline resistant bifidobacterial population was higher in the antibiotic group than in the control group (1.5 vs. 52% in sample t1) (Fig. 2).

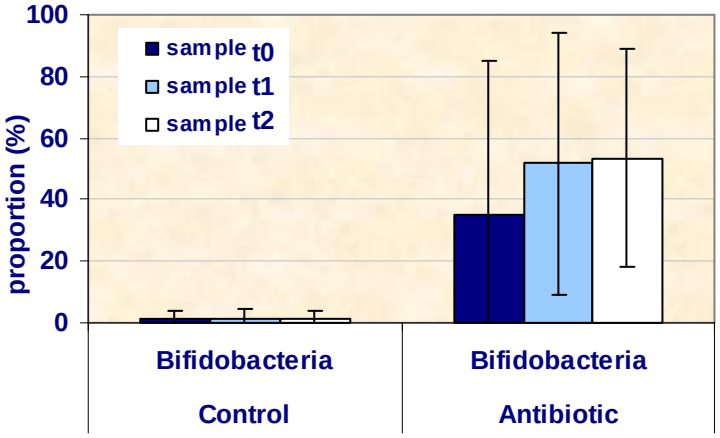


Fig 2. Proportion of tetracycline resistant bifidobacteria in the antibiotic and control groups.

The MICs of indigenous bifidobacteria to 13 tested antibiotics are shown in Table 1. Difference in MICs between antibiotic and control groups was seen in tetracycline and doxymycin, but not in other studied antibiotics. The isolates were uniformly susceptible to quinupristin/dalfopristin, ampicillin and vancomycin and generally resistant to streptomycin, while metronidazole resistance was variable. Atypical resistance to multiple antibiotics representing different antibiotic classes were rarely detected.

Table 1. MICs of the indigenous bifidobacterial isolates against 13 antibiotics.

	Median MIC (µg/ml)	
Antibiotic	Control group*	Antibiotic group**
Tetracycline	0.5	16
Doxymycin	0.5	8
Tigecycline	0.125	0.06
Erythromycin	0.125	0.06
Clindamycin	0.06	0.06
Quinupristin/dalfopristin	0.125	0.125
Streptomycin	64	32
Ampicillin	0.06	0.125
Piperacillin	0.125	0.25
Cefoxitin	1	2
Ceftriaxone	0.5	1
Vancomycin	0.5	0.5
Metronizadole	16	4

* 28 isolates
** 9 isolates
- only isolates representing distinct genotypes were included.

Based on the *tet* gene PCR, resistance could be associated with the presence of *tet*(W). In two subjects, strains (*B. longum* and *B. adolescentis* strain pairs) representing the same genetic fingerprints but different tetracycline susceptibilities were detected. A mutation causing lack of functionality in the *tet*(W) was observed in one of the susceptible strains. Several antibiotic group subjects had faecal *B. animalis* subsp. *lactis* Bb-12-like isolates with reduced tetracycline susceptibility (Bb-12 is originally intermediately resistant to tetracycline). This was unlikely due to the acquisition of novel tetracycline resistance determinants since only *tet*(W), which is also present in the ingested *B. animalis* subsp. *lactis* Bb-12, was found in the resistant isolates. Thus the concomitant ingestion of probiotic *B. animalis* subsp. *lactis* Bb-12 with the antibiotic did not generate a safety risk regarding the possible GI-transfer of tetracycline resistance genes to the ingested strains. However, doxycycline therapy had a drastic effect on the diversity and tetracycline susceptibility of intestinal indigenous *Bifidobacterium* populations and increased at least transiently the pool of tetracycline resistant commensal bacteria in the intestine. The long-term effects of antibiotic therapy on commensal bacteria warrant further investigations.

References:

Aires, J., Doucet-Populaire, F., and Butel, M.J. (2007). Tetracycline resistance mediated by *tet*(W), *tet*(M), and *tet*(O) genes in *Bifidobacterium isolates* from humans. *Applied Environmental Microbiology*, 73, 2751-2754.

Delgado, S., Florez, A. B. & Mayo, B. (2005). Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. *Current Microbiology*, 50, 202-207.

Mättö, J., van Hoek A.H.A.M., Domig, K.J., Saarela, M., Belen Florez, A., Brockmann, E., Amtmann, E., Mayo, B., Aarts, H.J.M. & Danielsen, M. Susceptibility of human and probiotic *Bifidobacterium* spp. to selected antibiotics as determined by the Etest method. *International Dairy Journal*, in press.

Moubareck, C., Gavini, F., Vaugien, L., Butel, M. J. & Doucet-Populaire, F. (2005). Antimicrobial susceptibility of bifidobacteria. *Journal of Antimicrobial Chemotherapy,* 55, 38-44.

Roberts, M.C. (2005) Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters* 245, 195-203.

Transfer of ermB-plasmid from lactobacillus to enterococcus faecalis in the Intestine of gnotobiotic rats

Louise Feld (1), Morten Danielsen (2), Karin Hammer (3) and Andrea Wilcks (1)

1 Department of Microbiology and Risk Assessment, Danish Food Institute, Technical University of Denmark, Søborg, Denmark.
2 Chr. Hansen, Hørsholm, Denmark.
3 Center for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, Lyngby, Denmark.

Objectives: A wild-type Lactobacillus strain recently isolated from cheese was found to carry a small plasmid harbouring the erythromycin resistance gene ermB. This plasmid could be transferred at relatively low frequency to different strains of enterococci in vitro. We wanted to investigate whether transfer could also occur in a mammalian gastrointestinal tract and if erythromycin treatment would influence on this.

Methods: A worst-case scenario using germfree rats was set up. The rats were initially dosed with the recipient Enterococcus faecalis and in the following week the drinking water was replaced with the Lactobacillus donor suspension. Parallel with donor administration, one group of rats received erythromycin at a high concentration, one group received erythromycin at a medium concentration and a third group did not receive the antibiotic. The rats were followed two additional weeks and the presence of donors, recipients and transconjugants (TC) in faecal and intestinal samples were enumerated by selective plating.

Results: TC were detected in rats from all three groups two days after donor introduction. However, when erythromycin was present the increase in numbers of TC occurred much faster i.e. approximately one day versus 4-5 days to reach a stabilized population level. Additionally, the size of the TC population, which established in the rats was approximately four log units higher in the treated than in the untreated groups.

Conclusions: The concentration of TC in vivo was surprisingly high compared to the relatively low transfer frequency in vitro. Furthermore, treatment with erythromycin favoured the establishment of TC.

Identification and molecular analysis of tetracycline and erythromycin resistance genes from lactic acid bacteria isolated from an italian dairy product

C. Devirgiliis, D. Coppola, S. Barile, A. Caravelli and G. Perozzi
INRAN, National Research Institute on Food and Nutrition, Roma, Italy

Background: the use of antibiotics as growth promoters in livestock has led to selection and spread of antibiotic resistance genes in commensal bacteria. Although horizontal transmission of antibiotic resistance genes from non pathogenic hosts to bacterial pathogens has not yet been demonstrated conclusively, this issue raises several questions concerning safety of fermented food products for human consumption. In Italy, as well as in other mediterranean countries, fermented foods employing natural starter cultures represent a significant fraction of the dairy and meat products, especially those carrying the official EU designation "PDO" (Protected Designation of Origin). Objectives: our aim was to isolate Lactobacilli and other LABs from an italian PDO dairy product, to investigate the presence of tetracycline, erythromicin and kanamycin resistance genes and to evaluate their potential horizontal transfer to other commensal bacteria.

Methods: homogenates of raw milk, natural whey starter and final fermented product were plated on MRS agar plates supplemented or not with tetracycline (8 µg/ml), erythromicin (4 µg/ml) and kanamycin (256 µg/ml). Isolated LABs were identified by Amplified Ribosomal DNA Restriction Analysis (ARDRA) and typed using rep-PCR fingerprinting. MICs for the three antibiotics were obtained for each isolate and the presence of antibiotic-resistance genes was confirmed by PCR, using primers designed on highly conserved DNA sequences. Total DNA was also extracted from the fermented cheese and the presence of antibiotic-resistance genes was directly analyzed by PCR. Results and conclusions: we have isolated and analyzed over 800 independent colonies from products made in three different cheese factories. Higher microbial titers and wider biodiversity were observed in the microbial population isolated from milk and natural whey with respect to the final product. Molecular analysis of tetracycline and erythromycin resistant LABs, which were found exclusively among the isolates from raw milk and natural whey, demonstrated the presence of tetM and ermB genes, leading to high MIC values. Moreover, analysis of a TetM-positive L. paracasei strain revealed the presence of a conjugative transposon. Further experiments are in progress to investigate the possible association of TetM with this transposon. Overall, our results suggest that the technological procedures necessary for cheese manufacturing, such as high temperature treatments, operate a strong negative selection against the LAB species most frequently carrying antibiotic resistance genes

Probiotics and Irritable Bowel Syndrome: Rationale, Mechanisms and Efficacy

Michael Camilleri, M.D.

Atherton and Winifred W. Bean Professor,
Professor of Medicine and Physiology, Mayo Clinic College of Medicine
Consultant in Gastroenterology, Mayo Clinic
Rochester, Minnesota, U.S.A.

Objective

To review the rationale and current evidence for the efficacy of probiotics in the treatment of irritable bowel syndrome.

The following specific questions will be addressed:

- Are bacterial flora altered in IBS?
- Is infection a significant risk factor for IBS? How prominent is the inflammation in IBS?
- Do probiotics change bacterial flora?
- Do probiotics adhere to mucosa?
- What are the potential mechanisms of action of probiotics in IBS? Immune function, motor, secretory, sensory, and fermentation?
- What is the efficacy of probiotics in IBS?

Introduction

Probiotics are preparations that contain viable microorganisms that confer potential health benefits by preventing or treating specific pathologic conditions. They are currently easily available, do not require prescription, and are used extensively for the relief of abdominal symptoms. The scientific basis of probiotic use has been investigated recently, and clinical studies have documented efficacy in treatment of irritable bowel syndrome (IBS).

Are bacterial flora altered in IBS?

One study suggested that Veillonella spp. are increased in C-IBS, and lactobacilli decreased in D-IBS

Is infection a significant risk factor for IBS? How prominent is the inflammation in IBS?

A meta-analysis and earlier epidemiological studies suggested that prior gastroenteritis was a very significant risk factor in the development of IBS with odds ratio of 7.3. However, more recent studies suggest the relative risk is closer to 2.2 and the follow up of patients with prior gastroenteritis may have a short lived form of IBS, with no increased risk beyond three months.

The magnitude of the inflammatory response is variable with inconsistent changes in T lymphocytes and mast cells, even in studies where the antecedent infection was amoebic colitis

Do probiotics change bacterial flora?

Not all probiotic preparations are equal; colonization or enrichment of colonic flora should be demonstrated preferably in health and in the disease of interest. The medium of

administration of the probiotic also varies e.g. the colonization from pasteurized yogurt is unclear.

Do probiotics adhere to mucosa?

Several studies in vitro and in animals show probiotics adhere to colonic epithelial cells and prevent other bacteria from accessing the intercellular space and mesenteric lymph nodes.

What are the potential mechanisms of action of probiotics in IBS?

a. **Immunology:** One study in patients showed that bifidobacteria increase the IL10/12 ratio in peripheral blood mononuclear cells; similarly, several probiotic species have been shown to alter mucosal cytokines. It is important to note that the literature suggests that peripheral blood mononuclear cell or dendritic cell responses do not predict mesenteric lymph node lymphocyte or dendritic cell responses. On the other hand, probiotics have been shown to enhance human enterocyte immune responses, and to modulate intestinal immune cells that mediate responses to enteric pathogens and specific antigens like flagellin. The cytokine responses appear to be strain specific in incubation studies of human colonic mucosa. Circulating cytokine levels in patients with IBS do not show consistently significant changes.
b. **Motor and secretory: In a randomized controlled study of the combination probiotic:** VSL#3 in patients with D-IBS, colonic transit was significantly reduced though there was no deleterious effect on stool consistency. These data suggested that the probiotic combination may alter motor function. A subsequent in vitro study of muscle strips taken from guinea pig ileum or colon demonstrated significant relaxatory effects of VSL#3, Lactobacillus, Bifidobacterium, and Streeptococcus species. Intestinal microflora also significantly affect small intestinal MMC periodicity and transit.
Probiotics also affect secretion: In a model of neonatal stress in rats, probiotics reduced colonic chloride secretion and permeability to a large molecule, horse radish peroxidase. Finally, probiotics upregulate the synthesis and secretion of mucus which has lubricant and other surface protective properties in the colon. The upregulation appears to be greater for lactobacillus that bifidobacterium species.
c. **Sensory:** Lactobacillus acidophilus NCFM strain upregulates the expression of opiate and CBR2 (cannabinoid type 2 receptors) in epithelial cells of mice and rats. This is associated with a 20% increase in the threshold for pain, an effect that is related to the dose of lactobacilli administered. Another species, Lactobacillus farciminis suppressed stress induced hypersensitivity, and increased colonic mucosal permeability. The effect appears to be in part caused by the NO produced by this probiotic, which is associated with inhibition of contraction of colonic epithelial cell cytoskeleton and the subsequent opening of tight junctions that increased the mucosal permeability. A third Lactobacillus (reuteri) decreased the heart rate/pain response to high pressure, noxious colorectal distension in rats. Interestingly, this effect was also noted with killed bacteria or conditioned medium suggesting that live organisms may not be essential for the effects of probiotics.
d. **Fermentation:** The intraluminal milieu in the colon is the result of the interaction of the microflora and food substrates. Colonic bacteria normally metabolize nutrient substrates reaching the colon with the formation of gas and production of short chain fatty acids. SCFA induce propulsive contractions to accelerate transit, or enhance fluid and sodium

absorption in the colon. SCFAs also release 5-HT from the mucosa and this may have secondary effects on other colonic functions such as secretion, motility or sensation. Lactobacilli and Bifidobacteria subspecies deconjugate and absorb bile acids; this may reduce bile salt load to the colon and may reduce mucosal secretion of mucin and fluids.

An important but forgotten field of investigation is the potential for gas production and its role in symptom generation in IBS. Breath test studies and more direct measurements with ileocolonic intubations showed that 2-20% of ingested starch reaches the colon even in healthy people and hydrogen production, especially of mono- and disaccharides, results in significant rates of hydrogen production. In IBS patients, the colonic excretion of hydrogen and methane were greater in IBS than controls and were normalized by an exclusion diet. Although a subsequent RCT of Lactobacillus plantarum 299v did not show any change in symptom score, hydrogen production or overall gas production rate, further studies are needed to determine whether this might be an explanation for the most consistent beneficial effect of probiotics on flatulence and bloating in IBS (see below).

What is the efficacy of probiotics in IBS?

Therapeutic trials show the potential benefit of Bifidobacteria or Lactobacilli species alone, or the specific probiotic combination, VSL#3, on symptoms in IBS without induction of significant changes in bowel function. The largest RCT to date (n=330) demonstrated a beneficial effect of a Bifidobacterium species, though the lack of a significant dose response is not completely understood, and it was attributed to a problem with formulation. The most consistent effects of single or combination probiotics appear to be on flatulence, bloating and excess/abundant gas production. Effects on pain are inconsistent either because the symptom was incorporated into a composite endpoint with other symptoms like bloating which is itself improved, or because studies are generally too small or the endpoint was at 4 weeks. In a pediatric RCT, Lactobacillus GG reduced frequency but not severity of pain in IBS, and had no effect on pain associated with dyspepsia.

The potential therapeutic effects of probiotics in post-infectious IBS have not been reported. From two trials reported in collagenous colitis, which may inform as a surrogate for a low inflammatory state in human colon, it does not appear that probiotics are effective.

Conclusions

Potential mechanisms supporting beneficial effects of probiotics in IBS include a combination of immune function, motility, secretion, sensation and the intraluminal milieu. Further studies on production and metabolism of gas in response to carbohydrate loads are needed.

The literature on randomized controlled trials confirms the potential benefit of probiotics in IBS, especially in the relief of flatulence and bloating; evidence supports several species, e.g. Bifidobacteria alone, Lactobacilli, or combinations, including VSL#3. However, the studies have generally been small and of short duration. Safety is paramount in irritable bowel syndrome. Probiotics deserve further study.

MICROBIOLOGICAL CHARACTERISTICS OF SOME ITALIAN PROBIOTICS

Lorenzo Drago – Clinical Microbiology – DISP LITA – University of Milan

The concept of probiotics dates 100 years, when Metchnikoff suggested that consumption of lactic acid bacteria may be of benefit to the human host. However, not until the mid-1960s the term probiotic come into vogue. Nowadays, probiotics are known to clinicians as well as to the general public. While probiotics have proven benefits, the optimism associated with their use is counterbalanced by the fact that many so-called "probiotic" products are unreliable in content and unproven clinically. Therefore much remains to be done to gain the acceptance of the broader medical community. Probiotics are generally defined as non-pathogenic organisms that when ingested in adequate amounts are capable to confer a health benefit to the host. There are several generally accepted characteristics that define probiotic microorganisms as stated by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO): they should be taxonomically classified and deposited in an internationally recognized culture collection; remain viable and stable after culture, manipulation, and storage before consumption; survive gastric biliary and pancreatic digestion; induce a host response once ingested by adhering to gut epithelium or other mechanisms; yield a functional or clinical benefit to the host when consumed; and, obviously be safe, including not only assessment of side effects during human studies and epidemiological surveillance of adverse incidents in consumers, but also determination of antibiotic resistance patterns since beneficial and commensal bacterial populations may play a role in the transfer of antibiotic resistance to pathogenic and opportunistic bacteria.

In the recent past, several data regarding the quality of probiotic products available on the market have evidenced a lack of compliance with FAO/WHO guidelines, particularly for species identification, stability during storage and ability to survive to acidity. Studies on patterns of resistance of several probiotics have evidenced that at present, multiresistance seems to be uncommon among lactic acid bacteria and bifidobacteria species, but an increasing number of strains displaying atypical resistance levels to some antibiotics (especially tetracycline and erythromycin) are being isolated. Many of these carry antibiotic-resistance genes, which are thought to be acquired by horizontal transmission.

As far as Italian products are concerned, a study performed in our laboratory has evaluated some *in vitro* characteristics of probiotic products by determining stability after storage, survival at low and high pH values and in presence of different types of bile, and ability to adhere to colonic cells. In respect to our previous paper, we have observed an improvement in assuring viability of products until their expiry date, since at that time, most of the examined products contained a microbial load similar to what stated on the label. Differences among lactobacilli, bacilli and bifidobacteria in

adherence to colonic cells have been confirmed by our study, as evidenced by other authors. A different ability to survive and to grow in presence of a low pH and bile has been observed among the tested strains.

In conclusion, in the last years many efforts have been sustained to improve quality of probiotic products; however, precise guidelines describing uniform analytical methods for evaluation of probiotic properties are still requested.

XXX International Congress on Microbial Ecology and Disease (SOMED) held in Rome, September 16-18, 2007, jointly with the 4th Probiotics, Prebiotics and New Foods International Congress.

Extended Abstract

Why not all probiotics are equal

Gregor Reid

Canadian Research and Development Centre for Probiotics, Lawson Health Research Institute, 268 Grosvenor Street, London, Ontario, N6A 4V2, Canada

"All animals are equal but some animals are more equal than others."

- George Orwell

ADHESINS SELECTED IN THE ENVIRONMENT AND BACTERIAL PATHOGENICITY

LUIGI VEZZULLI, BARBARA REPETTO, ELISABETTA PEZZATI, GIOVANNI GIUSTO, MONICA STAUDER, ANETA KARBOWIAK AND CARLA PRUZZO

DIBIO, GENOVA UNIVERSITY, CORSO EUROPA 26, 16123 GENOVA

Pathogenic bacteria and the marine environment

A surprising number of pathogenic bacteria, both indigenous and deriving from animal and human faeces, are present in marine environments. They are found either freely suspended in the water, or associated with biotic and abiotic surfaces, or hidden in sediments, or within the body and cells of other organisms. Growing evidence of intercellular communication and genetic exchange shows that, within the aquatic habitat, microbes undergo a large variety of interactions that can also have repercussion on pathogenicity for humans. Pathogens depend partially or entirely for their existence on several physical, chemical, or biological factors. Many pathogens are strictly vector-dependent while others are not. For both types, environmental factors can affect directly, or indirectly, survival, persistence and ability to produce disease. Temperature is a key factor in aquatic environments where a temperature increase means more evaporation, causing increased humidity, an increased concentration of nutrients and a general change in ecology. A rise in sea surface temperature in a given area occurring shortly before an increase in the number of people infected with cholera has been suggested as a possible impact of climate change on disease spreading (Colwell, 1996). Furthermore, reports of marine-related illnesses along the east cost of the United States had increased over the past 25 years in correlation with El Niño events (Harvell *et al.*, 1999). Sunlight can affect the persistence and spread of a pathogen if it is associated with phytoplankton and/or algae. For example, when algae and phytoplankton increase in bio-mass, zooplankton blooms rapidly follow. Bacteria associated with zooplankton also increase. In laboratory microcosm experiments, seroconversion of *V. cholerae* non-O1 to O1 and vice versa have been demonstrated (Colwell *et al*., 1995; Bik *et al*., 2005). Seroconversion has been observed in *V. cholerae* under different temperature and salinity conditions, suggesting that the phenomenon may occur more commonly than known before in brackish, estuarine or sea water throughout the year; this also indicates that environmental factors have a direct influence on the pathogenicity of *V. cholerae.*

Role of bacterial adhesion in the marine environment: the *V. cholerae*-chitin study case

Bacterial adhesion is a very important event in the colonization of animate and inanimate substrates and provides a link between the behaviour of microorganisms in natural environments and their pathogenicity potential. In fact, it is well known that this phenomenon is a prerequisite for epithelial cell colonization by both indigenous and pathogenic bacteria. On the other hand, marine bacteria that fail to attach to surfaces present in the aquatic environment fail to flourish and are washed away.

In the aquatic environment *V. cholerae* binding to chitin exemplifies for microbial ecology a successful bacteria-substrate interaction with complex and significant influence on the lifestyle of the bacterium. Chitin is one of the most abundant polymers on earth and possibly the most abundant in the aquatic environment, where its association with *V. cholerae* has provided the microorganism with a number of advantages, including food availability, adaptation to environmental nutrient gradients, tolerance to stress, and protection from predators.

Bacterial binding to chitin is a complex process involving hydrophobic and ionic bonds, forces responsible for the primary reversible phase of attachment, and specific cell ligands that are responsible for subsequent firm anchoring to substrate. The presence of pili is associated with the ability of bacterial cells to colonize surfaces. Mannose-sensitive hemagglutinin (MSHA) is a type 4 pilus produced by *V. cholerae* O1 El Tor and O139 promoting adherence to chitin beads (Meibom

et al, 2004) and crustaceans (Chiavelli *et al*, 2001 Meibom *et al*, 2004). In *V. cholerae* El Tor, a type 4 PilA-containing pilus, the expression of which is induced by chitin (also designated chitin-regulated pilus, ChiRP), has been reported to contribute to colonization of chitin (Meibom *et al*, 2004). More recently, the toxin-coregulated pilus (TCP), that is required for intestinal colonization (Kirn *et al*., 2000) and cholera toxin gene acquisition by phage infection (Waldor and Mekalanos, 1996) has also been shown to have a role in biofilm formation on chitin surfaces by *V. cholerae* El Tor (Reguera and Kolter, 2005).

Bacterial ligands mediating adhesion to chitin by a specific binding to N-acetyl glucosamine (GlcNAc) have been shown to be present in *V. cholerae* classical strains. A cell-associated GlcNAc specific hemagglutinin (HA) was purified from *V. cholerae* by affinity chromatography on a chitin column, followed by BioGel filtration (Sasmal *et al,* 1996). Chitin binding proteins (CBPs) associated with the cell wall have been described by Tarsi and Pruzzo (1999), who extended previous studies on *Vibrio harveyi* (Montgomery and Kirchman, 1993) and *Vibrio alginolyticus* (Pruzzo *et al*, 1996), by identifying two surface proteins involved in adhesion of *V. cholerae* classical strains to chitin beads: a 36-kDa protein, the binding of which to chitin is completely inhibited by GlcNAc and a 53-kDa protein, the binding partly reduced by the sugar. Employing TnphoA mutants, Zampini et al (2005) showed that the 53-kDa protein is also involved in adherence of *V. cholerae* to the copepod *Tigriopus fulvus*. A putative 53 kDa chitinase mediating adherence to chitin surfaces *via* GlcNAc binding, and termed GlcNAc-binding protein A (GbpA), has been described in a *V. cholerae* strain by Kirn *et al* (2005).

The analysis of the capability to adhere to chitin particles of strains belonging to different *Vibrio* species (*Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio nereis, Vibrio metschnikovii, Vibrio anguillarum, and Vibrio splendidus*) (Table 1), and SDS-PAGE analysis of their sarkosyl-insoluble membrane proteins able to bind chitin have shown that the presence of CBP is a common feature among vibrios. Due to the presence of such ligands, vibrios can efficiently compete with other marine bacteria for colonization of the nutritive chitin containing substrates.

Adhesins, environmental selection and pathogenecity

Growing evidence has been accumulated that adhesion mechanisms selected in the environment may have important implication for the pathogenecity of marine bacteria. An outstanding example is provided by the presence of *V. cholerae* O1 year-round via its commensal association with plankton as established by Colwell and coworkers using direct detection methods (Huq *et al*, 1990). Since then, other studies have further documented the association between *V. cholerae*, other vibrios and non *Vibrio* bacteria and chitinous plankton in both the marine (Huq *et al*., 2005) and freshwater (Shukla *et al.,* 1995) environment. The molecular basis of *V. cholerae* association with plankton crustaceans was recognised to reside, at least in part, in its capability to bind chitin. This interaction has been demonstrated to have important implications in the epidemiology of cholera. Chitin-adsorbed *V. cholerae* O1 survived exposure to acid better than non-adsorbed vibrios, and have an increased resistance to the effects of high temperature, low pH, and desiccation (Castro-Rosas and Escartin 2005). From different studies emerged that the association of *V. cholerae* with zooplankton, and their eggs that are dispersed in the water, is a key factor in deciphering the seasonal and geographic patterns of cholera epidemics and pandemics (Lipp *et al*., 2002). Recently, the *V. cholerae*-chitin interaction has given rise to further concern with regard to human health from the work of Meibom *et al (2005)* who showed that *V. cholerae* can acquire new genetic material by natural transformation during growth on chitin. This discovery provides another mechanism for this organism to acquire genes effectively for both adapting to the aquatic habitat and for infecting humans (Bartlett and Azam, 2005). Ligands produced by *V. cholerae* that are involved in intestinal colonization, TCP and MSHA, have been recently implicated in chitin binding and biofilm formation on chitin containing surfaces (Montgomery and Kirchman, 1993; Chiavelli *et al*., 2001). Recently, studies conducted by Zampini *et al* (2005) and Kirn *et al*. (2005) showed that a 53kDa

surface membrane protein involved in bacterial attachment to chitin particles and copepods (Tarsi and Pruzzo, 1999), also mediates adherence to cultured intestinal epithelial cells.

As a whole, these findings suggest that persistence of *V. cholerae* in the environment has deep influence on its capability to infect humans and to cause disease. The ability to use the same structure to interact with different substrates, in the environment and human host, may be a common property of pathogenic bacteria that have environmental reservoirs, and may represent a discriminating feature between harmless and potentially pathogenic environmental bacteria.

References

Bartlett DH, Azam F: Chitin, cholera, and competence. *Science* 2005, 310(5755):1775 – 1777.

Bik EM et al. Genesis of the novel epidemic *V. cholerae* O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. *EMBO journal*, 1995, 14:209-16.

Castro-Rosas J, Escartin EF: Increased tolerance of *Vibrio cholerae* O1 to temperature, pH, or drying associated with colonization of shrimp carapaces. *Int J Food Microbiol* 2005, 102(2):195-201.

Chiavelli DA, Marsh JW, Taylor RK: The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. *Appl Environ Microbiol* 2001, 67(7):3220-3225.

Colwell RR: Global climate and infectious disease: The cholera paradigm. *Science* 1996, 274(5295):2025 – 2031.

Colwell RR et al. Serogroup conversion of *V. cholerae*. *Canadian journal of microbiology*, 1995, 41:946-50.

Harvell CD et al., Emerging marine diseases—climate links and anthropogenic factors, *Science* 285 (1999) (5433), pp. 1505–1510

Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK: The *Vibrio cholerae* chitin utilization program. *Proc Natl Acad Sci U S A* 2004, 101(8):2524-2529.

Huq A, Colwell RR, Rahman R, Ali A, Chowdhury MAR, Parveen S, Sack DA, Russek-Cohen E: Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. *Appl Environ Microbiol* 1990, 56:2370-2373.

Kirn TJ, Lafferty MJ, Sandoe CMP, Taylor RK: Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae*. *Mol Microbiol* 2000, 35(4):896-910.

Kirn TJ, Jude BA, Taylor RK: A colonization factor links *Vibrio cholerae* environmental survival and human infection. *Nature* 2005, 438(7069):863-866.

Lipp EK, Huq A, Colwell RR: Effects of global climate on infectious disease: the cholera model. *Clin Microbiol Rev* 2002, 15(4):757-770.

Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK: Chitin induces natural competence in *Vibrio cholerae*. *Science* 2005, 310(5755):1824-1827.

Montgomery MT, Kirchman DL: Role of chitin-binding proteins in the specific attachment of the marine bacterium *Vibrio harveyi* to chitin. *Appl Environ Microbiol* 1993, 59(2):373-379.

Pruzzo C, Crippa A, Bertone S, Pane L, Carli A: Attachment of *Vibrio alginolyticus* to chitin mediated by chitin-binding proteins. *Microbiology-UK* 1996, 142:2181-2186.

Reguera G, Kolter R: Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. *J Bacteriol* 2005, 187(10):3551-3555.

Sasmal D, Guhathakurta B, Bhattacharya SK, Pal CR, Datta A: Role of cell-associated N-acetyl-D-glucosamine specific haemagglutinin in the adhesion of *Vibrio cholerae* O1 to rabbit intestinal epithelial cells in vitro. *FEMS Immunol Med Microbiol* 1996, 13:101–105.

Shukla BN, Singh DV, Sanyal SC: Attachment of non-culturable toxigenic *Vibrio cholerae* O1 and non-O1 and *Aeromonas* spp. to the aquatic arthropod geris-spinolae and plants in the river-ganga, Varanasi. *FEMS Immunol Med Microbiol* 1995, 12(2):113-120.

Tarsi R, Pruzzo C: Role of surface proteins in *Vibrio cholerae* attachment to chitin. *Appl Environ Microbiol* 1999, 65(3):1348-1351.

Waldor MK, Mekalanos JJ: Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 1996, 272(5270):1910-1914.

Zampini M, Pruzzo C, Bondre VP, Tarsi R, Cosmo M, Bacciaglia A, Chhabra A, Srivastava R, Srivastava BS: *Vibrio cholerae* persistence in aquatic environments and colonization of intestinal cells: involvement of a common adhesion mechanism. *FEMS Microbiol Lett* 2005, 244(2):267-273.

APPARENT ANTIBIOTIC MISUSE IN ENVIRONMENTAL ECOSYSTEMS AND FOOD

Bezirtzoglou E*, , Alexopoulos A.and Voidarou C.

Democritus University of Thrace, Faculty of Agricultural Development, Department of Food Science and Technology, Laboratory of Microbiology, Biotechnology and Hygiene. Orestiada, GR68200, Greece. *Corresponding Author: empezirt@agro.duth.gr

Antimicrobial resistance is actually a major public health problem. Antibiotic effectiveness is threatened because of an increase in antibiotic resistant bacteria [1,2]. When antibiotics are given to battle bacterial infections, most of the bacteria are killed. However, it is well known that antimicrobial drugs can produce alterations on host's indigenous microbiota selecting resistant organisms, which can appear as opportunistic pathogens [3,4]. Additionally, antibiotics are fed to animals not only for treating an eventual infection but mainly to promote the faster flesh growth of the animal for commercial purposes.

Antibiotic resistance has showed as well in plant pathogenic bacteria [5]. Furthermore, hospital, industrial and domestic sewage worsen the situation.Surviving resistant bacteria may be passed on other hosts in different ways or on their mutations to new multiplying bacterial generations. As a result, 'pathogenicity islands' are formed harboring multiple drug resistance genes.

On the present paper, in order to estimate the antibiotic resistance profile in our country, environmental: such as soil and water, food: such as honey, ham, dairy traditional foods including fisheries and finally human intestinal flora samples were studied for antimicrobial resistance development.

In order to investigate the antibiotic resistance extent, environmental, biological and samples from foods were examined and specifically water, soil, honey, fish, ham, milk as well as samples from the human intestinal flora. All isolated bacterial strains from the above samples were tested for their antimicrobial activities in Mueller–Hinton agar by applying the antibiotic discs and detected using the disk diffusion method, according to the standards by the National Committee for Clinical Laboratory Standards(6)(7)(8)(9)(10)(11)(12). The following concentrations in antibiotic discs were used: Amoxicillin with Clavulanic Acid (30μg), penicillin G (10units), neomycin (30μg), tetracycline (30μg), doxycycline (30μg), erythromycin (15μg), tylosin (30μg) , metronidazole (15μg), ofloxacin (5μg), vancomycin (30μg), oxytetracycline (30μg), ceftiofur (30μg), ampicillin (10μg), sulfamethoxazole with trimethoprim (25μg), ciprofloxacin (5μg), cephalexin (30μg). Testing for *Campylobacter sp.* was supplemented with horse blood. The Bauer–Kirby technique was used to determine

susceptibility to antibiotics for *L. monocytogenes.* Antibiotic resistance on specific antibiotics for each pathogen were also studied. A multiresistance antibiotic profile was effective for most bacterial strains, and pronounced resistance profiles were observed for the commonly used antibiotics as ampicillin, penicillin, cephalothin and streptomycin followed by ceftriaxone and gentamycin. Albeit this high observed resistance profile, amikacin, aztreonam, chloramphenicol and tylosin conserved an almost absent resistance. Tylosin presented important resistance in soil samples. Fish samples showed a lower multiresistant profile, while mussels showed increased resistance to most of the antibiotics. Antibiotics commonly used for therapeutic purposes, as well as antibiotics added to feed stuff of animals for increasing animal flesh production should contribute to the extensive spreading of antibiotic resistance in food and the environment. Systematically monitoring and surveillance of the microbiological quality of selected ecosystems by implementation of a public education campaign must be done, in order to preserve food quality, optimizing sewage treatment and safeguard the public health.

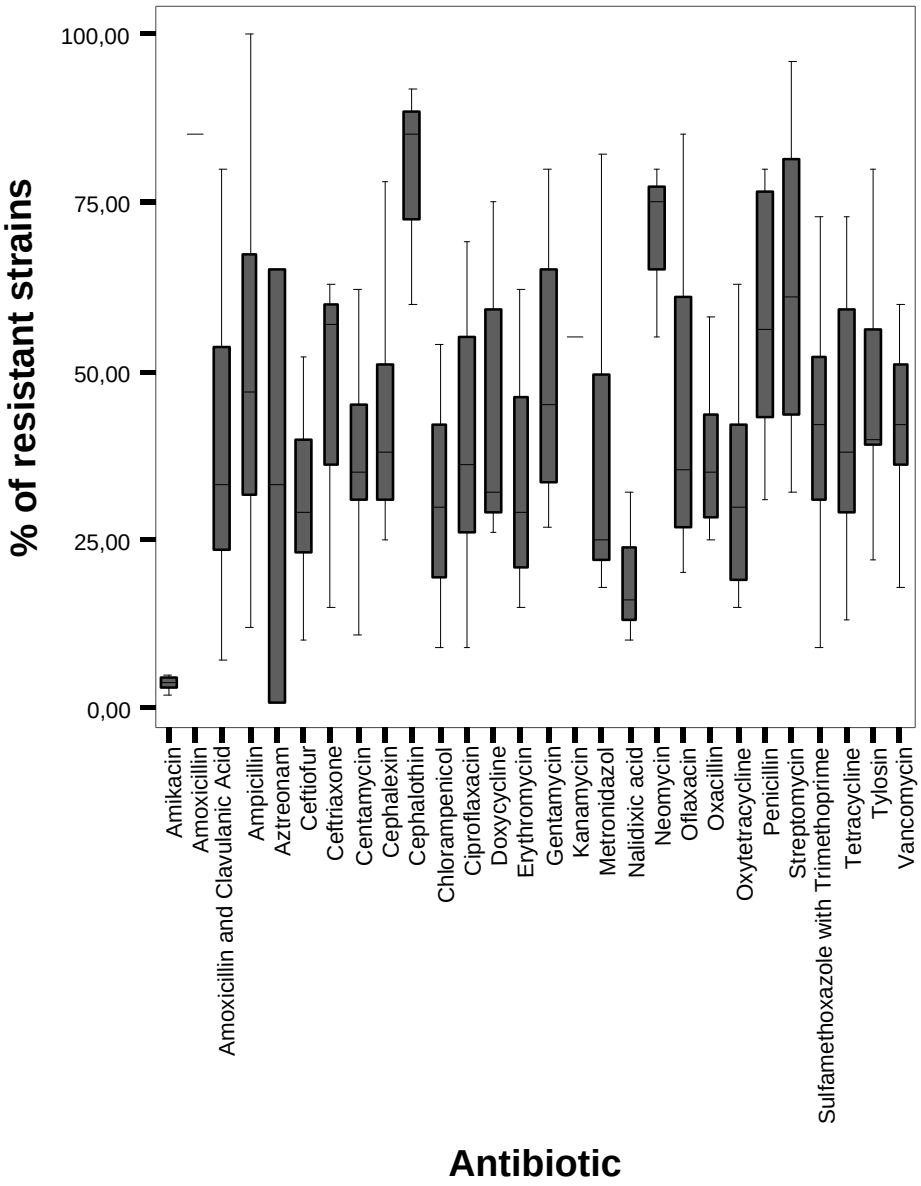
REFERENCES

1. Martnez JL,Baquero F. 2002. Interactions among strategies associated with bacterial infection:Pathogenicity,epidemicity and antibiotic strategy. Clin Microbiol Rev 15(4), 647-679.
2. Wright GD, Sutherland AD. 2007. New strategies for combating multidrug resistant bacteria. Trends Mol Med , May 8 [Epub ahead of print] 1-16.
3. Armstrong JL, Shigeno DS, Calomiris JJ and Seidler RJ. 1981. Antibiotic-Resistant Bacteria in Drinking Water. Appl Environ Microbiol 42(2), 277-283.
4. Bezirtzoglou E. 1997. The intestinal microflora during the first weeks of life. Anaerobe 3, 173-177.
5. Kumar K, Gupta SC, Baidoo SK,Chandler Y,Rosen CJ. 2005. Antibiotic uptake by plants from soil fertilized with animal manure. J Environ Qual 34(6), 2082-2085.
6. APHA, 1990 . Standard methods for the examination of water and waste water. 17th edition. Washington D.C. USA.
7. Facklam RR. Streptococci and aerococci. In: Lennette EH, Balows A, Hausler Jr WJ ,Truant JP(ed), Manual of Clinical Microbiology, 3rd ed, ASM, Washington DC.
8. Garcia-Garrote F, Cercenado E, Bouza E. 2000. Evaluation of a new system, VITEK 2, for identification and antimicrobial susceptibility testing of enterococci. J Clin Microbiol. 38(6), 2108-11.
9. Eisner A, Gorkiewicz G, Feierl G, Leitner E, Kofer J, Kessler HH, Marth E. 2005. Identification of glycopeptide-resistant enterococci by VITEK 2 system and

conventional and real-time polymerase chain reaction. Diagn Microbiol Infect Dis. 53(1),17-21.

10. Bezirtzoglou E., Romond C. 1990. Rapid identification and enumeration of C. perfringens in the human faecal flora, Micr. Ecol. H. Dis. (MEHD) 3, 159-163.
11. National Committee for Clinical Laboratory Standards 1997: Methods for antimicrobial susceptibility testing of anaerobic bacteria, 4th Ed. Approved standard. NCCLS publication no. M11-A4. National Committee for Clinical Laboratory Standards, Wayne, PA.
12. Charteris, WP, Kelly, PM, Morelli, L, and Collins, JK. 2001. Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. J. Food Prot. 64, 2007–2014.

Figure : Prevalence of resistant strains* isolated from environmental, biological and food samples (N= 1320), according to the antibiotic substance.



* List of Strains:
B. cereus, B. subtilis subsp. subtilis, C. perfringens, Campylobacter coli, Campylobacter jejuni, E. coli, Enterococcus sp., L. paracasei subsp. paracasei, L. plantarum, S. aureus, S. aureus subsp. anaerobius, S. faecalis, S. faecium, S. pyogenes, S. typhimurium, Salmonella sp.

Colonization of rectal and vaginal epithelium by three different *Lactobacillus* strains administered orally.

Piotr B. Heczko[1], Magdalena Strus[1], Piotr Kochan[1], Zbigniew Chełmicki[2], Artur Chełmicki[2], Tomasz Gosiewski[1], Andrzej Pałucha[3]

[1] Chair of Microbiology, Jagiellonian University Medical College, Krakow, Poland
[2] Clinic of Gynaecological Endocrinology, Silesian Medical University, Katowice, Poland
[3] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

Introduction

A phenomenon of migration or translocation of certain human pathogens like *Escherichia coli* or *Streptococcus agalactiae* from intestinal reservoir to vaginal orifice has been found and documented some decades ago. Less is known about similar movement of the normal microbial gut flora, e.g. like as members of the *Lactobacillus* genus. Still, there are evidences for translocation of lactobacilli from rectum to vagina coming from clinical studies conducted by Hilton in 1992 on prevention of vaginal candidosis by consumption of yogurt and more recent studies by Reid and colleagues (2003, 2004) on oral application of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 strains to prevent bacterial vaginosis. A more recent but indirect evidence comes from observations done by Antonio and co-workers (2005) that increased numbers of lactobacilli in rectum are related to decreased risk of bacterial vaginosis.

Aims

Aims of our human observation studies were to confirm if orally applied *Lactobacillus* strains belonging to three different species and showing different adherence toward vaginal and colon epithelial cells in vitro will be able to colonize rectal and vaginal epithelium in vivo and whether this colonization is related to decreased vaginal pH and Nugent score values and an increase in total vaginal *Lactobacillus* populations.

Materials and Methods

The study was performed on 37 healthy women volunteers who were enrolled on the basis of an initial gynaecological examination, history of previous episodes of vaginal inflammation and elevated pH and Nugent score values. The subjects were once a day swallowing a mixture of three probiotic *Lactobacillus* strains of vaginal origin: *L. fermentum 57A, L. plantarum 57B, L. gasseri 57C* contained in a dietary supplement preparation called PrOVag produced by Biomed company from Krakow. The capsules were administered during two consecutive inter- menstrual periods.

Rectal and vaginal colonization by the ingested *Lactobacillus* strains was followed by taking swabs from rectum and vagina during 6 consecutive clinical visits. The swabs were placed in a transport medium and then plated and quantitatively cultured both for lactobacilli and

vaginal pathogens. Moreover, vaginal pH and Nugent score values were measured during each visit.

To assess colonization, *Lactobacillus* colonies picked up from vaginal and rectal cultures grown on selective media were initially characterized as belonging to *L. plantarum, L. gasseri* and *L. fermentum* species using phenotyping methods. *L. gasseri* and *L. fermentum* isolates were further identified using pulse-field gel electrophoresis. Characterization of *L. plantarum* strains was performed by the multilocus sequence typing method (MLST) previously described by de las Rivas (2006). Polymorphic sites of six *L. plantarum* housekeeping genes (gyrB, gdh, mutS, ddl, purK and pgm) were sequenced and the resulted sequences compared in order to identify the applied strain *L. plantarum* 57B.

Results

Altogether, 24 women completed the study. Their *Lactobacillus* microflora of rectum and vagina and their vaginal pH and Nugent score were checked during 1 clinical visit before and during 6 visits after ingestion of the tested strains. The strains appeared both in rectal and vaginal cultures however with a different frequency related to species: *L. fermentum* 57A which shows a high adherence rates to colon epithelial cells *in vitro* was present less frequently in vaginal cultures while *L. gasseri* adhering more frequently to vaginal cells was found more often in vaginal cultures.

Colonization with the tested strains was followed at first by an increase in the total numbers of lactobacilli in rectal swabs which occurred usually in around 10 days after starting the ingestion of the tested strains. Changes in the parameters related to vaginal health were as follows: decreased pH and Nugent score values in vagina and increased numbers of total lactobacilli in vagina appeared later, in about 50 days after starting the oral administration of the tested strains and lasted for about 2 weeks after its stopping; a return to initial values was seen 2-3 days after the first menstruation post-cessation of the supplement.

Conclusions

- Oral application of vaginal probiotic *Lactobacillus* strains belonging to 3 different species results in colonization of rectal and then vaginal epithelium in a few weeks after starting regular once a day administration.
- Colonization rates of the rectal or vaginal epithelium by these strains are related to their various grades of adherence affinities tested *in vitro*.
- Colonization is related to improvement of general parameters of the vaginal health: lowering pH and Nugent score values and increase in total *Lactobacillus* counts in vagina.
- These changes become evident in about 6 weeks after starting the ingestion of the tested *Lactobacillus* strains and disappear in about 2 weeks after its cessation i.e. usually after the second menstruation.

Literature

Antonio, MAD, Rabe LK, Hillier SL. Colonization of the rectum by Lactobacillus species and decreased risk of bacterial vaginosis. J Infect Dis 2005,192:394-398.

Hilton E, Isenberg HD, Alperstein P, France K, Borenstein MT. Ingestion of yogurt containing Lactobacillus acidophilus as prophylaxis for candidal vaginitis. Ann Intern Med 1992,116:353-357.

de Las Rivas B, Marcobal A, Munoz R. Development of a multilocus sequence typing method for analysis of Lactobacillus plantarum strains. Microbiology 2006;152:85-93.

Reid G, Charbonneau D, Erb J, Kochanowski B, Beuerman D, Poehner R, Bruce AW. Oral use of Lactobacillus rhamnosus GR-1 and L. fermentum RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunol. Med. Microbiol. 2003,35:131-134.

Reid G, Burton J, Hammond JA, Bruce AW. Nucleic acid-based diagnosis of bacterial vaginosis and improved management using probiotic lactobacilli. J Med Food. 2004,7:223-228.

MICROFLORA ASSOCIATED CHARACTERISTICS IN CHILDREN WITH CELIAC DISEASE

Norin E[1], Tjellström B[1, 2], Stenhammar L[2], Midtvedt T[1]

[1]Department of Cell and Molecular Biology, Karolinska Institute, Stockholm;

[2]Department of Paediatrics, Linköping University & Norrköping Hospital, Norrköping; Sweden

The aim of our study was to investigate the functional status of the intestinal microflora in children with coeliac disease (CD), untreated and treated ones, and to compare these with healthy control children, by analysis of short chain fatty acid pattern (SCFAs) in faecal samples.

The study comprised 110 coeliac children of both sexes of whom 36 children were newly diagnosed and still not had been turned on to a gluten free diet. The results from the two CD groups were compared with SCFA pattern in faecal samples from 42 healthy control children. CD was diagnosed according to current criteria formulated by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition.

There was a significant difference between CD children and controls regarding acetic acid and i-butyric acid and i-valeric acid ($p < 0.05$). The other SCFAs studied did not show any significant differences between the groups.

We concluded that the result of this study indicates that there is an interesting difference in the metabolic activity of the intestinal bacterial flora in children with CD compared to healthy controls – irrespectively dietary treatment. Since SCFA function reflects the gut flora and dietary cross-talks, we speculate on possible implications for the pathogenesis of CD.

Antagonist effect of naturally occurring peptide in celiac disease

Marco Silano, Francesca Maialetti, Maria Rita Dessì°, Olimpia Vincentini, Mariachiara Petrassi, Massimo De Vincenzi.
Istituto Superiore di Sanità, Rome, Italy
°Università Tor Vergata, Rome, Italy

Celiac disease (CD) is an autoimmune permanent enteropathy, triggered in susceptible individuals, by the ingestion of gluten, a storage protein fraction present in wheat grain, and by similar proteins of rye and barley (1). Approximately 96% of CD patients express the HLA class II molecule DQ2, while the remainder mostly express the less common haplotype DQ8 (2). All the intestinal gluten-sensitive T cell clones are DQ restricted, reflecting the critical role of this molecule in the pathogenesis of CD. After being bound to DQ, certain deamidated gluten peptides are recognized by interferon-γ (IFN-γ) producing T-cells, thus driving the inflammatory response that causes the histological features of celiac intestinal mucosa—villous atrophy, hyperplasia of the crypts and lymphocyte infiltration (3).

We have previously described a decapeptide (p10mer, sequence QQPQDAVQPF) from the alcohol soluble protein fraction of durum wheat that prevents peripheral celiac lymphocytes from activation by gliadin peptides (4). In this work, we have focused on the production of two key cytokines, IFN-γ and IL-10, by small intestinal CD4+ cells from celiac children following exposure of these cells to gliadin peptides and peptide 10mer to investigate the possibility that the p10mer sequence is able to promote a shift from a Th1-type towards a Th-2 type of pathogenic immune response in celiac disease. We assumed that T-cell proliferation and INF- gamma production provides a measure of Th-1response and IL10 of Th2. Moreover we describe the ability of this decapeptide to protect the enterocytes of coeliac mucosa from the gliadin peptides- induced apoptosis.

METHODS

The mucosa cells collected from 8 coeliac patients were cultured in RPMI medium with 1 x 10^6/ml autologous peripheral blood mononuclear cells (PBMCs) and 10 U/ml human recombinant IL-2 (Peprotech, London, UK). Cells were restimulated every 7

days with deamidated PTgli (200 μg/ml), feeder cells and IL-2. T-cell proliferation , INF-γ and IL-10 concentration in culture medium were measured after 24 to 96 hs by BrdU cell proliferation test (Chemicon International, Temencula, CA, USA) and commercial ELISA kits (Biosource, Camarillo, CA, USA) respectevely, according to ther manufactures' instructions.

DNA fragmentation as index of cell apoptosis was assayed on 4 μm tissue sections TUNEL using a commercial kit according to manufacturer's instruction (Roche, Basel, Switzerland). Two colour immunofluorescence staining was used to determine if the TUNEL+ cells were epithelial cells or CD3+ T-lymphocytes.

RESULTS

Whereas the incubation with PT –gli (500 mg/ml) increased T-cell proliferation measured by the incorporation of the 5-bromo-2- deoxyuridine to an SI of 3.9± 0.16 at day 4, the simultaneous incubation with 10mer (10μg/ml) resulted in an SI 1.05±0.09, similar to that T cells incubated with culture medium only.

The relase of IFN-gamma in the culture medium from celiac mucosa lymphocytes, measured after the exposure to PT- gli resulted in a substantial increase (122±3.7pg/ml at 96' hs vs 70.9±5.7for the negative control; $p<0.001$). The simultaneous to exposure peptide 10mer totally abolished it (71.6±2.9 pg/ml at 96' hs)

IL-10 is produced by CD4+ T cells when activated in order to control the immune response and prevent it from expanding excessively. The differences seen in its release at 24 hs after the various treatments is apparently related to this effect. When lymphocytes were exposed to medium alone, no immune activation of lymphocytes occurred and the IL-10 production remained low at 24 hrs . But when dPTgli, both alone or simultaneously with dp10mer, were present in culture medium, IL-10 production increased, presumably to modulate the immune response. The exposure of mucosal gluten-specific T cells to dPTgli failed to maintain over the course of 96 hrs the IL-10 concentration that we found was initially elevated in the culture medium (26.1±1.12 pg/ml after 24 hours vs. 20.1±1.01 pg/ml at 96 hours, $p< 0.001$). In contrast, the simultaneous treatment of T cells with dPTgli and d10mer increased the release of IL-10 over the course of time (24.6±1.16 pg/ml after 24 hours vs. 31.4±0.83 pg/ml at 96 hours, $p< 0.001$). Interestingly, the individual exposure of T lymphocytes to dp10mer alone also resulted in an increasing release of IL-10 over the incubation time (15.4±0.96 at 24 hours vs 28.1±0.95 at 96 hs, $p < 0.001$) (Fig 3A).

The protective effect of 10mer is evident when the mucosa specimens were incubated with PT-gli peptide 10mer. In fact, in the absence of peptide 10mer, a number of apoptotic cells in the coeliac mucosa, were detected whereas no apoptotic cells are observed in the presence of peptide 10mer.
Moreover, when the mucosa specimens are incubated with PT-gli alone, a very heavy FAS staining is evident, whereas no FAS expression by enterocytes is detectable when 10mer is present in the culture medium.

CONCLUSION

This is the first report about the antagonist effect against gliadin peptides toxicity of a peptide whose sequence is present in a cereal avoided in the gluten-free diet. This finding suggest a new therapeutic strategy for CD, consisting of the identification of non toxic sequences in the cereals and the over-expression of the genome encoding for these sequences. However, further studies are required to evaluate the protective effects of this peptide *in vivo* and the safety of such modified grains.
The mechanism responsible for the antagonist effect of this peptide against gliadin peptides toxicity is yet to be elucidated, but it is likely that 10mer interferes with T cell activation by gliadin peptides, as exerted by artificially designed antagonist ligand peptides.

REFERENCES

1. Jabri B, Kasarda DD, Green PH. Innate and adaptive immunity: the yin and yang of celiac disease. Immunol Rev 2005;206:219-31.
2. Tollefsen S, Arentz-Hansen H, Fleckenstein B, et al. HLA-DQ2 and -DQ8 signatures of gluten T cell epitopes in celiac disease. J Clin Invest 2006;116:2226-36.
3. Jabri B, Sollid LM. Mechanisms of disease: immunopathogenesis of celiac disease. Nat Clin Pract Gastroenterol Hepatol. 2006;3:516-25.
4. Silano M., Di Benedetto R., Trecca A. et al. A decapeptide from durum wheat prevents celiac peripheral lymphocytes from activation by gliadin peptides Pediatr Res 2007; 61; 67-71.

Microbial biofilms in the human gastrointestinal tract

S. Macfarlane

Microbial biofilms are widespread in nature occurring in soils, sediments and aquatic systems as well as in various sites associated with the human body. Over recent years, a substantial amount of information has been acquired regarding the composition, structure and metabolism of oral biofilms, however, there has been a paucity of studies on biofilms growing in the gastrointestinal tract. Despite this, it is likely that the biofilms that form on the gut mucosa and on the surfaces of particulate material in the gut lumen, are highly evolved assemblages resembling those found in the oral cavity (1).

Until quite recently, it was thought that the healthy oesophageal microbiota was relatively simple, and was mainly composed of low numbers of facultative anaerobes such as streptococci and lactobacilli originating from the oral cavity. However, culturing and sequencing of bacteria obtained from aspirates and the oesophageal mucosa of patients with Barrett's oesophagus (BO) and healthy controls (2), it was found that a more complex heterogeneous community of microorganisms exists in this part of the upper gut. BO is a complication of gastro-oesophageal reflux disease in which the usual squamous mucosa of the distal oesophagus transforms into a columnar lined epithelium. Patients with BO have a greatly increased risk of developing oesophageal adenocarcinoma, which is now the seventh commonest cause of cancer death in the UK. We have found higher numbers of bacteria and a greater species diversity on the distal oesophageal mucosa of Barrett's patients, compared to healthy controls, and have shown that the microorganisms often occur in biofilms and in microcolonies on the epithelial surface. High numbers of nitrate-reducing species such as campylobacter and veillonella were commonly isolated from the oesophageal mucosa of Barrett's patients, though not from control subjects, and due to the potential for production of N-nitroso compounds and nitric oxide from nitrite, it is thought that these organisms may be involved in DNA damage and cancer induction (2).

Due to the short retention time of intestinal contents, gastric acid and other defence mechanisms, the human stomach is usually colonised by low numbers of bacteria, including lactobacilli, streptococci and *Helicobacter pylori.* Percutaneous endoscopic gastrostomy

(PEG) feeding is a common form of enteral nutrition used for long-term support to enhance the nutritional status of patients unable to ingest food through the normal route, in which the feeding tube passes through the abdomen into the stomach. Significant microbial overgrowth can occur in the stomach of these PEG tube patients and extensive microbial biofilms have been shown to form on the surfaces of PEG tubes (3) Facultative anaerobes were shown to predominate on PEG tubes and in gastric aspirates from enteral-fed patients, however, enterococci, campylobacters, corynebacteria and the yeast kloeckera were only found growing on the surfaces of PEG tubes, while bifidobacteria and klebsiella were only found in the gastric aspirates (3).

In the large gut, biofilms also occur as mixtures of living and dead bacteria in the mucus layer lining mucosal surfaces, and on food residues in the gut lumen. Bacteroides and bifidobacteria were found to be the predominant bacteria attached to particulate matter in stools and were shown to be phenotypically similar in composition to non-adherent microbiotas (4). Biofilm populations were found to be more efficient in digesting polysaccharides than the non-adhering communities, which were able to break down oligosaccharides most rapidly. However, short-term fermentation experiments showed that the two communities were metabolically distinct in that the principal fermentation product formed by strongly adherent bacteria was acetate, whereas the non-adherent populations produced higher levels of butyrate.

Ulcerative colitis (UC) is one of the two main forms of idiopathic inflammatory bowel disease (IBD). There is evidence, mainly from animal studies, that commensal gut microorganisms are involved in either the initiation or maintenance of UC. Recent interest has focused on the role of mucosal biofilms which may be important in disease aetiology due to their close proximity to gut tissues and the host’s immune system. Confocal laser scanning microscopy combined with FISH is a useful tool for analysing the three dimensional structure of gastrointestinal biofilms. Using this technique bacteria growing on the rectal mucosa were shown to be distributed throughout the mucus layer (5), often occurring in microcolonies. Viable staining of these microcolonies showed increased numbers of living cells close to the epithelial surface, which may result in high levels of immunogenic substances at the mucosal

surface, stimulating inflammatory processes. Several studies have shown that a dysbiosis in bacterial populations occurs in patients with UC compared to healthy controls. Based on chemotaxonomic analysis of mucosal isolates, a 30-fold reduction in bifidobacterial species was found, with an increase in more proinflammatory bacteria such as peptostreptococci and *E. coli* (5).

Maintenance treatments for UC usually involve the use of immunosuppressants, anti-inflammatory drugs, antibiotics and surgery, however, some of these drugs can have deleterious side-effects restricting their use in some patients. So far, antibiotics have been of limited value since the bacteria that cause UC are unknown, while microorganisms growing in biofilms are considerably more resistant to antimicrobial substances than free-living cells. Although little is known of how diet can effect the composition of mucosal communities, in one study feeding a prebiotic mixture to patients for two weeks prior to colonoscopy was shown to increase more than 10-fold numbers of mucosal bifidobacterial and eubacterial in the proximal and distal colon (6). Therefore the use of prebiotics, probiotics or synbiotics may be useful as therapies for correcting the dysbiosis that occurs on the mucosal surface in IBD (7). To this end, we carried out a RCT involving a synbiotic in patients with active UC (8). The synbiotic was composed of a probiotic *Bifidobacterium longum* isolated from healthy rectal mucosa and the prebiotic Synergy 1^{TM} (Orafti) a combination of oligofructose and inulin. At the end of the study, a significant increase (42-fold) in bifidobacterial numbers on the rectal mucosa was found in the synbiotic group compared to the controls, together with reductions in inflammatory infiltration and ulceration, and regeneration of normal epithelium.

References

1. Macfarlane S, Dillon JF (2007) *J Appl Microbiol* 102, 1187-1196.
2. Macfarlane S *et al.* (2007) *Clin Infect Dis* 45, 29-38.
3. O'May GA *et al.* (2005) *J Clin Microbiol* 43, 3059-3065.
4. Macfarlane S, Macfarlane GT (2006) *Appl Environ Microbiol* 72, 6204-6211.
5. Macfarlane S *et al.* (2004) *Clin Infect Dis* 38, 1690-1699.
6. Langlands SJ *et al.*(2004) *Gut* 53,1610-1616.
7. Macfarlane S *et al.* (2005) *BJN* 93, S67-S72.
8. Furrie E *et al.* (2004) *Gut* 54, 242-249.

Switch from planktonic life to biofilm formation: a major event in pneumococcal pathogenesis

Trappetti C 1, F Iannelli 1, A Kadioglu 2, PW Andrew 2, G Pozzi 1, MR Oggioni 1

1 Universita di Siena, Siena, Italy; 2 University of Leicester, Leicester, UK.

Objectives. Gene expression analysis of Streptococcus pneumoniae during infection in mice showed two distinct gene expression patterns depending on the disease model. Objective of the work was to identify in vitro situations with comparable gene expression pattern, to develop tools for analysis of these situations to be used as validated tools for the characterisation of pneumococcal physiology during infection.
Methods. Quantitative real time RT PCR was used gene expression analysis and a novel biofilm model for pneumococcal biofilm was developed.
Results. Bacteria in pneumonia and meningitis showed the same gene expression pattern as in static in vitro biofilm, including a characteristic up regulation of the competence related quorum sensing genes. In contrast bacteria in blood showed a pattern comparable to growth in liquid culture, characterised in turn by down regulation of the competence regulon. In vitro mature was found to be dependent on peptide cell-cell signalling and accordingly, the use of the competence peptide CSP during pneumococcal lung infection rendered bacteria more virulent. In contrast, intravenous administration of CSP rendered pneumococci less virulent in a sepsis model. When pneumococci grown in a biofilm were used for challenge they were more effective in inducing meningitis and pneumonia, while cells from liquid culture were more effective in inducing sepsis.
Conclusion. The main finding of this work is the correlation of the physical state of pneumococci to acute infections; during sepsis pneumococci are in a planktonic state, while during tissue infection, such as pneumonia or meningitis, pneumococci are in a biofilm-like state.

The role of multispecies microbial biofilms in the occlusion of biliary stents

[1]Guaglianone E, [2]Cardines R, [2]Mastrantonio P, [3]Di Rosa R, [4]Penni A, [4]Puggioni G, [5]Basoli A, [5]Fiocca F, [1]Donelli G.

[1]Department of Technologies and Health and [2]Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità; [3]Chair of Clinical Immunology, 2nd Faculty of Medicine, [4]Department of Clinical Medicine and [5]Department of General Surgery "P. Stefanini", University of Rome "La Sapienza", Rome, Italy.

Introduction

Endoscopic stenting is a standard palliative approach for the treatment of a variety of diseases involving biliary obstruction (1). However, the major limitation of this approach is represented by stent occlusion followed by life-threatening cholangitis, often requiring stent removal and replacement with a new one (2). Although it is generally believed that microbial colonization of the inner surface of the stent plays an important role in initiating the clogging process, so far available data are not enough for a full understanding of this phenomenon (3,4).
In fact, it is known that when a biliary stent is inserted across the sphincter of Oddi, the loss of the antimicrobial barrier represented by the sphincter itself and the low pressure in the common bile duct, allow reflux of duodenal content thus promoting an ascending microbial colonization (5). The sessile mode of growth and the exopolysaccharide production, which leads to the subsequent establishment of a thick biofilm, provides microorganisms with an efficient protection from both antibacterial agents and phagocytic cells. The aim of this study was to analyze the structure of the microbial biofilm grown in the lumen of 15 clogged biliary stents and to identify the microbial species involved in the clogging process.

Material & Methods

Stents.
Fifteen biliary stents were removed from patients who had undergone endoscopic stent insertion due to malignant or benign bile duct obstruction. Immediately after removal, segments of approximately 1 cm were cut under sterile conditions from the distal, proximal and central portions of the stents, put into sterile tubes and immediately sent to the lab.

Microbiological analysis.
For the isolation and identification of aerobic microorganisms, the segments obtained from the distal end of stents were bisected along their long axis, placed into PBS (pH = 7.4) and sonicated in ice for 10 min at 2 μA (Soniprep 150, MSE); 0.1 and 0.01 ml of the suspension were plated on non-selective media and incubated for 24-48 h under aerobic conditions. Isolated microorganisms were counted and identified at the species level according to standard biochemical tests.
For the isolation and identification of anaerobic bacteria, all procedures were performed in an anaerobic cabinet. Each segment of the proximal portion of the stents was bisected along its major axis and the inner luminal surface of one section of the stent was scraped with a sterile wire loop to remove the sludge and adherent bacteria. Then, the suspension was serially diluted (1:10) in PBS and one hundred microliters of each dilution was spread on prereduced Columbia agar plates supplemented with 5% sheep blood, 0.1% vitamin K_1 and hemin and incubated anaerobically at 37°C for 72 hours. The other half of the stent was transferred into prereduced BHI broth, vortexed and incubated anaerobically for 7 days. After appropriate dilutions, samples were streaked onto Columbia blood agar plates to determine the bacterial density (CFUs). Individual colonies were selected on the basis of their morphology and plates were also incubated aerobically to exclude the aerobic growth. Anaerobes were identified by using the RAPID ID 32A kit (Bio Mèrieux).

Scanning electron microscopy.
Segments cut from the central part of stents and bisected as already mentioned were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.1% ruthenium red (Sigma) at room temperature for 30 min. Following postfixation in 1% OsO_4 for 30 min, samples were dehydrated through graded ethanols, critical point dried in hexamethyldisilazane (Polysciences Inc., Warrington, PA, USA) and gold coated by sputtering. Samples were examined with a Cambridge 360 scanning electron microscope.

Results and Conclusions

At macroscopical level, all the examined stents resulted to be more or less occluded by a heterogeneous sludge. Microbiological analysis revealed the presence of a mixed microbial colonization. Isolates belonging to both aerobic and anaerobic bacterial species, as well as to fungi, were identified.
Among the aerobic bacteria, the gram-positive *Enterococcus faecalis* was the most frequently isolated species followed by the gram-negative *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp. and *Enterobacter* spp.
Bacteroides spp. and *Fusobacterium* spp. among gram-negatives and *Clostridium* spp. among gram-positives were the most frequently isolated anaerobes. As previously suggested, anaerobes may play an important role in the blockage of biliary stents (6).
Candida albicans and *Candida parapsilosis* represented the only two isolated fungal species.
Scanning electron microscopy investigations, as previously reported (7-11), revealed that sludge present in the stent lumen consist of a rich and assorted microbial flora including enterococcal (**Fig 1a**) and fungal (**Fig 1b**) species mixed to a large amount of amorphous material containing dietary fibers (**Fig 1c**) and crystals of bile salts (**Fig 1d**). Observation of the surface of the clogging material in direct contact with the bile flow showed the presence of a dense amorphous material (**Fig 1e**), probably mucus, in which several bacterial species were immersed to form a dense microbial biofilm (**Fig 1f**).

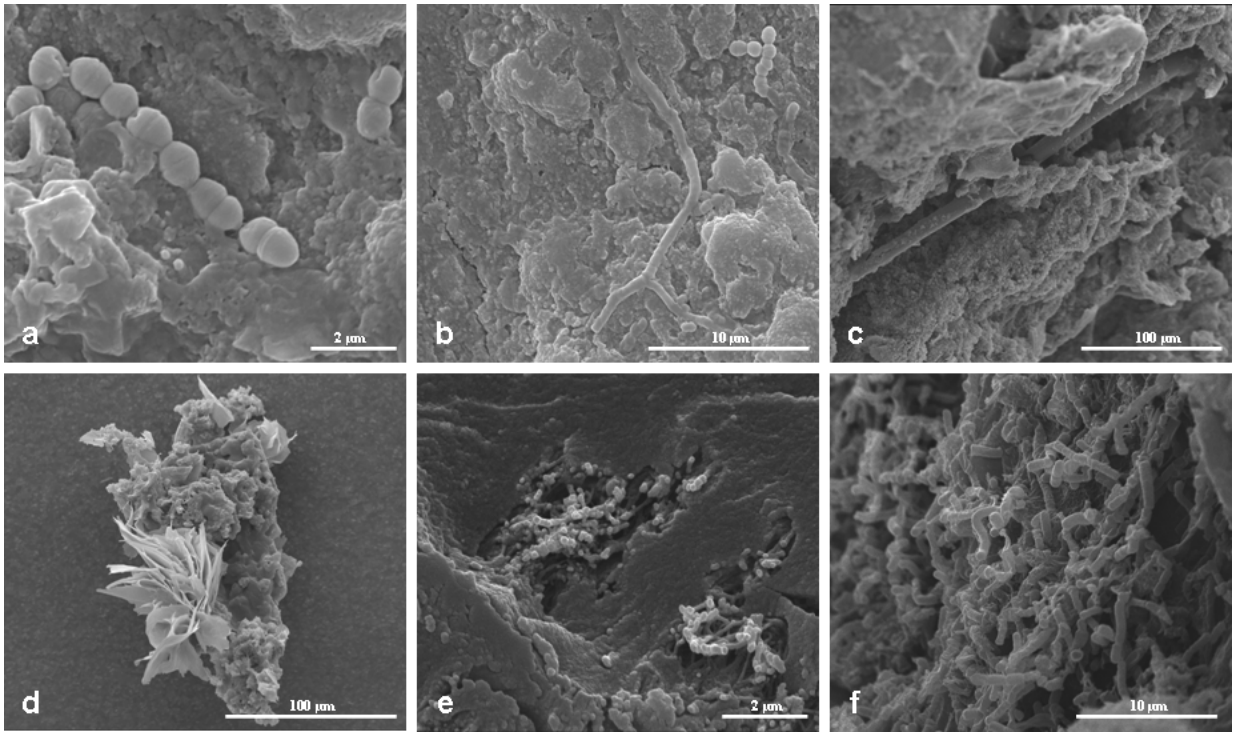


Figure 1. Scanning electron micrographs of biliary stent clogging material.

References

1. **Soehendra, N. and V. Reynders-Frederix**. 1980. Palliative bile duct drainage - a new endoscopic method of introducing a transpapillary drain. Endoscopy **12**:8-11.

2. **Dowidar, N., H. J. Kolmos, H. Lyon, and P. Matzen**. 1991. Clogging of biliary endoprostheses. A morphologic and bacteriologic study. Scand. J. Gastroenterol. **26**:1137-1144.

3. **Leung, J. W., T. K. Ling, J. L. Kung, and J. Vallance-Owen**. 1988. The role of bacteria in the blockage of biliary stents. Gastrointest. Endosc. **34**:19-22.

4. **Donelli, G., E. Guaglianone, R. R. Di, F. Fiocca, and A. Basoli**. 2007. Plastic biliary stent occlusion: factors involved and possible preventive approaches. Clin. Med. Res. **5**:53-60.

5. **Sung, J. Y., J. W. Leung, E. A. Shaffer, K. Lam, M. E. Olson, and J. W. Costerton**. 1992. Ascending infection of the biliary tract after surgical sphincterotomy and biliary stenting. J. Gastroenterol. Hepatol. **7**:240-245.

6. **Leung, J. W., Y. Liu, R. C. Chan, Y. Tang, Y. Mina, A. F. Cheng, and J. Silva, Jr.** 2000. Early attachment of anaerobic bacteria may play an important role in biliary stent blockage. Gastrointest. Endosc. **52**:725-729.

7. **Basoli, A., F. Fiocca, R. Di Rosa, L. Baldassarri, and G. Donelli**. 1999. Biliary stent occlusion: a microbiological and scanning electron microscopy (SEM) investigation, p. 69-80. *In* E. Zanella (ed.), Advances in Abdominal Surgery. Kluwer Academic Publishers, Dordrecht, The Netherlands.

8. **Di Rosa, R., A. Basoli, G. Donelli, A. Penni, F. M. Salvatori, F. Fiocca, and L. Baldassarri**. 1999. A microbiological and morphological study of blocked biliary stents. Microbial Ecology in Health and Diseases **11**:84-88.

9. **van Berkel, A. M., M. J. van, A. K. Groen, and M. J. Bruno**. 2005. Mechanisms of biliary stent clogging: confocal laser scanning and scanning electron microscopy. Endoscopy **37**:729-734.

10. **van Berkel, A. M., M. J. van, V. H. van, A. K. Groen, and K. Huibregtse**. 2000. A scanning electron microscopic study of biliary stent materials. Gastrointest. Endosc. **51**:19-22.

11. **Speer, A. G., P. B. Cotton, J. Rode, A. M. Seddon, C. R. Neal, J. Holton, and J. W. Costerton**. 1988. Biliary stent blockage with bacterial biofilm. A light and electron microscopy study. Ann. Intern. Med. **108**:546-553.

A new bacterial biofilm model for chronic CF lung infections

Soeren Molin, Janus Haagensen

BioCentrum-DTU, 2800 Lyngby, Denmark

It is generally accepted by many that the opportunistic pathogen Pseudomonas aeruginosa survives and persists in CF lungs as a chronic infection through the development of biofilms, and that these biofilms protect the bacteria from antibiotics and attacks by the immune defence system. We have for several years studied P. aeruginosa biofilms in vitro using reference strains such as PAO1 and PA14, and from these investigations there now is a general picture of the biofilm development as it proceeds in laboratory flow cells. Testing this model of biofilm formation in samples from CF lungs of chronically infected patients has produced conflicting results, which suggest that the biofilm state in the CF lung is tightly associated with alginate over-production (the mucoid variants), and that the infecting bacteria have mutations that prevent them from developing biofilms with similar properties as those we have observed in vitro with laboratory strains. Moreover, antibiotic resistance does not seem to be associated with the mucoid biofilm type found in the CF lung. We consequently propose that 1) the biofilm life style in CF lungs is confined to the presence of mucoid variants, 2) that it is not possible to extrapolate from in vitro biofilm systems using reference strains to CF lung biofilms, and 3) that increased antibiotic resistance is not directly associated with mucoidy and biofilm formation. In stead, we propose that the population heterogeneity observed in the CF lung bacterial samples reflects the presence of many different niches in the infected lungs in which different selective conditions drive the adaptation and evolution of subpopulations of the infecting genotype(s).

EFFECT OF VEGETAL FOODS AND MOLECULAR BIOMARKERS

Marisa Porrini
Dept. Food Science and Microbiology – Division of Human Nutrition
University of Milan

There is a large amount of evidence, from epidemiologic, experimental and clinical trial data, indicating that a plant-based diet can reduce the risk of chronic diseases, particularly cancer and heart diseases. It is now clear that there are components in a plant-based diet other than traditional nutrients that can contribute in protecting the organism. Different classes of these biologically active compounds, known as "phytochemicals", have been identified and studied, however whole foods seem often more active than single molecules.

The procedure to follow to confirm the hypothesis of the association between dietary intake and protection from disease is complex and includes epidemiologic surveys, experimental studies and dietary intervention studies.

The essential feature of an intervention study is the use of appropriate biomarkers to measure: exposure to agents implicated in the aetiology of a particular disease or protecting against disease, individual differences in susceptibility, early stages in the disease process, or features of the disease itself. If the endpoint of an intervention study is an index of the probability of disease occurring, measurable by means of a molecular biomarker, then studies can be carried out over a shorter time-scale and with fewer subjects than if the disease itself is the endpoint (1).

A biomarker assay should be reliable and reproducible, and it should have a clear and quantitative relationship to the specific aspect of the disease process it is supposed to represent (2).

With the aim to contribute to clarify the role of fruit and vegetable consumption on health, we performed several intervention studies using some molecular biomarkers to investigate antioxidant and protective properties: lipid peroxidation products, such as malondialdehyde and isoprostanes; endogenous DNA oxidation in white blood cells; ex vivo resistance of lymphocyte DNA to oxidation by H_2O_2 (by the Comet assay); markers of inflammation (e.g. TNF-α) and growth factors (e.g. IGF-1). We enrolled healthy volunteers and adopted a crossover design (that is dietary supplement vs. placebo or controlled diet) with a washout period between the two treatments.

In table 1, results of studies with different tomato products are summarized. It can be seen that, independently from the amount and type of tomato products consumed, we always found an increase in lycopene concentration in both plasma and lymphocytes and an increase in lymphocyte protection from oxidative insult (from H_2O_2 and Fe). Plasma lycopene concentration was inversely correlated to lymphocyte DNA damage after the oxidative treatment. However, apart from lycopene, there was also an increase in plasma and lymphocyte content of vitamin C, phytoene and phytofluene, suggesting that all these compounds can contribute to the protection observed.

Interestingly, in the study with the Lyc-o-mato drink we found a significant decrease in IGF-1 levels after Lyc-o-mato intake in those subjects who had the highest increase in lycopene concentration (high respondent subjects) and high basal IGF-1 levels at recruitment (7). Thus, subjects with high levels of IGF-1 (considered a risk factor for cancer development) may benefit from the consumption of lycopene containing products. A decrease in TNF-α was also observed after the intake of the Lyc-o-Mato drink (8).

Altogether these results suggest that the regular consumption of tomato derivatives increase cell protection and can modulate different function associated to health.

Blood orange juice was used in two different studies. The first had the aim to evaluate the effect of regular consumption of blood orange juice on plasma antioxidant compounds, antioxidant capacity, protection from DNA and lipid oxidative damage, and on markers of inflammation (9). It was performed on 16 healthy female subjects who consumed 600 ml of orange juice daily for 21 days. The study demonstrated that 3 weeks of blood orange juice (rich in vitamin C, carotenoids, and anthocyanins such as cyaniding 3-glucoside) consumption increased plasma antioxidant levels, but did not exert significant effects on several markers of oxidative stress (plasma antioxidant capacity,

plasma malondialdehyde, and urinary excretion of thromboxane [11-dehydro TXB2]). After the consumption of orange juice, lymphocyte resistance to the *ex vivo* oxidative stress induced by H_2O_2 increased significantly (about 63%) only in the group of subjects with high plasma vitamin C concentration. An inverse and significant (R = -0.49, p < 0.005) correlation between plasma vitamin C concentrations and DNA damage was observed, suggesting a protecting role for vitamin C.

Table 1- Intervention studies with different tomato products

Tomato intervention (reference)	**Lycopene intake (mg/die)**	**DNA Protection variation**	**Lycopene plasma (μmol/L)**	**Lycopene lymphocyte variation**	**Relation lycopene/DNA**	**Other antioxidants variation**
60 g puree 3 wks (3)	≈ 16 mg	+30-40% (H_2O_2)	+0.5 (185%)		r=-0.8 P<0.01 (Plasma)	
25 g puree 2 wks (4)	≈ 7 mg	+50% (H_2O_2)	+0.4 (323%)	+ 81%	r=-0.8 P<0.001 (Plasma)	
15 g paste, 100 g raw, 60 g sauce 3 wks (5)	≈ 7 mg (≈14 mg Vit.C)	+ 24% (Fe2+)	+ 0.2 (53%)			**Vitamin C** *Plasma:* +35% *Lymphocyte:* +236%
Lyc-o-Mato drink 250 ml 26 days (6)	≈ 6 mg (+ 8 mg other carot.)	+ 42 % (H_2O_2)	+ 0.2 (68%)	+ 105%		**Phytoene,Phyitofluene, β-carotene** *Plasma:* +92%, +61%, +28% *Lymphocyte:* +159%, +84%, +51%

Afterwards, we performed another study to evaluate the effect of the intake of a single portion of blood orange juice (BOJ, 300 ml, providing 150 mg vitamin C) on lymphocyte DNA damage, compared with a drink supplemented with the same amount of vitamin C (C-drink) or sugars (S-drink) (10). Plasma vitamin C concentration increased similarly following BOJ or C-drink intake and was not affected by the S-drink. DNA damage significantly decreased 3 h after BOJ intake (about 18 %; P,0·01) and remained constant at 24 h (about 16 %; P,0·01) (table 2). No effect of the C-drink and S-drink was observed. These results provide further evidence that whole foods can increase cell resistance to oxidative stress better than single compounds. In fact, the protection observed on lymphocyte DNA at 3 h corresponded to an increase in plasma vitamin C concentration, but at 24 h vitamin C returned to its baseline value, suggesting that different compounds were also involved. This observation is supported by the fact that the same amount of vitamin C, provided as a supplement, did not affect cell DNA damage, despite the similar increase in plasma vitamin C.

Table 2 – Plasma vitamin C and DNA damage after 300 ml of blood orange juice

Time (h)	**Plasma vitamin C (μmol/l)**			**DNA damage (% DNA in tail)**		
	BOJ	**C-drink**	**S-drink**	**BOJ**	**C-drink**	**S-drink**
0	76.9 ± 22.1^a	73.4 ± 13.0^a	73.3 ± 14.0^a	62.9 ± 6.3^a	55.6 ± 12.8^a	56.9 ± 6.3^a
3	99.2 ± 18.9^b	103.3 ± 19.8^b	74.7 ± 16.6^a	51.9 ± 9.9^b	59.4 ± 13.2^a	54.3 ± 6.0^a
24	73.1 ± 17.3^a	76.1 ± 13.1^a	65.5 ± 13.2^a	52.0 ± 14.5^b	56.7 ± 15.0^a	58.1 ± 5.5^a

Another class of vegetables that received recently great attention, are cruciferous vegetables, whose consumption has been associated with decreased cancer risk. We were interested in evaluating the effect of the regular consumption of broccoli (200 g daily for 10 days) in subjects with different exposure to oxidative stress due to smoking habits. The portion of broccoli provided a consistent amount of isothiocyanates (200 μmol), vitamin C (about 100 mg) and folates, and small amounts of carotenoids (3.6 mg lutein and 1.4 mg β-carotene). We evaluated lymphocyte resistance to induced oxidative stress, endogenous level of oxidized DNA bases, GST activity, relationship between these variables and GST polymorphisms. The results obtained demonstrated that the regular consumption of this vegetable produce a significant increase in carotenoids, folates and isothiocyanates in plasma. Furthermore, a significant increase in the protection of cellular DNA from induced damage and a decrease in endogenous levels of oxidised bases (mainly guanine) were observed. This may be due to a direct effect of the antioxidant compounds introduced with broccoli but also to an indirect action of isothiocyanates. These compounds in fact seem to act as modulators of the detoxifying endogenous system and for this reason should be particularly important for smokers, continuously exposed to toxic species.
In conclusion, results from our study strongly support the protective role of fruit and vegetable and the importance of their regular consumption.

REFERENCES

1. Collins AR. Antioxidant intervention as a route to cancer prevention. Eur J Cancer 2005;41:1923-1930.
2. Griffiths HR et al. Biomarkers. Mol Asp Med 2002;23:101-208.
3. Riso P, Pinder A, Santangelo A, Porrini M. Does tomato consumption effectively increase the resistance of lymphocyte DNA to oxidative damage? Am J Clin Nutr 1999;69:712-8.
4. Porrini M, Riso P. Lymphocyte lycopene concentration and DNA protection from oxidative damage in women after a short period of tomato consumption. J Nutr 2000;130:189-92.
5. Riso P, Visioli F, Erba D, Testolin G, Porrini M. Lycopene and vitamin C concentration increase in plasma and lymphocytes after tomato intake. Effects on cellular antioxidant protection. Eur J Clin Nutr 2004 ;58:1350-1358.
6. Porrini M, Riso P, Brusamolino A, Berti C, Guarnieri S, Visioli F. Daily intake of a formulated tomato drink affects carotenoid plasma and lymphocyte concentrations and improves cellular antioxidant protection. Brit J Nutr 2005;93:93-99.
7. Riso P, Brusamolino A, Martinetti A, Porrini M. Effect of a tomato drink intervention on Insulin-like Growth Factor (IGF)-1 serum levels in healthy subjects. Nutr Cancer 2006;55(2):157-162.
8. Riso P, Visioli F, Grande S, Guarnirei S, Gardana C, Simonetti P, Porrini M. Effect of a tomato-based drink on markers of inflammation, immunomodulation, and oxidative stress. J Agric Food Chem 2006;54:2563-2566.
9. Riso P, Visioli F, Gardana C, Grande S, Brusamolino A, Galvano F, Galvano G, Porrini M. Effects of blood orange juice intake on antioxidant bioavailability and on different markers related to oxidative stress. J Agric Food Chem 2005 Feb 23;53(4):941-947.
10. Guarnieri S, Riso P, Porrini M. Orange juice *v.* vitamin C: effect on hydrogen peroxide-induced DNA damage in mononuclear blood cells. Brit J Nutr 2007;97:639:643.

Post-prandial glucose and insulin response is improved by bread fortified with germinated wheat seedlings

G. Andersen, P. Köhler, V. Somoza

German Research Centre for Food Chemistry, Lichtenbergstrasse 4, 85748 Garching, Germany

Former studies have shown that the amount of dietary fibre in wheat can be increased by germination. The aim of the present project was to investigate whether the intake of bread fortified with wheat seedlings (30 % dry weight) has a positive impact on selected parameters of glucose metabolism in healthy volunteers. At the beginning of this longitudinal study, a 75 g-OGTT (oral glucose tolerance test) was performed. Subsequently, the subjects received 300 g of a control wheat bread or a bread containing wheat seedlings daily for 9 days. After the experimental diets, again a 75 g-OGTT was performed. Venous blood samples were collected at the time points of 0, 30, 60, 120 and 180 min and selected parameters of glucose homeostasis in the plasma (glucose, insulin, C-peptide, free fatty acids, gastric inhibitory polypeptide) were analyzed.
The intake of 300 g of the control bread per day for 9 days led to a significant decrease of fasting plasma insulin levels. Also, the insulin levels after intake of the glucose solution were decreased. Insulin sensitivity was markedly increased as determined by the HOMA-index. The intake of the control bread did not show any effect on plasma glucose levels, neither in the fasting state nor after the uptake of the glucose solution.
In contrast to this, the daily intake of 300 g bread containing wheat seedlings for 9 days resulted in a significant decrease of fasting plasma glucose levels, although no difference in the dietary fibre content had been present. In addition, peak plasma glucose levels were reduced after the uptake of 75 g glucose. All other markers of glucose homeostasis analyzed remained unchanged, compared to the results obtained after administration of the control bread. Notwithstanding, insulin sensitivity was improved after the intake of bread containing wheat seedlings.

Whole grain health benefit: the human studies

Adele Costabile

Department of Food Biosciences, The School of Chemistry, Food Biosciences, and Pharmacy, The University of Reading, RG6 6 AP, Reading United Kingdom

Whole grain intake is associated with beneficial health effects and epidemiological studies have shown that it is protective against cancer, diabetes, obesity and in particular cardiovascular disease (CVD). Whole grains are rich in fermentable carbohydrates such as dietary fibre, resistant starch and oligosaccharides and one proposed protective mechanism is the effect on human gut microbiota. Diet-microbe interactions within the colon are now thought to play important roles in regulating mucosal physiology and may provide protection from invading pathogens, impact on liver function, bone health, satiety and chronic diseases like some cancers, inflammatory conditions and heart disease. Many of these health promoting activities are likely to be mediated by dominant members of the gut microbiota which have co-evolved with the human colon. Bacteria now seen as beneficial for human health include species belonging to the genera *Bifidobacterium* and *Lactobacillus*. Similarly, maintenance of stable and diverse populations of commensal bacteria e.g. *Eubacterium* spp., *Atopobium* spp., and certain *Bacteroides* spp., characterizes the gut microbiota in health and no doubt contributes greatly towards improved colonisation resistance and protection against gastrointestinal disorder. Functional foods targeting the human colon aim to stimulate beneficial genera either directly by providing growth substrates which selectively promote the growth of an individual's autochthonous bifidobacteria and lactobacilli *in vivo* within the colon (prebiotics) or indirectly by introducing live exogenous bacteria in specially formulated foods (probiotics). Currently, no information exists on the prebiotic potential of whole grain wheat. Whole grain cereals comprise three distinct physiological regions, the endosperm, germ and bran. The grain endosperm is composed mainly of starch, whose digestibility and subsequent fermentability will be affected by food processing (e.g. heating, drying, acid/enzymatic digestion). Grain germ, which is a minor fraction of the grain in wheat, is made up of a complex mixture of lipids, proteins and some mainly soluble carbohydrates, while wheat bran is composed of non-digestible mainly insoluble and poorly fermented carbohydrates such as cellulose, hemicellulose, arabinoxylan as well as polyphenolic lignins all together indicated as dietary fibre. Whole grains contain many compounds such as antioxidants, lignans, vitamins and minerals that may protect against chronic disease. Particularly in cereal products, dietary fibre is composed of different compounds that may be co-responsible for many of its physiological effects. An important amount of phenolic compounds (500-1500 mg/kg), mainly ferulic acid, is linked to the dietary fibre and this may explain why wheat dietary fibre has a marked antioxidant activity. Dietary fibre *in toto* (carbohydrate and phenolic compounds) mediates their biological activity on host health through the colonic microbiota. There is currently no information on the

impact of specific whole grain cereals on the microbial ecology of the human colon, and how this may impact upon chronic disease risk.

In the present study, the efficacy of whole grain wheat compared to wheat bran alone to beneficially modulate the gastrointestinal microbiota and their activities was determined. The objective was to assess the ability of WG compared to WB to selectively increase numbers of bifidobacteria and alter colonic metabolic output. We present the findings of a double blind, placebo controlled cross-over study where 31 healthy subjects were randomised in 2 groups and were fed either 48 g/d wheat breakfast cereal WG or WB breakfast cereal as placebo for 3 weeks. After a 2-week wash-out phase volunteers were then crossed over to the other breakfast cereal treatment for another 3 weeks. Fasting blood, 24h urine and single stool samples were collected before and after treatment with the cereals, and changes within the gut microbiota and its metabolic output in terms of short chain fatty acid profiles and plasma ferulic acid.

In conclusion, this human study, for the first time, demonstrated the differential impact of whole grain wheat and wheat bran on the microbial ecology of the human gut. Additionally, it has established a prebiotic mode of activity for the WG breakfast cereal investigated with increased populations of bifidobacteria and lactobacilli compared to starting levels and the wheat bran. Finally, the increase of the ferulic acid concentration in fasting plasma suggests that the WG intake caused a continuous release of antioxidant in the bloodstream. This study has established a prebiotic mode of action for a whole grain cereal, which together with antioxidant activities may contribute to the underlying mechanisms of protective health effects of whole grain wheat.

Role of carnitine in butyrate metabolism

G. D'Argenio

Carnitine transporters have recently been implicated in susceptibility to inflammatory bowel disease (IBD). Because carnitine is required for beta-oxidation, it was suggested that decreased carnitine transporters, and hence reduced carnitine uptake, could lead to impaired fatty acid oxidation in intestinal epithelial cells, and to cell injury. This issue has been investigated by examining the expression of the carnitine transporters OCTN2 and ATB0+, and butyrate metabolism in colonocytes in a rat model of IBD induced by trinitrobenzene sulfonic acid (TNBS). The finding is that Octn2 and Atb0+ expression is decreased in inflammatory samples at translational and functional level. Butyrate oxidation, evaluated based on CO2 production and acetyl-coenzyme A synthesis, is deranged in colonocytes from TNBS-treated rats. Treatment with carnitine-loaded liposomes corrects the butyrate metabolic alterations in vitro and reduces the severity of colitis in vivo. These results suggest that carnitine depletion in colonocytes is associated with the inability of mitochondria to maintain normal butyrate beta-oxidation. These data indicate that carnitine is a rate-limiting factor for the maintenance of physiological butyrate oxidation in colonic cells. This hypothesis could also explain the contradictory therapeutic efficacy of butyrate supplementation observed in clinical trials of IBD.

Functional milk as adjuvants therapy of chronic hepatitis C

Filomena Morisco, Barbara Russo*, Giuseppe Maio**, Vincenzo Lamura***, Daniela Amoruso, Paola Vitaglione, Vincenzo Fogliano, Carlo Carella***, Nicola Caporaso*.
Dipartimento di Scienza degli Alimenti e *Dipartimento di Medicina Clinica e Sperimentale, Università di Napoli Federico II, ** A.O.G. Rummo, Benevento; *** Dipartimento di Medicina clinica e Sperimentale, Seconda Università di Napoli, Napoli

It is well known that the use of ribavirin in association with interferon, for the treatment of HCV infection, shows severe side effects, that in most cases leads to dosage reduction or premature discontinuation of the drug.

It has been reported that antiviral treatment for hepatitis C virus infection causes a moderate-severe bone loss in association to backpain and sometimes fractures.

Loss of bone mineral density during antiviral treatment is probably due to a multifactorial mechanism: 1) patients ongoing antiviral therapy have probably lower dietary calcium intake due to anorexia and dyspepsia; 2) it has been hypothizes but not demonstrated a vitamin D deficiency; 3) ribavirin could impair intestinal calcium up-take, as in malabsorption syndrome; 4) finally, recently in vitro study reported that ribavirin reduce the proliferation and the differentiation of metabolically active osteoblasts.

The aim of our project was: 1) to develop and produce Foods for Special Medical Purpose (FSMPs) able to improve calcium bioavailability; and 2) to value the effect of the daily supplementation of the FSMPs on calcium up-take in patients with chronic hepatitis C ongoing antiviral treatment.

In our study, we decided to use two different FSMPs using partially skimmed milk added with oligofructose (FSMP no.1) or galacto-oligosaccarides + chaseinophosphopeptides (FSMP no.2), respectively.

The FSMP no.1 is a milk yet commercially known as Fibresse, FSMP no 2 is a new milk designed end produced by our department in collaboration with PARMALT.

FOS (oligofructose) and GOS (galacto-oligosaccarides) are prebiotic substances with “bifidogenic effect”. Moreover, they increase bioavailability of minerals, particularly calcium. CPP

(chaseinophosphopeptides) are peptides derived from chasein, with high grade of phosphorilation. This characteristic makes them able to complex with ionised calcium, increasing the up-take. For these reasons their use was suggested not only in prevention of bone diseases, but also in prevention of dental caries.

We decided to enroll patients with chronic hepatitis C able to be treated with PEG-IFN and ribavirin and showing urinary calcium excretion higher than >100 mg/24h (sign of good calcium availability)

The patients have been randomized in three groups to receive :

A. No FSMP (control group)

B. milk added with FOS (Fibresse)

C. milk added with GOS and CPP (new milk)

All patients of the group B and C received the same milk daily dose (250 ml), during the first six months of therapy.

The efficacy of FSMPs was assessed at 6 months of treatment with antiviral drugs and functional milk, comparing the modification of 24h urinary calcium excretion among the 3 groups.

Serum vitamin D levels were evaluated at baseline, after six months of treatment with antiviral drugs and functional milk, and after further six months with only IFN and ribavirin administration.

At 6 months in all groups we obersved a decrease of calcium urinary excretion.

A medium decrease was 55% in the control group, and decisively less severe in the groups using FSMP (respectively 26% for patients using FOS milk and 14% in patients using GOS and + CPP milk).

At baseline vitamin D blood level was inadequate in almost all patients studied but increased during the period of milk supplementation.

In conclusion, the regular intake of the milk added with FOS or GOS + CPP improves the calcium up-take in patients with chronic hepatitis C during antiviral treatment. The assessment of vitamin D status can be recommended in all patients with chronic viral hepatitis.

References

1. Solis-herruzo JA, Castellano G, Fernadez I, Munoz R, Hawkins F. Decreased bone mineral density after therapy with alpha interferon in combination with ribavirin for chronic hepatitis C. J Hepatol 2000 Nov; 33(5): 812-7.
2. Moreira RO, Balduino A, Martins HS Reis JS, Duarte ME, Farias ML, Borojevic R. Ribavirin, but not interferon alpha-2b, is associated with impaired osteoblast proliferation and differentiation in vitro. Calcif Tissue Int. 2004 Aug; 75 (2): 160-8.
3. Ohta A, Ohtsuki M, Baba S, Takizawa T, Adachi T, Kimura S. Effects of fructo-oligosaccharides on the absorption of iron, calcium and magnesium in iron-deficient anemic rats. J Nutr Sci Vitaminol 1995; 41:281-291.
4. Othani H, Kihara Y, Park M. The immunoenhancing property of a dietary casein phosphopeptide preparation in mice. Food Agric. Immunol. 2000;12: 65-173.
5. Schieffke I, Fach A, Wiedemann M, Aretin AV, Schenker E, Borte G, Wiese M, Moessner I. Reduced bone mineral density and altered bone turnover markers in patients with non-cirrhotic chronic hepatitis B or C infection. World J Gastroenterol 2005; 11(12): 1843-47.

Satiating effect of new dietary fibre-rich foods

Paola Vitaglione and Luca Scalfi

Department of Food Science, University of Naples, 80055 Portici (Napoli), Italy

To control the worldwide increasing of overweight and obesity as well as the associated metabolic syndrome (glucose intolerance, insulin resistance, central adiposity, mild dyslipidemia and hypertension) the WHO recommends a lifestyle modification approach. This includes, together with the increase of physical activity, nutritional advices based on the reduction of diet calories (WHO, 1998).

Appetite management is an actual approach to moderate energy intake through the modulation of calories consumed in each eating occasion and the frequency of eating occasions.

Several studies have shown the ability of some food components and properties to influence the mechanisms controlling both the size (satiation) and the frequency (satiety) of meals.

Dietary fibres are food constituents with well documented effects (Howarth *et al.*, 2001; Burton-Freeman, 2000), although the mechanisms remain still poorly characterized. Satiation may be mediated through the intrinsic properties of dietary fibre-containing foods, such as a decrease in energy density, a prolonged chewing and mastication period, or through its gelling properties in the stomach causing the distension of the gastric antrum with the consequent increase of fullness sensation (Slavin, 2005; Hoad *et al.*, 2004). The increase of satiety, with attendant greater reductions in energy intake, through a delayed gastric emptying as well as the increase of plasma concentration of anorexigenic gut-derived peptides (PYY and glucagon-like peptide-1) and the reduction of serum levels of ghrelin (an orexigenic hormone), have been attributed to fermentable dietary fibres, such as glucans (Cani *et al.*, 2004).

Concerning the satiating efficacy of different types of dietary fibre, due to the complexity of numerous biological and behavioural processes that modulate hunger and satiety, the experimental evidences are still not conclusive. Anyway the knowledge of the mechanisms that regulate hunger and satiety as well as the existence of validated biomarkers of satiation/satiety (de Graaf *et al.*, 2004) and of methodology to measure the sensations of hunger/satiety (method of preloads and use of Visual Analogue Scale) in humans (Porrini *et al.*, 1995), make an achievable task for food technologists and nutritionists the development of new dietary fibre-rich foods having high satiating effect useful in the prevention of non-communicable chronic diseases, in which metabolic syndrome play a fundamental role.

Preliminary results about satiating efficiency of new beta-glucan-rich foods, specifically designed to improve blood lipids in hyperlipidemic subjects, will be shown.

References

- World Hearth Organization. Obesity: preventing and managing the global epidemic. Geneva, Switzerland: World Hearth Organization, 1998.
- Howarth NC, Saltzman E, Roberts SB. Dietary fiber and weight regulation. Nutr Rev 2001; 59: 129-139.
- Burton-Freeman B. Dietary fiber and energy regulation. J Nutr 2000; 130: 272S-275S.
- Slavin JL. Dietary fiber and body weight. Nutrition 2005; 21: 411-418.
- Hoad CL, Raiman P, Spiller RC, *et al.* In vivo imaging of intragastric gelation and its effects on satiety in humans. J Nutr 2004; 134: 2293-2300.
- Cani PD, Dewever C, Delzenne NM. Inulin-type fructans modulate gastro-intestinal peptides involved in appetite regulation (Glucagon-like peptide-1 and Ghrelin) in rats. Br J Nutr 2004; 92: 521-526.
- Porrini M, Crovetti R, Testolin G, Silva S. Evaluation of satiety sensations and food intake after different preloads. Appetite 1995; 25: 17-30.
- de Graaf C, Blom W AM, Smeets P AM, *et al.* Biomarkers of satiation and satiety. Am J Clin Nutr 2004; 79: 946-961.

Lipid metabolism and bowel health: an up-date on knowledge about probiotic/prebiotic supplementation and cholesterol metabolism.

Arrigo F.G. Cicero
"GC Descovich" Atherosclerosis and Metabolic disease Research Center
"D. Campanacci" Clinical medicine and Applied biotechnology Dept.
Alma Mater Studiorum University of Bologna

The increase of cholesterolemia over the optimal level is related to a proportional increase in the risk of cardiovascular events (NCEP, 2001). The scientific (and marketing) interest in the search of new dietary supplements (including nutraceuticals and functional foods) with antihypercholesterolemic properties is exponentially augmenting, also because there are always more support to the idea that their use could be useful in all subjects for which cholesterolemia or estimated global cardiovascular disease risk are not so high to justify a pharmacological treatment with an antihypercholesterolemic drug (Patch et al, 2006). The preclinical scientific evidences strongly support the use of both probiotics and prebiotics in the treatment of mild-to–moderate hypercholesterolemia (Pereira et al, 2002), but clinical evidences are often contrasting, especially as it regards probiotics. As it regards prebiotics, the reasons of the observed differences in cholesterol reduction are mainly related to the different chemical structure of the different fibres. However, "dietary fiber" is a collective term for a variety of plant substances that are resistant to digestion by human gastrointestinal enzymes (Eastwood et al, 2003). Dietary fibers can be classified in two major groups depending on their solubility in water. The structural or matrix fibers (lignin, cellulose, and some hemicelluloses) are insoluble, whereas the natural gel-forming fibers (pectin, gums, mucilages, and the remainder of the hemicelluloses) are soluble. Studies have focused on soluble fibers such as oats, psyllium, pectin, and guar gum, and qualitative reviews suggested that these fibers lower total and LDL cholesterol (Truswell, 1995; Glore et al, 1994). Water insoluble wheat fiber and cellulose have no significant effect unless they displace foods supplying saturated fats and cholesterol (Kris-Etherton et al, 1988). Among soluble fibers, psyllium appears to be the one with the highest antihypercholesterolemic efficacy (Brown et al, 1999). Beyond the antihypercholesterolemic effect, psyllium appears to have even other positive effects that could be beneficial for the management of patients at high cardiovascular risk, such as those affected by metabolic syndrome (MS). In fact, it showed a slight antihypertensive effect (Burke et al, 2001), an antihyperglycemic action (Sierra et al, 2002; Anderson et al, 1999) and a slowing effect on gastric emptying that is supposed (but yet not demonstrated) to be beneficial in weight loss dietary regimens (11,12).

Guar gum appears to have a positive metabolic effect on cholesterolemia and insulinemia (13), and a slowing effect on gastric emptying (14), as well. Directly comparing the metabolic effect of psyllium and guar in the setting of a randomized clinical trial we observed that both kinds of fiber are able to significantly improve BMI (-7.2% vs –6.5%), FPG (-27.9% vs –11.1%, respectively), FPI (-20.4% vs –10.8%), HOMA Index (-39.2% vs –

16.7%), LDL-C (-7.9% vs –6.5%,), and Apo B (-10.5% vs –5.6%) after 6 months of treatment. Apart from the speed and efficacy, only the 6 months psyllium supplementation exerted a significant improvement in plasma TG concentration (-13.3%) and in SBP (-3.9%) and DBP (-2.6%), two main components of the metabolic syndrome (MS) (Cicero et al, 2007).

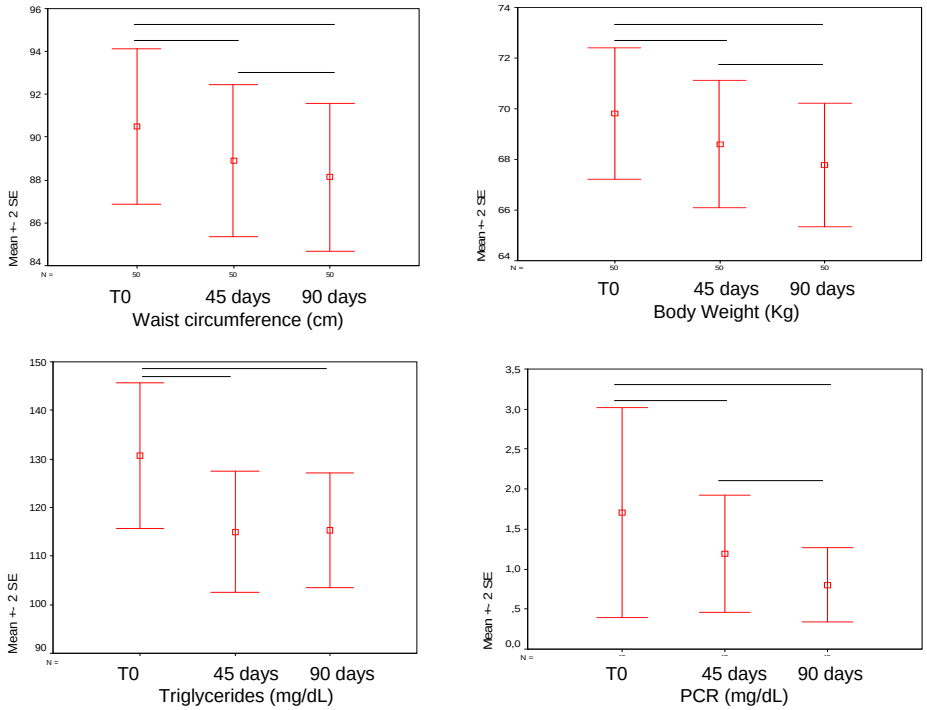
The probiotics could also act on cholesterol metabolism, but clinical data are less evident (Greany et al 2007). This property has been clearly observed in different animal models for the most part of the usually colonising different parts of our bowel (*Streptococcus, Lactobacillus* and *Bifidobacterium)*. Lactobacilli in particular seem to directly sequestrate the bowel cholesterol (both from dietary and bile origin), thus inhibiting its absorption and the entero-hepatic cycle, but also deconiugating the bile salts. The fermentation of non digestible carbohydrates by the bacteria also contribute to the cholesterolemia reduction by producing shot-chain fatty acids that slightly inhibit the liver synthesis of cholesterol (Liong et al 2005). Studies carried out on humans confirm this effect when *Bifidobacteria* are added to fermented dairy products, while *Lactobacillus plantarum* could have also other positive action modulating other cardiovascular risk factors such as blood pressure, fibrinogenemia, leptninemia and the plasma level of some adhesion molecules (Nguyen et al, 2007). In a recent study carried out on subjects affected by irritable bowel syndrome and metabolic syndrome, we observed that *Lactobacillus acidophilus* is able to significantly reduce waist circumference, triglyceridemia and PCR plasma level, three main components of the metabolic syndrome (unpublished data) (Figure 1). Further research is needed to evaluate which kind of prebiotics is more efficacious in which kind of patient and to evaluate the long-term efficacy of this kind of treatment on the reduction of plasma lipids.

References

1. NCEP expert panel. Expert Panel on detection, evaluation and treatment of high blood cholesterol in adults. Executive summary of the third report of the National Cholesterol Education Program (NCEP) (Adult Treatment Panel III). JAMA 2001;285(19):2486-97.
2. Patch CS, Tapsell LC, Williams PG, Gordon M. Plant sterols as dietary adjuvants in the reduction of cardiovascular risk: theory and evidence. Vasc Health Risk Manag. 2006;2(2):157-62.
3. Pereira DI, Gibson GR. Effects of consumption of probiotics and prebiotics on serum lipid levels in humans. Crit Rev Biochem Mol Biol. 2002;37(4):259-81.
4. Eastwood MA, Passmore R. Dietary fibre. Lancet 1983;2:202-6.
5. Truswell AS. Dietary fibre and plasma lipids. Eur J Clin Nutr 1995;49(suppl):S105-9.
6. Glore SR, Van Treeck D, Knehans AW, Guild M. Soluble fiber and serum lipids: a literature review. J Am Diet Assoc 1994;94:425-36.
7. Kris-Etherton PM, Krummel D, Russell ME, et al. The effect of diet on plasma lipids, lipoproteins, and coronary heart disease. J Am Diet Assoc 1988;88:1373-400.
8. Brown L, Rosner B, Willett WW, Sacks FM. Cholesterol-lowering effects of dietary fiber: a meta-analysis. Am J Clin Nutr 1999;69:30-42.

9. Burke V, Hodgson JM, Beilin LJ, Giangiulioi N, Rogers P, Puddey IB. Dietary protein and soluble fiber reduce ambulatory blood pressure in treated hypertensives. Hypertension 2001;38:821-826.
10. Sierra M, Garcia JJ, Fernandez N, Diez MJ, Calle AP. Therapeutic effects of psyllium in type 2 diabetic patients. Eur J Clin Nutr 2002;56(9):830-42.
11. Anderson JW, Allgood LD, Turner J, Oeltgen PR, Daggy BP. Effects of psyllium on glucose and serum lipid responses in men with type 2 diabetes and hypercholesterolemia. Am J Clin Nutr 1999;70(4):466-73.
12. Delargy HJ, O'Sullivan KR, Fletcher RJ, Blundell JE. Effects of amount and type of dietary fibre (soluble and insoluble) on short-term control of appetite. Int J Food Sci Nutr 1997;48(1):67-77.
13. Turnbull WH, Thomas HG. The effect of a *Plantago ovata* seed containing preparation on appetite variables, nutrient and energy intake. Int J Obes Relat Metab Disord 1995;19(5):338-42.
14. Landin K, Holm G, Tengborn L, Smith U. Guar gum improves insulin sensitivity, blood lipids, blood pressure, and fibrinolysis in healthy men. Am J Clin Nutr 1992;56(6):1061-5.
15. van Nieuwenhoven MA, Kovacs EM, Brummer RJ, Westerterp-Plantenga MS, Brouns F. The effect of different dosages of guar gum on gastric emptying and small intestinal transit of a consumed semisolid meal. J Am Coll Nutr 2001;20(1):87-91.
16. Cicero AFG, Derosa G, Manca M, Bove M, Borghi C, Gaddi AV. Different effect of psyllium and guar dietary supplementation on blood pressure control in hypertensive overweight patients: a 6-month, randomized clinical trial. *Clinical and Experimental Hypertension* (Clin Exp Hypert) 2007; In press.
17. Greany KA, Bonorden MJ, Hamilton-Reeves JM, McMullen MH, Wangen KE, Phipps WR, Feirtag J, Thomas W, Kurzer MS. Probiotic capsules do not lower plasma lipids in young women and men. Eur J Clin Nutr. 2007; In press
18. Nguyen TD, Kang JH, Lee MS. Characterization of Lactobacillus plantarum PH04, a potential probiotic bacterium with cholesterol-lowering effects. Int J Food Microbiol. 2007 Feb 15;113(3):358-61.
19. Liong MT, Shah NP. Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. J Dairy Sci. 2005;88(1):55-66.

Figure 1 – Effect of a *Lactobacillus acidophilus* containing probiotic on some components of the Metabolic Syndrome in moderately hypercholesterolemic subjects with Irritable Bowel Syndrome (upper lines indicate statistically significant difference among evaluated periods)



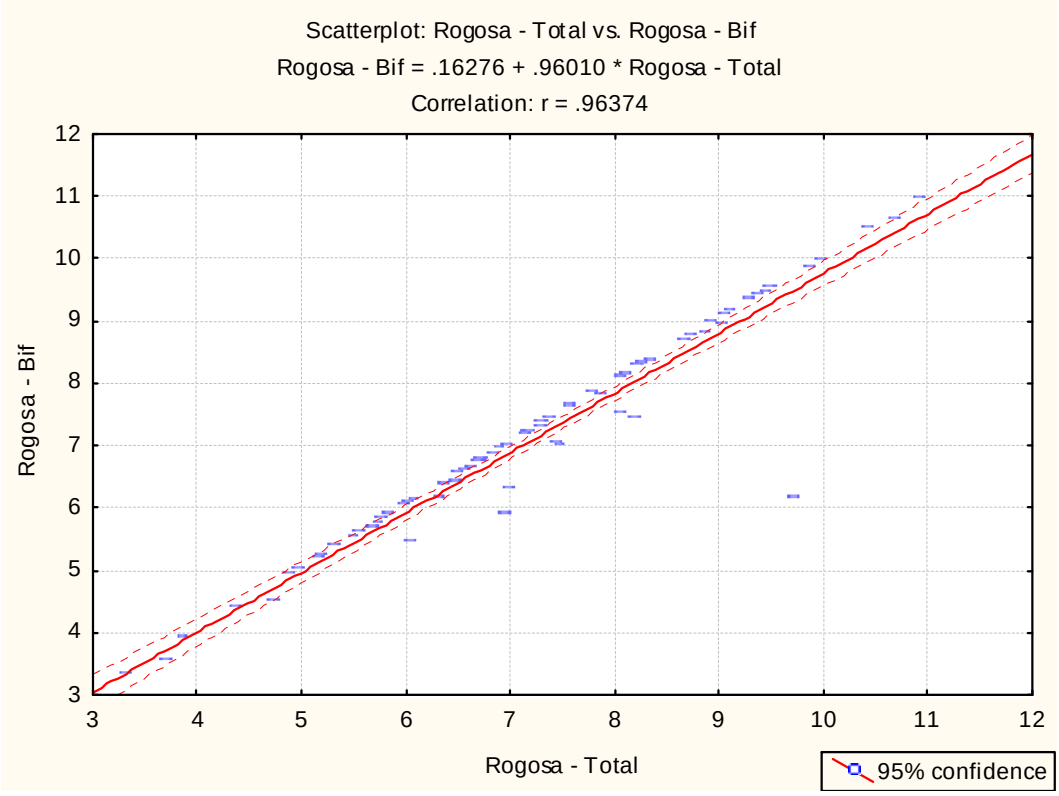
The Possible Role of the Intestinal Microflora of Infants in the Subsequent Development of Atopic Responses

Andrew B. Onderdonk, PhD
Brigham and Women's Hospital, Harvard Medical School, Boston, MA

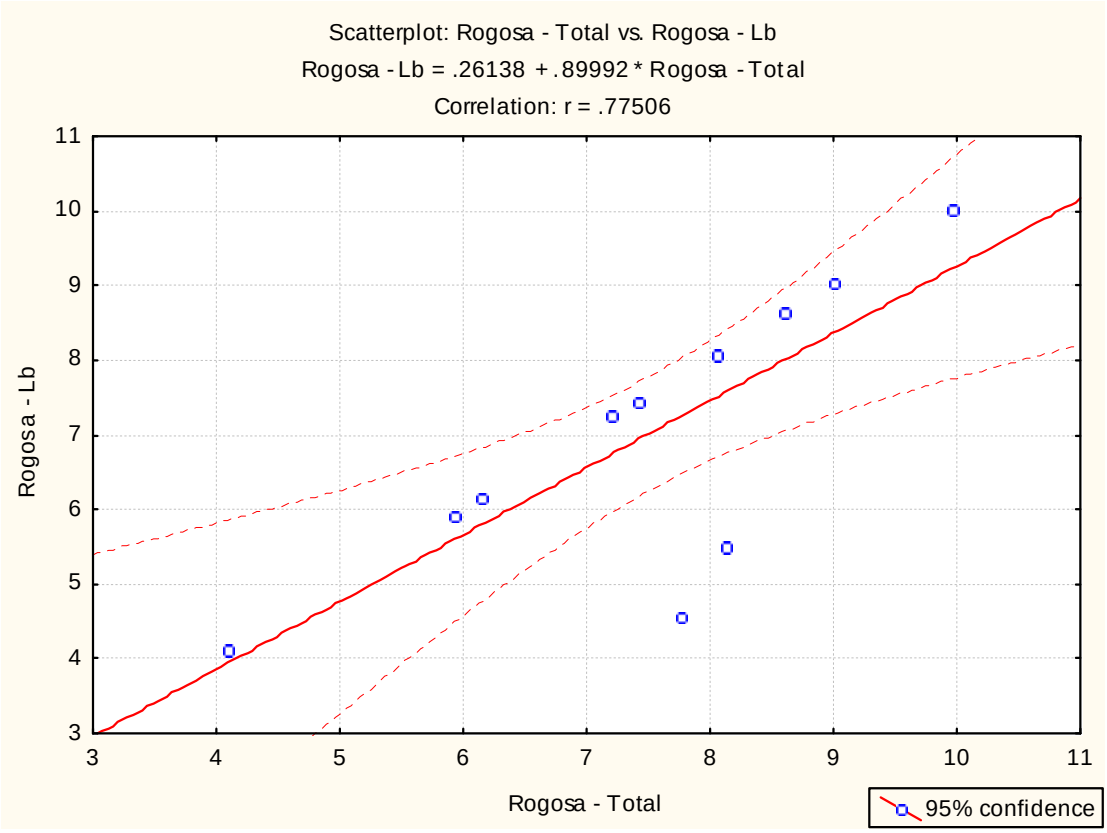
Analysis of the preliminary study data includes 78 stool specimens from mothers paired with 179 stool specimens for neonates obtained at 1, 4 and 6 months following delivery. Our preliminary work is focused on determining whether there are obvious associations between specific groups of organisms, particularly at the earliest sample time (1 month) after birth since this is when the intestinal microflora is beginning to develop and an autochthonous bacterial population is establishing within the large intestine.

Shown below is the correlation for neonate stool combined total counts for *Lactobacillus sp*. and *Bifidobacterium sp.*, compared to *Bifidobacterium* alone using two media, one that isolates both *Lactobacillus sp*. and *Bifidobacterium sp.* and one that isolates only *Bifidobacterium sp.*

.



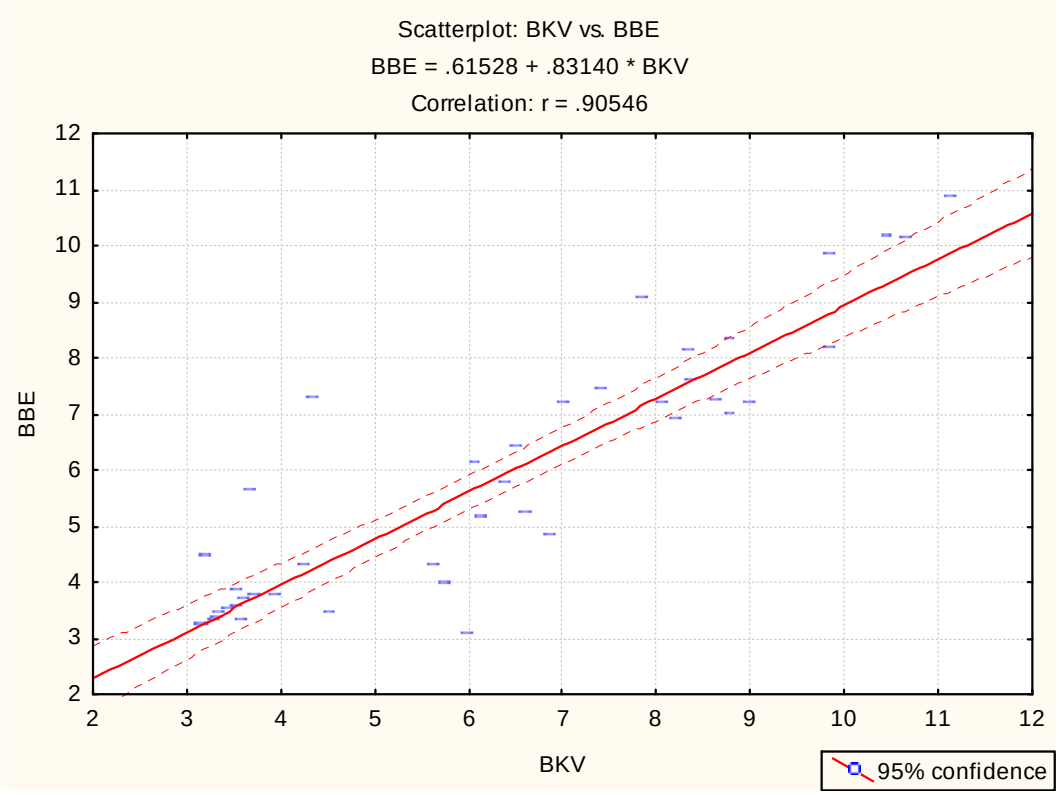
As can be seen, there is almost a perfect correlation between the combined total counts and the counts for *Bifidobacterium sp*. Contrast this observation with the following graph showing the total combined counts for both *Lactobacillus sp*. and *Bifidobacterium sp*. compared to the total counts for *Lactobacillus sp*. alone.



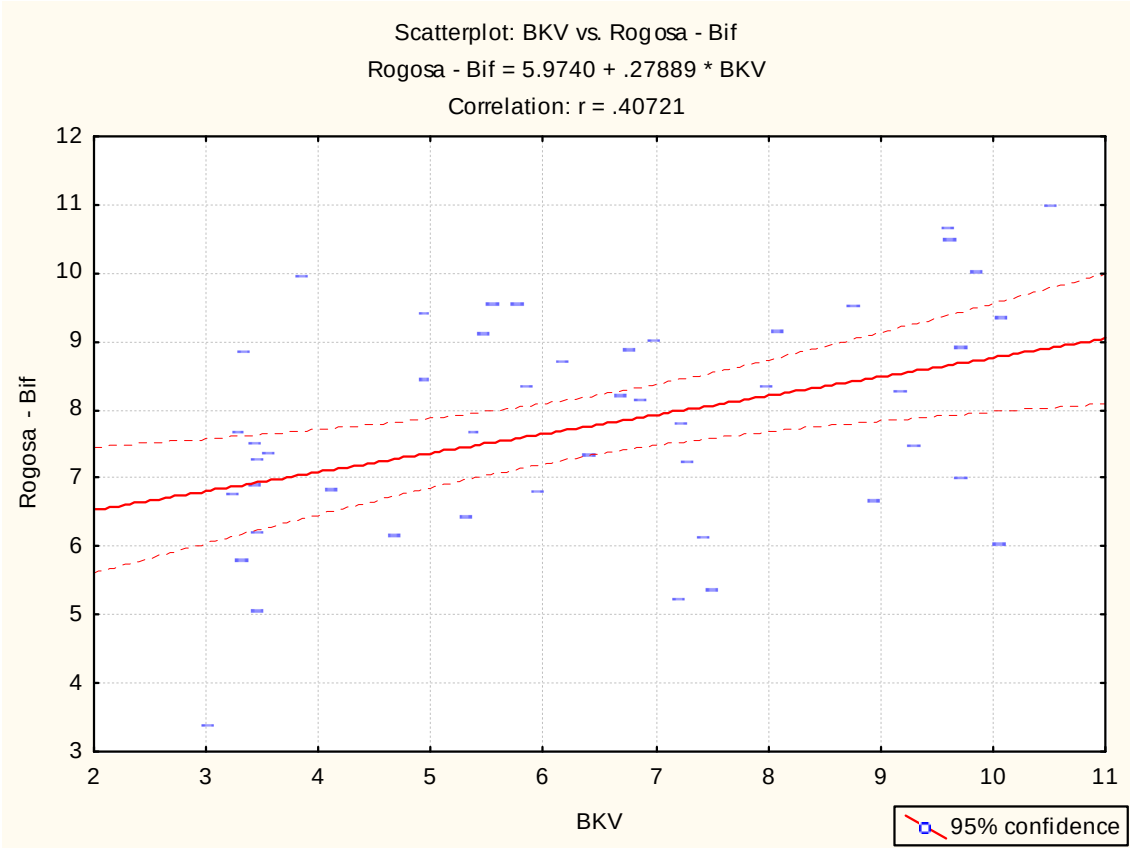
While there is a significant correlation between total counts and counts for *Lactobacillus sp*. alone, it is clear that early development of intestinal microflora is driven more by the presence of *Bifidobacterium sp*. than *Lactobacillus sp*. For purpose of the ongoing studies, observations such as this target *Bifidobacterium* sp. as major early members of the microflora that warrant further scrutiny to determine by molecular methods whether there are specific strains associated with this early development and also to determine whether the strains detected in infants are the same as those present in the mother.

In evaluating the earliest samples from neonates, there are similar findings between the total counts for Gram-negative anaerobes (*Bacteroides, Prevotella, Fusobacterium, Succinomonas* etc.) and *Bacteroides* sp. As can be seen in the following graph, there is an excellent correlation between total Gram-negative anaerobes and *Bacteroides* sp. during the early establishment of the intestinal microflora. As is the case for *Bifidobacterium* sp., the *Bacteroides* sp. are clearly a target for further evaluation by both

phenotypic methods (which species are most common?) and by molecular methods (how similar are the various strains isolated during these studies?)



Interestingly, an examination of the correlation between the various bacterial populations suggests that development of high numbers of one population at the one-month time point is not necessarily correlated with concomitant increases in a second population. For example, comparison of counts for *Bacteroides* sp. and *Bifidobacterium* sp. show little correlation with each other ($r<0.40$, see below). In contrast, it appears that once established, there is a significant correlation between these two populations for the four and six month stool samples (data not shown)



While these preliminary data provide some provocative information regarding the development of human intestinal microflora during the first year of life that will be useful both as stand alone data and within the context of the larger study, it is clear that the additional data derived from the immunology studies and ultimately, which children develop atopic responses, is paramount to understanding the role of the intestinal microflora in the development of the early systemic immune response.

Functional compounds in the newborn Formula

Olle Hernell Pediatrics,
Department of clinical sciences, Umeå university, Sweden

Infant formulas are regarded as products intended to satisfy totally the nutritional requirements of infants during the first 4 to 6 months of life, and to contribute a major part of the nutritional requirements throughout the first year of life. These substitutes for breast milk were developed from the milk of other mammals, particularly cow milk through numerous modifications, into the complex formulas that are available today. However even with modern infant formulas there are significant differences between breastfed and formula-fed infants, and some of these differences may persist beyond the weaning period. Thus, it is evident that chemical and nutrient composition similar to breast milk is in itself no longer sufficient. A better reference for infant formulas is biochemical, physiological and functional outcomes seen in healthy infants, exclusively breastfed for 4 to 6 months. To meat this challenge the most recent formula development has focused on which potential functional compounds in human milk are relevant - and possible to add to infant formulas to make the performance of the formula-fed infant closer to the breastfed infant? Examples of compounds and intended functions are: long-chain polyunsaturated fatty acids - neurodevelopment, free nucleotides - immune function, structured triglycerides - fat and calcium absorption, oligosaccharides - gut microbiota and stool consistency, probiotics – gut microbiota, allergy treatment and prevention.

Many proteins in human milk have more than one function. α-lactalbumin is a major whey protein in human milk and a rich source of tryptophan, typically a limiting amino acid in formula manufacturing, but may also have functional properties, e.g. probiotic- and mineral absorption-enhancing effects. Another major whey protein is lactoferrin to which numerous biological functions have been described, including trophic and antimicrobial effects. Human milk *k*-casein may operate as a decoy receptor for *H.* pylori via its carbohydrate moiety. There are several ways by which human milk proteins may exert biological functions, e.g. by the intact protein (resistant to proteolysis), by peptides formed during digestion, by glyco- or lipid ligands. The simplest way to include bioactive compound into formulas is by the use of enriched bovine milk fractions, e.g. enriched in lactoferrin, *a*-lactalbumin, glycomacropeptide (GMP) and other fractions. However, even with continued technical development there will be limitations. The function of a compound may be species specific as for lactoferrin, or the protein may be species specific as for the bile salt stimulated lipase. The latter, which is an enzyme present in human but not bovine milk, contributes to the efficient fat absorption in breastfed infants. However, it also seems to have significant antimicrobial effects, again illustrating that human milk proteins often serve more than one function. By use of modern recombinant and transgenic techniques it will be possible in the future to produce recombinant human milk proteins and include these into infant formulas, which will be the ultimate way to achieve the goal of making the formula-fed infant perform as close as possible to the exclusively breastfed infant. For both lactoferrin and bile salt-stimulated lipase clinical trials with the recombinant human proteins are in progress with promising results, which will be discussed.

Toward the European Indications for Complementary Feeding

Carlo Agostoni
Department of Pediatrics
San Paolo Hospital
University of Milan, Italy

Nutritional goals of Complementary Feeding

- Classic approach→ prevention of malnutrition and deficiency states.
- Most current guidelines on complementary feeding →not evidence-based, but are based on ‘best practice’.

Complementary Feeding :

A commentary by the ESPGHAN Committee on Nutrition.

ESPGHAN Committee on Nutrition:
C Agostoni;T Decsi; M Fewtrell; O Goulet; S Kolacek, B Koletzko;
K Fleischer Michaelsen; L Moreno; J Puntis, J Rigo; R Shamir;
H Szajewska; D Turck; J van Goudoever

JPGN: 2007, in press

Complementary Feeding

- Why
- When
- What
- The future

WHY

- Optimal duration of exclusive breastfeeding→how long?
- The volume of human milk ingested by exclusively breastfed infants at about 6 months becomes insufficient to meet the requirements of calories, protein, iron, zinc and some fat-soluble vitamins (A and D).

ESPGHAN CoN, 2007

- gastrointestinal and renal function are sufficiently mature by around 4 months of age to enable term infants to process some complementary foods
- there is a range of ages when infants attain the necessary motor skills to cope safely with complementary feedings.

ESPGHAN CoN, 2007

WHEN

- Exclusive breastfeeding for about 6 months is a desirable goal.
- In any case, CF should not be introduced in any infant before 4 completed months (17 weeks) and all infants should start CF by 6 months (26 weeks).
- Although there are theoretical reasons why different complementary foods might benefit breast or formula-fed infants, to devise and implement separate recommendations for the introduction of solid foods for breast fed and formula fed infants may present practical problems.

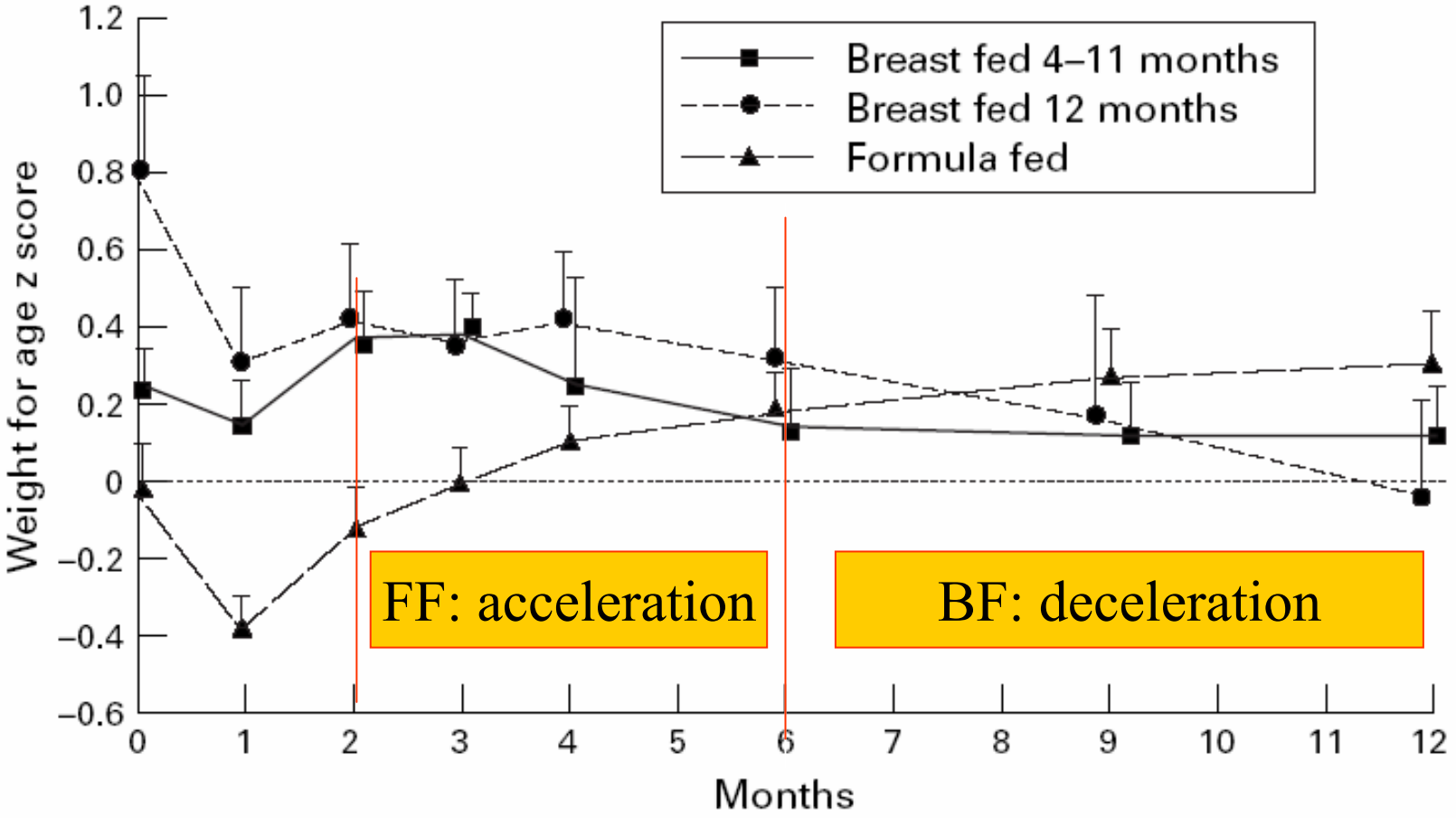
ESPGHAN CoN, 2007

MANDATORY PREREQUISITE:

CORRECT EVALUATION OF INFANT GROWTH

- to prevent inappropriate dietary supplementations
- to maintain prolonged breastfeeding
- to appropriately check the growth rates of BF infants

Growth patterns of breastfed and formula-fed Italian infants: an Italian Study
Agostoni C et al, Arch Dis Child 1999; 81: 395



Data consistent with the only “randomized” study design
Kramer et al, Pediatrics 2002;110:343

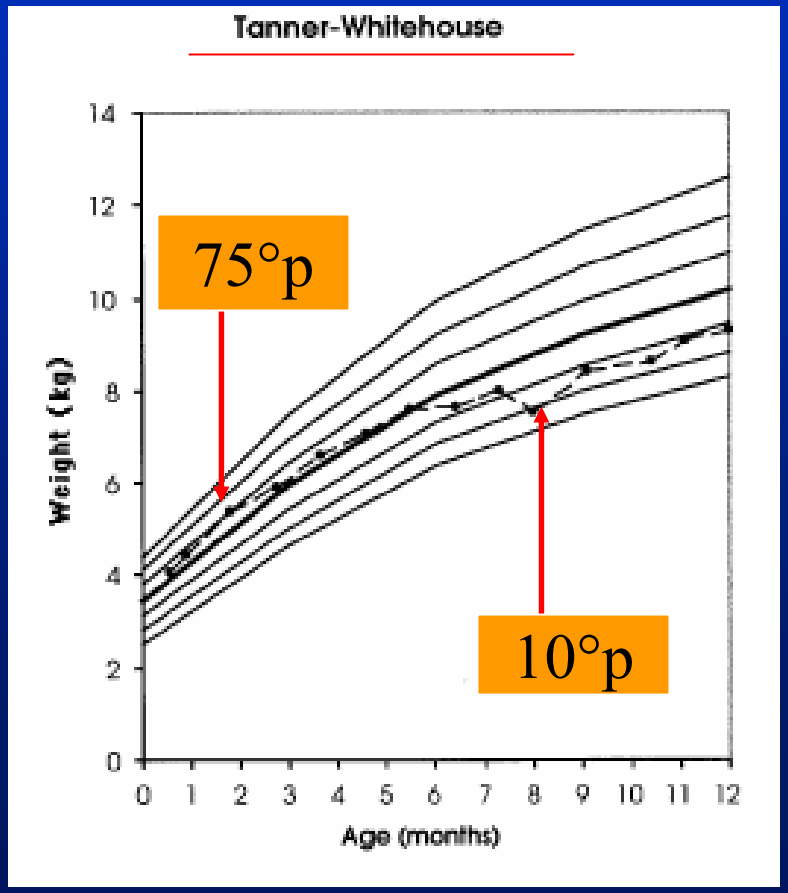
Acta Pædiatr 91: 1296–1300. 2002

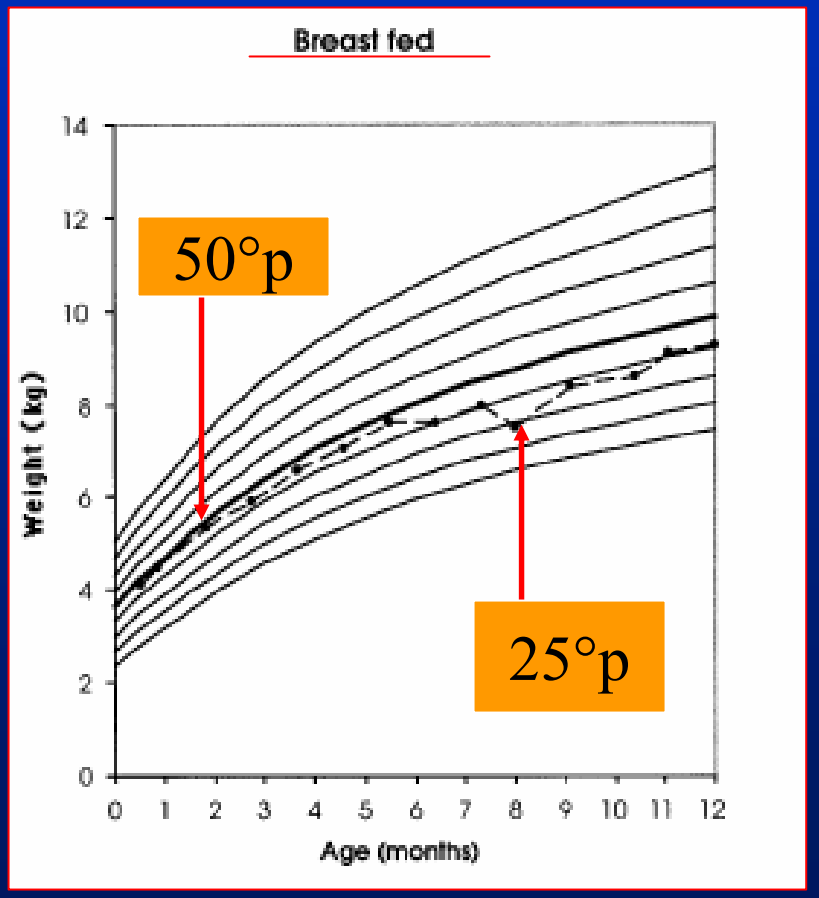
Taylor&Francis
healthsciences

Weight reference charts for British long-term breastfed infants

TJ Cole[1], AA Paul[2] and RG Whitehead[3]

Centre for Paediatric Epidemiology and Biostatistics[1], Institute of Child Health, London; Elsie Widdowson Laboratory[2], MRC Human Nutrition Research, Cambridge; Church End[3], Weston Colville, Cambridge, UK





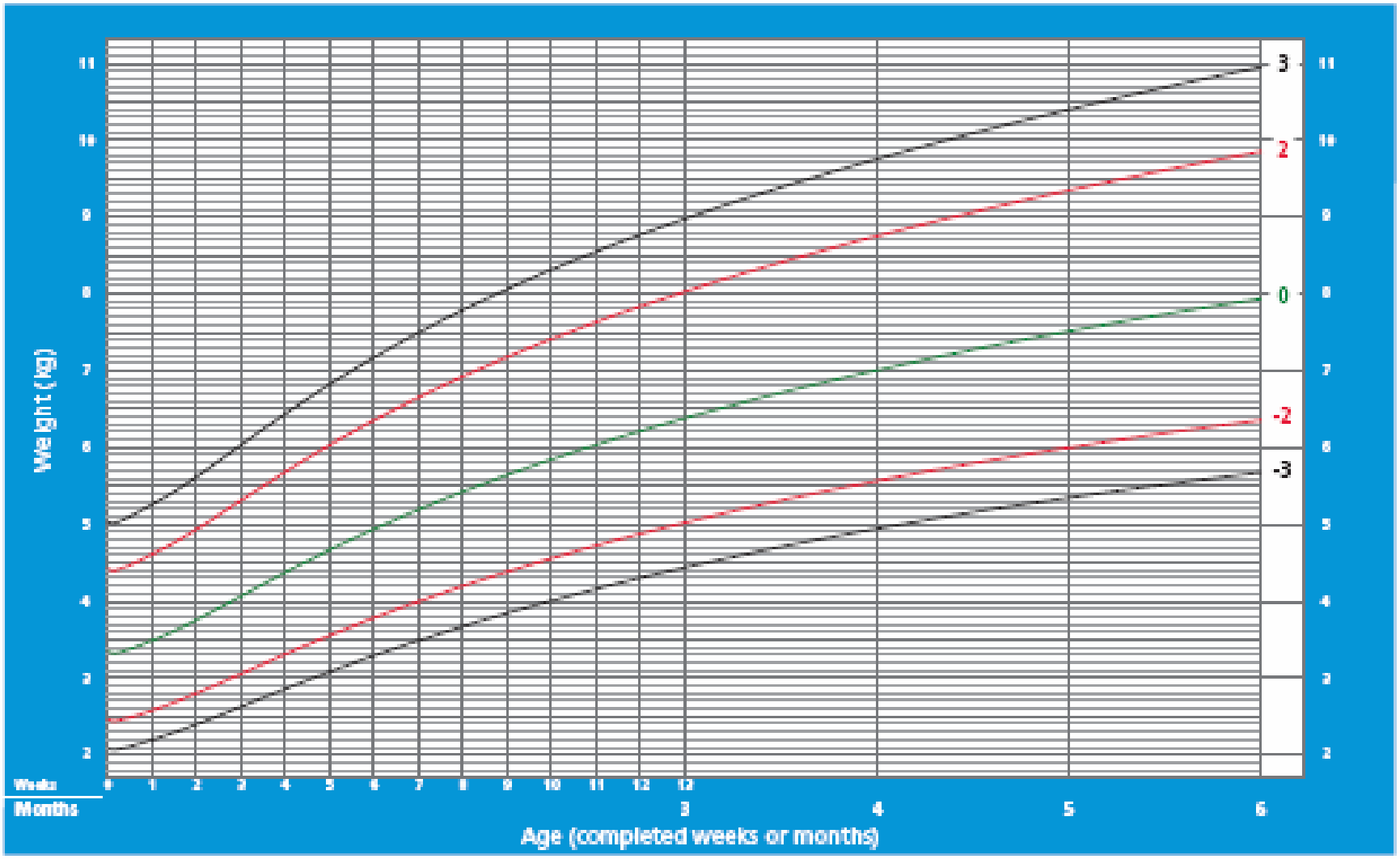
WHO Anthro 2005.lnk

http://www.who.int/childgrowth/en/

Weight-for-age BOYS

Birth to 6 months (z-scores)

World Health Organization



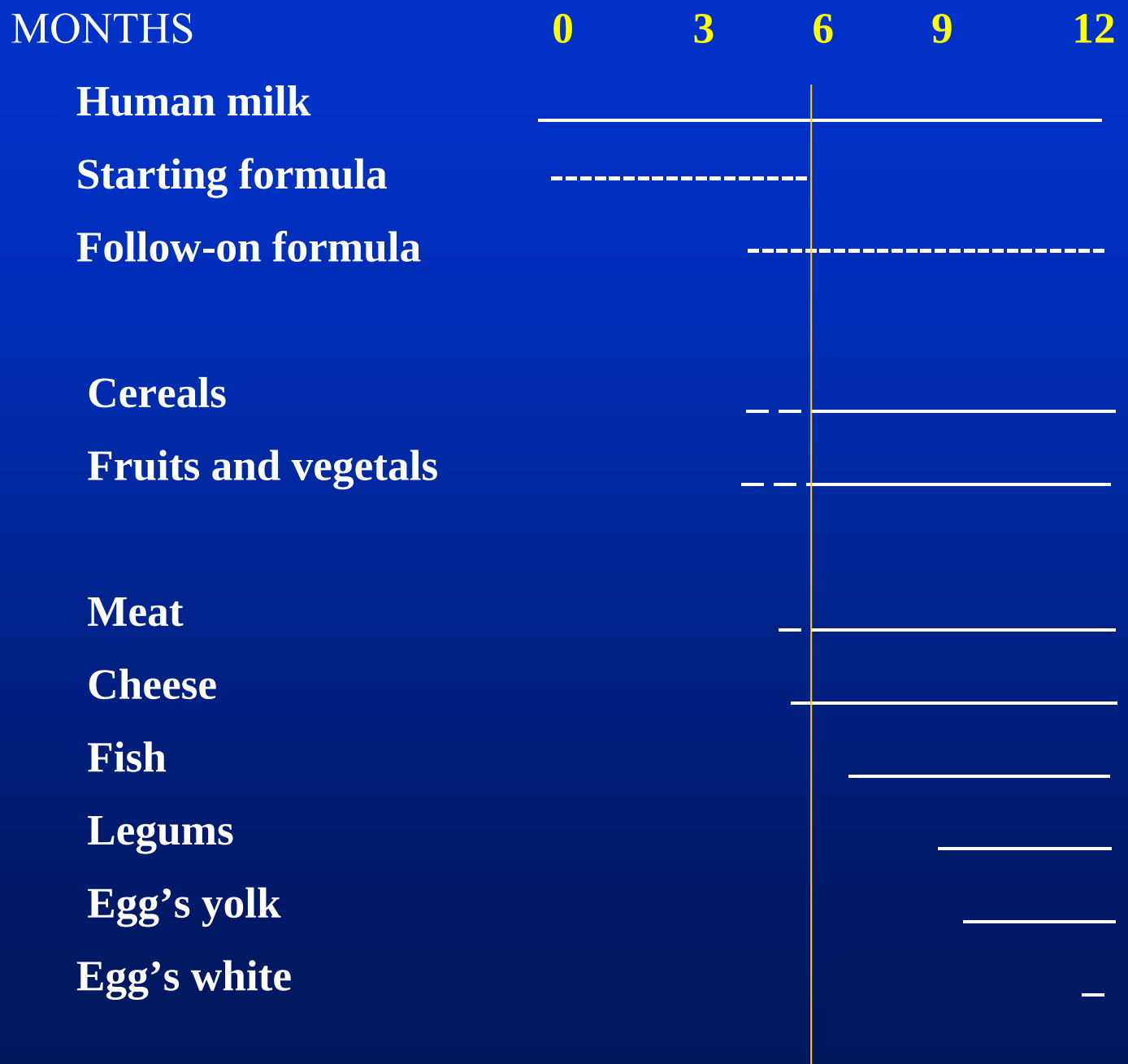
WHO Child Growth Standards

WHAT

- Dietary schedules in most countries take origin from cultural factors and available foods.
- The composition of diet during the complementary feeding period, as well as the type of milk feeding, may have health effects not just in the short- term, but also in the medium and long-term

ESPGHAN CoN, 2007

Complementary feeding: what?



A traditional, *updated* schedule

A timing for the introduction of potentially allergenic foods?

Taking into account the available data on delaying or eliminating specific foods and also the potential wider nutritional consequences, there is no convincing scientific evidence that avoidance or delayed introduction of potentially allergenic foods, such as fish and eggs, reduces allergies, either in infants considered at-risk for the development of allergy, or in those not considered to be at risk .

ESPGHAN CoN, 2007

Journal of Pediatric Gastroenterology and Nutrition
34:337–345 © April 2002 Lippincott Williams & Wilkins, Inc., Philadelphia

Medical Position Paper

Iron Metabolism and Requirements in Early Childhood: Do We Know Enough?: A Commentary by the ESPGHAN Committee on Nutrition

The available literature does not show a causal relationship between *moderate* IDA and impaired cognitive development, even if such an association is plausible based on studies of the role of iron in brain development and function. Until further knowledge is available, measures should be taken to prevent iron deficiency, for example, promoting exclusive breast-feeding, using iron-fortified formula when formula is required, postponing introduction of whole cow milk until the end of the first year of life, and promoting iron-rich complementary foods.

Low fat diet > 2-3 yrs of age: what The case of whole cow's milk

There are considerable differences between countries in recommendations on the age at which cows' milk with reduced fat intake can be introduced.

The main consideration has been that low fat milk might limit energy intake and thereby growth.

It is acceptable to add small volumes of cows' milk to complementary foods, but it should not be used as the main drink before 12 months.

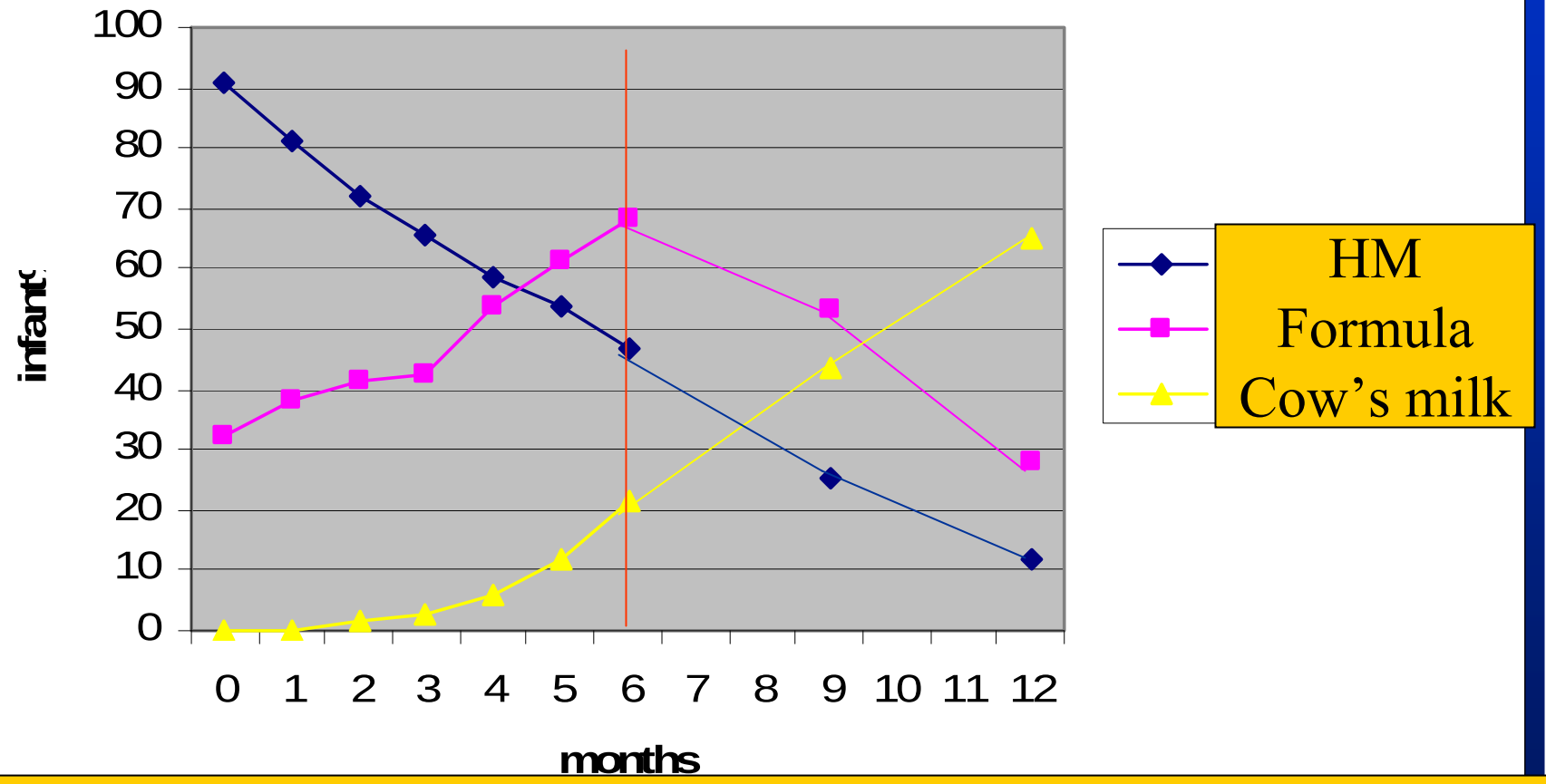
The ESPGHAN Committee concluded in 1994 that fat intake should not be actively reduced before the age of 3 years, but no lower limit for fat content was suggested

The preferential use of cows' milk with a reduced fat content (1.5-2%) was recommended from 2-3 years of life onwards

ESPGHAN CoN, 2007

Milk intakes in the first 12 months in Italy

(Puer Project: Giovannini M et al, Acta Paediatr 2003; 92: 357)

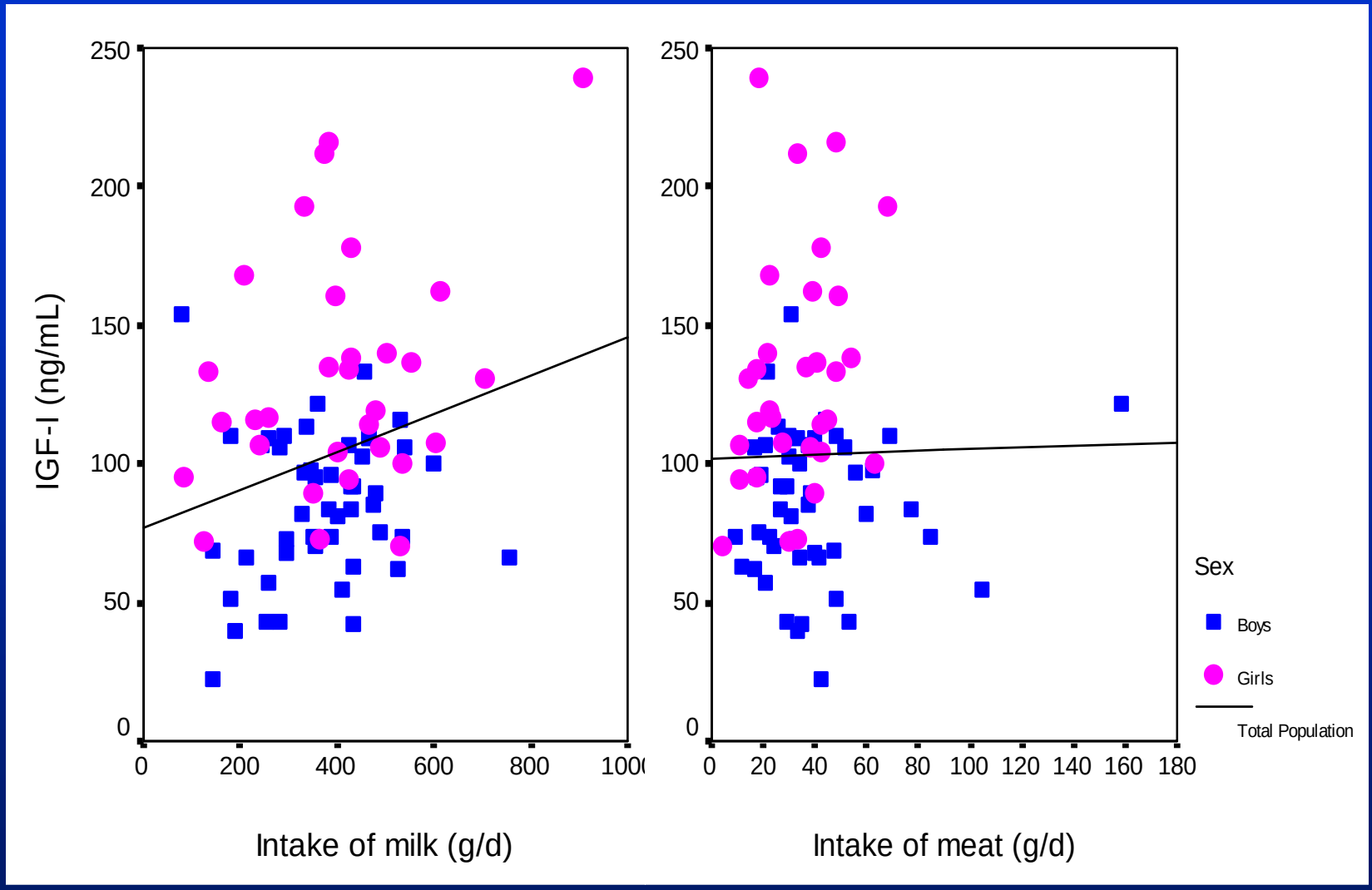


500 mL whole cow's milk supply 18 grams of proteins

Italian Recommended Dietary Allowances (Revised, 1996)

Age	En kcal, range	Protein (adjusted for quality)	
mos	min F/M→max F/M	g/kg/d	%* (x kg)
6-9	653/710 → 950/1027	2.0	8 (x 8)
9-12	739/797 → 1133/1056	1.8	7.6 (x 10)
12-18	854/922 → 1190/1277	1.4	6 (x 11)
18-24	950/1008 → 1306/1382	1.4	6 (x 12)

*calculated



r=0.24, p=0.03

r=0.12, p=0.31

Increase in milk intake from 200 to 600 ml equal to a 30% increase in IGF-1

Future obese?

Better future for her?

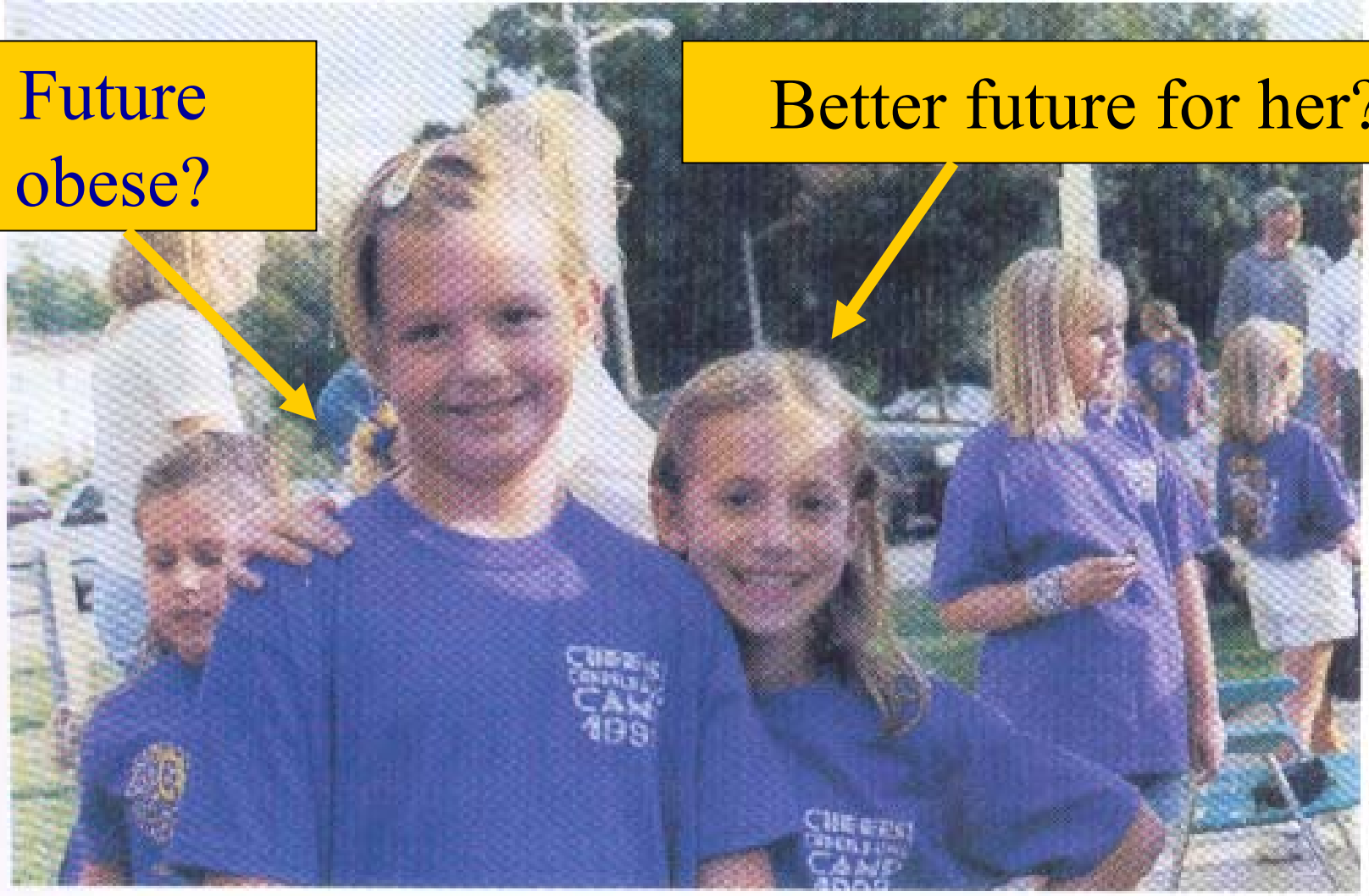


FIGURE 3–11 Both girls pictured are the same age. However, the child on the left consumed a high-protein diet over her lifetime. Genetics and protein consumption both impact overall height and growth rates.

Krause's Food, Nutrition & Diet Therapy, 10th Ed. 2000

Gluten

- Both early (<4 months) and late (≥7 months) introduction of gluten should be avoided
- Gluten should be introduced gradually whilst the infant is still breast-fed.
- Avoiding early (<4 months) introduction of gluten in at risk infants may also reduce the risk of developing diabetes.

ESPGHAN CoN, 2007

Special dietary habits

- If infants and young children are on a vegetarian diet, it is important that the diet include a sufficient amount (about 500 ml) of milk and dairy products.
- During the first years of life a vegan diet (one with no animal products) is dangerous because of the risk of B12 deficiency which can seriously affect neuro-cognitive development, and it should be discouraged.

ESPGHAN CoN, 2007

THE FUTURE

- RCT of the effect of introducing complementary feeding at different ages
- RCT of specific complementary foods/nutrients - suggestions could include meat, LCPUFA
- Studies to determine whether the complementary feeding period is a critical window for long-term programming, including the development of allergy-related disorders, early overweight and obesity.
- Studies examining the effects of complementary foods on later food preferences and intake

ESPGHAN CoN, 2007

Short-term and long-term effects of long-chain polyunsaturated fatty acids in infant nutrition

Tamás Decsi, Department of Paediatrics, University of Pécs, Pécs, Hungary

General background

The human brain grows very quickly during the perinatal period. While most of the lipids which are to be deposited in the growing central nervous system can be synthesised by the infant from other substrates, the essential fatty acids (EFAs) linoleic acid (C18:2n-6, LA) and alpha-linolenic acid (C18:3n-3, ALA) cannot be synthesised by the human organism and thus must be taken up with the diet. The long-chain polyunsaturated fatty acid (LCPUFA) metabolites of LA and ALA, i.e. arachidonic acid (C20:4n-6, AA) and docosahexaenoic acid (C22:6n-3, DHA) comprise substantial part of the lipids of retinal and neuronal tissues. The LCPUFAs to be deposited in the growing membranes must originate either from endogenous synthesis from the precursor EFAs, or from dietary intake of preformed LCPUFAs.

Aim of the presentation

The effects of supplying preformed dietary LCPUFA to the diet of full-term infants will be reviewed on the basis of recently published randomised controlled trials (RCTs) and systematic reviews (SRs) of RCTs. We recently systematically reviewed the effects of supplementing LCPUFA to the diet of expecting women elsewhere [Decsi & Koletzko, 2005].

LCPUFA supplementation and infant growth

We recently contributed with the results of our own RCT [Decsi & Koletzko, 1995] to a SR [Makrides et al, 2005] on the effect of supplementing infant formula with LCPUFA on growth. Body weight, body height and head circumference was evaluated on the basis of the data of about 600 supplemented and 300 control infants investigated in 11 RCTs at the age of 4 months and in 9 RCTs at the age of 12 months. No significant effect of supplementation on infant growth was revealed [Makrides et al, 2005].

Effect of LCPUFA supplementation on the development of visual perception

In a Cochrane Collaboration SR, Simmer [2001] concluded that: ”Minor effects on visual evoked potential acuity have been suggested but appear unlikely when all studies are reviewed.” However, the last study evaluated by Simmer [2001] was published in 1999, and several RCTs have been published thereafter.

Uauy et al [2003] systematically reviewed 7 RCTs published up to 2001 and reporting data on visual acuity in infants fed formula with or without preformed dietary DHA. The authors calculated various DHA equivalent doses by assuming different conversion rates of dietary ALA to DHA. The results indicated a strong and significant effect of DHA equivalent dose on magnitude of the visual acuity response (e.g. $r = 0.82$, $p = 0.001$). Morale et al [2005] combined data from 4 RCTs to assess the effect of the duration of LCPUFA supply on visual acuity and concluded that “A continued benefit from a supply of LCPUFAs is apparent in infants through 52 weeks of age, suggesting that the brain may not have sufficient stores of LCPUFAs from an early postnatal supply to support the optimal maturation of the visual cortex”.

Very recently, Eilander et al [2007] systematically reviewed data of the literature and reported improvement of visual development measured by electrophysiological tests as a result of supplementing 100 mg DHA and 200 mg AA to full-term infants.

Effect of LCPUFA supplementation on cognitive development

The Cochrane Collaboration SR [Simmer, 2001] concluded: "A beneficial effect on information processing is possible but larger studies over longer periods are required to conclude that LCPUFA supplementation provides a benefit when compared with standard formula". Several studies published since 1999 appear to support the concept of a beneficial effect of DHA on cognitive development.

Uauy et al [2003] tabulated 10 studies investigating some aspect of neurodevelopment in infants fed formula supplemented with LCPUFA and in controls, whereas four years later Eilander et al [2007] included only 9 studies in their SR. In spite of the slightly different criteria of selection, the general message of these two SRs can be read as significant beneficial effect of LCPUFA supplementation in some, but by far not in all studies. The considerably different sources and doses of the LCPUFA supplemented renders it impossible to mathematically aggregate ("meta-analyse") the data obtained in the original RCTs. However, the at least five studies reporting significant benefits of supplementation [Agostoni et al, 1995; Forsyth et al, 1996; Willats et al, 1998; Birch et al, 2000; Bouwstra et al 2003 & 2005] appear to furnish evidence for the rationale to include LCPUFAs into fat blends of formulae for full-term infants.

LCPUFA supplementation and the prevention of allergy and asthma

The observation that fish oil supplementation to expecting mothers resulted in a nearly 10% absolute reduction in the prevalence of any wheeze and an about 8% absolute reduction in prevalence of wheeze lasting longer than 1 week [Mihrshahi et al, 2003] gave rise to studies on the effect of LCPUFA supplementation in infancy on prevalence allergic symptoms. For instance, Pastor et al [2006] reported significantly lower incidence of bronchiolitis at the ages of 5, 7 and 9 months in infants fed formula supplemented with DHA and AA than in controls. However, there are apparently similar studies without appreciable beneficial effect of LCPUFA supplementation on the prevention of asthma or allergy [Marks et al, 2006; Almquist et al, 2007].

References

1. Agostoni C, Trojan S, Bellu R, Riva E, Giovannini M. Neurodevelpmental quotient of healthy term infants at 4 months and feeding practice: the role of long-chain polyunsaturated fatty acids. Pediatr Res 38: 262-266, 1995
2. Almqvist C, Garden F, Xuan W, Mihrshahi S, Leeder SR, Oddy W, Webb K, Marks GB; CAPS team. Omega-3 and omega-6 fatty acid exposure from early life does not affect atopy and asthma at age 5 years. J Allergy Clin Immunol 119: 1438-44, 2007
3. Birch EE, Garfield S, Hoffman DR, Uauy R, Birch DG. A randomized controlled trial of early dietary supply of long-chain polyunsaturated fatty acids and mental development in term infants. Dev Med Child Neurol 42: 174-181, 2000
4. Bouwstra H, Dijk-Brouwer DA, Wildeman JA, Tjoonk HM, van der Heide JC, Boersma ER, Muskiet FA, Hadders-Algra M. Long-chain polyunsaturated fatty acids have a positive effect on the quality of general movements of healthy term infants. Am J Clin Nutr 78: 313-318, 2003
5. Bouwstra H, Dijck-Brouwer DAJ, Boehm G, Boersma ER, Muskiet FAJ, Hadders-Alagra M. Long-chain polyunsaturated fatty acids and neurological developmental outcome at 18 months in healthy term infants. Acta Paediatr 94: 26-32, 2005
6. Decsi T, Koletzko B. Growth, fatty acid composition of plasma lipid classes, and plasma retinol and α-tocopherol concentrations in full-term infants fed formula

enriched with ω-6 and ω-3 long-chain polyunsaturated fatty acids. Acta Paediatr, 84: 725-732, 1995
7. Decsi T, Koletzko B. Omega-3 fatty acids and pregnancy outcome. *Curr Opin Clin Nutr Metab Care,* 8: 161-166, 2005
8. Eilander A, Hundscheid DC, Osendarp SJ, Transler C, Zock PL. Effects of n-3 long chain polyunsaturated fatty acid supplementation on visual and cognitive development throughout childhood: a review of human studies. Prostaglandins Leukot Essent Fatty Acids 76: 89-203, 2007
9. Forsyth JS, Willats P, DiModogno MK, Varma S, Colvin M. Do long-chain polyunsaturated fatty acids influence infant cognitive behaviour? Biochem Soc Trans 26: 252-257, 1996
10. Makrides M, Gibson RA, Udell T, Ried K; The International LCPUFA: Investigators. Supplementation of infant formula with long-chain polyunsaturated fatty acids does not influence the growth of term infants. Am J Clin Nutr 81: 1094-1101, 2005
11. Marks GB, Mihrshahi S, Kemp AS, Tovey ER, Webb K, Almqvist C, Ampon RD, Crisafulli D, Belousova EG, Mellis CM, Peat JK, Leeder SR. Prevention of asthma during the first 5 years of life: a randomized controlled trial. J Allergy Clin Immunol 118: 53-61, 2006
12. Mihrshahi S, Peat JK, Marks GB, Mellis CM, Tovey ER, Webb K, Britton WJ, Leeder SR; Childhood Asthma Prevention Study. Eighteen-month outcomes of house dust mite avoidance and dietary fatty acid modification in the Childhood Asthma Prevention Study (CAPS). J Allergy Clin Immunol 111: 162-8, 2003
13. Morale SE, Hoffman DR, Castañeda YS, Wheaton DH, Burns RA, Birch EE. Duration of long-chain polyunsaturated fatty acids availability in the diet and visual acuity Early Hum Dev 81: 197-203, 2005
14. Pastor N, Soler B, Mitmesser SH, Ferguson P, Lifschitz C: Infants fed docosahexaenoic acid- and arachidonic acid- supplemented formula have decreased incidence of bronchiolitis/bronchitis the first year of life. Clin Pediatr 45: 850-855, 2006
15. Simmer K: Longchain polyunsaturated fatty acid supplementation in infants born at term. Cochrane Database Syst Rev CD000376, 2001
16. Uauy R, Hoffman DR, Mena P, Llanos A, Birch EE: Term infant studies of DHA and ARA supplementation on neurodevelopment: results of randomized controlled trials. J Pediatr 143: S17-S25, 2003.
17. Willats P, Forsyth JS, DiModugno MK, Varma S, Colvin M: Effect of long-chain polyunsaturated fatty acids in infant formula on problem solving at 10 months of age. Lancet 352: 688-691, 1998

Probiotics and allergic rhinitis.

Giorgio Ciprandi

Allergic rhintis is characterized by a Th2-polarized immune response. Thus allergic children show a defect of IFN-□defect that induces an increased number of infections. Respiratory infections (RI), mainly involving the upper airways, are common in children and their recurrence constitutes a demanding challenge for the paediatrician (1). It has been proposed that to diagnose recurrent RI (RRI) at least one of the following criteria has to be present: i) > 6 annual RI, ii) > 1 monthly RI involving the upper airways from September to April, iii) > 3 annual RI involving the lower airways (2). Moreover, RRI represent a social problem both concerning the pharmaco-economy and the impact on the family and social milieu of the child. In addition, allergic children have more numerous and severe respiratory infections than non-allergic children (3).

It has been previously reported that the prophylactic use of a probiotic milk reduced the number and the severity of RI among children attending day care centres (4). Probiotics are microorganisms that exhibit a beneficial effect on the health of the host (5). The interaction between probiotics and host has a profound impact in many ways. One is to stimulate immune system by promoting T helper-1 (Th1) and T regulatory (Treg) immunity, and by decreasing T helper-2 (Th2) activity in allergic subjects (6). In this regard, *Bacillus clausii* is a probiotic capable of modulating the immune response (7,8). Particularly, it has been evidenced that *B clausii* stimulates Th1 and Treg immunity, promoting IL-12, IFN□, IL-10, and TGF□ synthesis, and down-regulates Th2 response, inhibiting IL-4 production, in allergic children with RRI (9).

The possible mechanism of action may be due to the probiotic modulatory activity on the immune response as previously reported in a study concerning allergic children with RRI (9). In fact, RRI may be consequent to a “relative” immaturity of the immune system that is typically Th2 polarized in first and second infancy (9). Probiotics are able to direct the immune response toward a physiologic Th1 oriented polarization that adequately fight infections mainly by IFN□. This phenomenon depends on the stimulation of Treg cells releasing cytokines important for stimulating the Th1 response. Moreover, previous studies demonstrated that *Bacillus clausii* stimulates the production of IgA (11). IgA synthesis is under the strict control of TGF□ that is just induced by Treg cells (12).

In conclusion, probiotics may be useful in treating both allergic rhinitis and in preventing RRI.

REFERENCES

1) Acute respiratory infections: the forgotten pandemic. Bull World Health Organ 1998; 76 (1): 101-103

2) Gruppo di studio di Immunologia della società Italiana di Pediatria: Le infezioni ricorrenti nel bambino: definizione ed approccio diagnostico. Riv Imunol Allergol Pediatrica 1988; 2: 127-134

3) Ciprandi G, Tosca MA, Fasce L. Allergic children have more numerous and severe respiratory infections than non-allergic children. Ped Allergy Immunol (in press)

4) Hatakka K, Savilahti E, Ponka A et al. Effect of long term consumption of probiotic milk on infections in children attending day care centers: double blind, randomised trial. BMJ 2001;322:1-5

5) Kaur IP, Chopra K, Saini A Probiotics: potential pharmaceutical applications. Eur J Pharm Sci 2002;15:1-9

6) Elson CO, Cong Y Understanding Immune-Microbial homeostasis in intestine. Immunologic Research 2002;26:87-94

7) Ciprandi G, Vizzaccaro A, Cirillo I, Tosca MA *Bacillus clausii* effects in children with allergic rhinitis. Allergy 2005;60:702-3

8) Ciprandi G, Vizzaccaro A, Cirillo I, Tosca MA *Bacillus clausii* exerts immuno-modulatory activity in allergic subjects: a pilot study. Eur Ann Allergy Clin Immunol 2005;37:129-33

9) Ciprandi G, Tosca MA, Milanese M, Caligo G, Ricca V Cytokines evaluation in nasal lavage of allergic children after bacillus clausii administration: a pilot study. Ped Allergy Immunol 2004;15:148-51

10) Duc LH, Hong HA, Barbosa TM, Henriques AO, Cutting SM. Characterization of Bacillus Probiotics Available for Human Use. Appl Env Microbiology 2004;70:2161-71

11) Fiorini G, Cimminiello C, Chianese R et al. B subtilis selectively stimulates the synthesis of membrane bound and secreted IgA. Chemioter 1985;4:310-2

12) Sporn MB, Roberts AB Transforming growth factor-beta recent progress and new challenges. J Cell Biol 1992;119:1017-21

Allergen Free Probiotics

Dr. Luca Mogna, Probiotical Srl, Novara

Food allergies and food intolerances are both types of “food sensitivities”.

Food allergies are anomalous immunological reactions (IgE-mediated) to generally safe foods. The food component which triggers this reaction (allergen) is generally a protein in the molecular weight range of 5 to 200 kDa. Many allergens may be found in foods and adverse reactions generally occur shortly after product ingestion. Most reactions are short-lived and relatively harmless, but severe allergic reactions leading to anaphylactic shock and death are not uncommon.

Even though their symptoms are similar to food allergies, food intolerances do not involve IgE production and adverse reactions may occur even hours after consumption. Food intolerance can develop towards a wide range of foods. Intolerances can be triggered by enzymatic deficiencies or biochemical reactions due to substances naturally present in the food or specifically used as additives. For example, lactose intolerance is due to a deficiency of the enzyme lactase, needed to break the disaccharide down into the single sugars, glucose and galactose.

Both food allergies and intolerances are constantly increasing in developed countries. It is estimated that 2-4% of adults and 6-8% of children up to 3 years of age suffer from these food insensitivities. Moreover, among allergic people between 20 and 30 per cent may have an adverse reaction to food which is not revealed by skin or blood tests.

Currently, the only way to treat food allergies is to avoid the foods which trigger these reactions.

The European Economic Community has defined a list of 12 classes of potential allergens (cereals containing gluten, crustaceans, eggs, fish, peanuts, nuts, soybeans, milk, celery, mustard, sesame, and sulphur dioxide at levels above 10mg/kg or 10 mg/litre expressed as SO_2) which are included in Annex IIIa of Directive 2003/89/EC, whose aim is to achieve a high level of health protection for consumers and guarantee the right to information through clear and complete product labelling.

The recent increase in allergic disorders has been attributed to a relative lack of microbial stimulation of the infantile gut immune system (Journal of Allergy and Clinical Immunology, January 2007). Perturbations in the gastrointestinal (GI) microbiota composition that occur as a result of antibiotics and diet in “westernized” countries are strongly associated with allergies and asthma (hygiene hypothesis) (Trends in Microbiology, December 2004). For this reason, many efficacy trials have been conducted and demonstrated the ability of probiotic strains to modulate immune responses. Furthermore, the capacity of probiotic bacteria to shift the equilibrium of the mucosal immune system towards Th1 response provides a strategy for treatment of these disorders (Immunology and Cell Biology, February 2000).

However, some clinical studies with probiotic supplements have identified unfavourable reactions, especially in children. The reason for these negative results has been investigated in some cases. Morisset M. et al. have analysed and confirmed that about 70% of probiotic supplements commonly used today in France contain milk and/or its derivatives. They also reported a case of an infant with cow’s milk allergy who experienced anaphylactic shock a few minutes after ingesting a probiotic supplement (Journal of Allergy and Clinical Immunology, March 2007).

The administration of probiotics which are not “Allergen Free” poses a serious risk, especially to children. In fact, the allergological profile of a child is generally not well known and unexpected adverse effects may occur after the intake of even traces of one or more allergenic molecules.

This further reinforces the need for safe and effective probiotics according to the European Directive. Safe probiotics should be produced by avoiding all potential allergens or raw materials derived from these substances by means of hydrolysis, heating and/or mechanical treatments.

The first step to achieve this objective is the identification of the primary allergenic or sensitizing raw materials typically used in the production of probiotic strains (e.g. as carbon sources, nitrogen sources, cryoprotectants, excipients, etc.). Some common examples are milk proteins, lactose, soy proteins, gluten-containing cereals and their respective derivatives.

The second step is the substitution of these materials at risk with alternative substances, particularly of vegetable origin. The use of vegetable raw materials, particularly those rich in bioactive peptides, provides an excellent nutritional source for probiotic bacteria and guarantees their healthy growth and more robust stability, even after freeze-drying.

The last step is the optimisation of all manufacturing phases as regards both the achievement of the highest industrial yield and especially an extremely vital biomass, indispensable to minimize the mortality and stress of the successive freezing phase. The main common industrial steps in the production of a probiotic microrganism are fermentation, concentration, cell washing, cryoprotection and freeze-drying.

The overall Allergen Free manufacturing process is able to guarantee safe consumption even to individuals with food allergies and/or food intolerances and also to improve stability of the freeze-dried probiotic in biobulk at room temperature due to the physiological integrity of the bacterial cells grown in media rich in bioactive peptides.

A food supplement containing one or more probiotic strains should be prepared using only other active components (e.g. prebiotic fibres, aminoacids, vitamins, minerals, etc.) and technological excipients which are Allergen Free and certified as such by their supplier.

Pediatric supplements should be optimized with particular attention to the following points:

- use of strains typical of the microbiota of children;
- formulation totally Allergen Free;
- the avoidance of fructose or saccharose for individuals suffering from hereditary fructose intolerance (HFI);
- use of only natural sweeteners, flavourings and, if present, colourants;
- organoleptic characteristics specifically targeted to appeal to children’s tastes (sweetness, fruit colour and flavour, etc.).

Many kind of Allergen Free product typologies such as sachets, unit dose vials, capsules and tablets (swallowable, chewable and modified release) may be manufactured.

The Quality System must guarantee the non-use of allergenic raw materials and their derivatives in all phases of the manufacturing process of the probiotic strain and the finished product. The application of an effective HACCP control plan must be able to monitor possible cross-contamination by means of ultra-sensitive and reliable analytical methods which are able to detect even traces of allergens in raw materials, process intermediates and finished products.

In this way technology, research and the quality system are indispensable for the production of safe and effective probiotic strains and the food supplements containing them.

PROBIOTICS AND IMMUNE SYSTEM…BEYOND THE PROBIOTICS

Annamaria Castellazzi, Chiara Valsecchi, Gianluigi Marseglia
Laboratory of Immunity and Nutrition
Dept. of Paediatric Science
University of Pavia

In order to study the activity of probiotic strains "in vitro", mainly in the field of immunological modulation, we kill the bacteria by irradiation, to avoid the possibility of overgrowth in culture medium. In this way we can demonstrate the activity , at molecular level, that the probiotic strains exert on cells of the innate and adaptive immune system.
The probiotic effect , "in vivo", regards the establishment of a normal intestinal permeability, when altered and the restoration of a normal intestinal micro-ecology when it is unbalanced.
For these effects the probiotic bacteria must reach the gut environment alive and colonize the gut mucosa., almost temporarily.
Moreover, it is now evident the important role of an array of bacterial surface molecules. for the immunomodulation activity that directs the immune response to the control of the intestinal inflammation through cytokines production.
Humans, but also other mammals, have a dynamic relationship with environmental bacteria. Newborns are rapidly colonize by the bacteria flora that quickly populate the gastro-intestinal tract. Bacterial density is well controlled, showing a gradient from the stomach to the colon and tolerance to the molecular pattern associated to bacteria (such as lipopolysaccharide LPS) appears very early in the perinatal period as demonstred by the study of Lotz et al, 2006 (1).
It has been recently reported by Mazmanian et al (2005) (2) that an immunomodulatory molecule , expressed by commensal bacteria is involved in directing the development of a normal immune system and response. *Bacteoides fragilis* produces a zwitterionic polysaccharide (ZPS) that activates CD4+ T cells and can correct the reduced proportion of CD4+ T cells in the splenic lymphocyte population and the dysregulated systemic cytokine production that are found in the absence of bacterial colonization. So, the ZPSs of symbiotic bacteria have emerged as the archetypal members of a family of health-promoting microbial molecules. The structure of ZPS presents both positive and negative charges in each repeating unit (3).
Bacterial polysaccharides are classical B cells antigens and are recognised by a B-cell Receptor (BCR) specific for each polysaccharide. Interaction between the polysaccharide and the specific BCR is sufficient to induce the signals required to stimulate clonal expansion of B cells and antibody production. However this pathway does not result in immunological memory. Purified polysaccharides induce specific IgM response, without a detectable IgG response. A failure to induce immunoglobulin class switching from IgM to IgG isotypes (excluding IgG3) and a lack of increased antibody production after rechallenge with antigen are hallmarks of a classic T-cell-independent immune response.
The conjugation of polysaccharides to proteins seems to allow carbohydrate specific responses that elicit T-cell help, and this technique has been used to improve the efficacy of vaccine (4). Polysaccharide-protein conjugates interact with a BCR in a way similar to polysaccharides, but, in addiction, they elicit T-cell help, through antigen presentation of the protein component to CD4+ T cells, which provide the necessary co-stimulation to induce memory B cells and memory T cells. Therefore antibody production and immunoglobulin class switching are achieved and the consequent immunological memory results in antigen specific immunity to both the polysaccharide and the protein.
Zwitterionic polysaccharides interact directly with CD4+ T cells in a manner similar to protein antigen. These polysaccharides are captured by Antigen Presenting Cells (APCs), degraded and presented to T-cells, leading to T-cells activation. In addition , zwitteronic polysaccharides elicit B-cells–dependent antibody responses similar to those elicited by conventional polysaccharides. Than

ZPSs constitute a structurally distinct category of carbohydrates and seem to elicit immune responses that are unique among bacterial polysaccharides . The important finding that APCs are required (5) for CD4+ T cell activation has a profound impact on the understanding why a commensal bacterium might have evolved the ability to induce protective T cell responses. Polysaccharide A (PSA) of *B. fragilis* , a zwitterionic polysaccharide, is internalised into the endosome of professional APCs as an intact polymer and it is degraded by an oxidation reaction (unlike proteins which are processed by proteases in the endosome) . This reaction requires the presence of nitric oxide synthase2 (NOS2) and the generation of nitric oxide (NO), which mediates the oxidative breakdown of PSA into smaller fragments. PSA fragments are loaded onto the MHC class II molecule HLA-DR which is present in the endosome. PSA fragments are presented in the context of MHC class II molecules to the T-cell receptor (TCR) of CD4+ T cells and activate these cells.

It is possible that immunomodulatory molecules of symbiotic bacteria evolved to induce immune responses that are distinct from (or perhaps opposite to) those induced by virulence factors of pathogens? An investigation into the mechanisms of protection against inflammation has recently shown that ZPSs induce CD4+ T cells to express the cytokine IL-10 (6). In response to treatment with ZPS , IL-10 is produced by an heterogeneous subpopulation of CD4+ T cells, $CD45RB^{low}$, that contains activated T cells, memory T cells and regulatory T cells (CD4+CD25+). As peripheral T regs are known to traffic to sites of inflammation where they control T cell reaction, T cell migration from lymph nodes to spleen may mediate the cellular signal-transduction mechanisms required for PSA-mediated immune development at extraintestinal sites.

Further studies are required to understand the role of PSA-induced IL-10 production in determining the balance of Th1 and Th2 cytokines and protection against disease.

Various and distinct immune mechanisms have evolved to control aberrant immune response to innocuous antigens. The source of the antigen (self or non-self) might be a key determinant of the response generated. The human immune system elaborates mechanisms to prevent or suppress inflammation in response to self antigens and these mechanisms have been extensively studied. By contrast the mechanism by which a host controls responses to foreign non-pathogenic molecules, such as commensal bacteria, food and inhaled antigens, remains less understood. Commensal bacteria may provide instruction for the development of the host immune system and the developmental signals might be crucial for establishing a correct and balanced immune system, the base for mammals health. In the past two decades it has been supposed that a reduction in exposure to infectious bacterial agents early in the life can be the cause of increased incidence of allergy in developed countries (hygiene hypothesis) (7). Several epidemiological studies have shown that the composition of the gut microflora differs between atopic and non-atopic individuals: children with allergies had lower rates of colonization by *Bacteroides* species and higher rates of colonization by anaerobic bacteria that children without allergies(8). It is possible that the development of tolerance to environmental antigens might require immune mechanisms that are mediated by symbiotic bacteria at mucosal surfaces. Identifying the molecular interaction between ZPSs and immune system during immune system development may bring more informations to understand the mechanism underlying the two distinct arms of immunological tolerance, that is tolerance to self antigens and tolerance to innocuous non-self antigens.

References

1. Lewelyn M, Cohen J. Superantigens: microbial agents that corrupt immunity. Lancet Infect. Dis. 2002 ; 2 : 156-162.
2. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 2005; 122: 107-118.
3. Tzianabos AO, Russell PR, Onderdonk AB, Gibson FC 3rd, Cywes C, Chan M, Finderg RW, Kasper DL. IL-2 mediates protection against abscess formation in an experimental model of sepsis. J. Immunol 1999; 163: 893-897.

4. Kelly DF, Moxon ER, Pollard AJ. *Haemophilus Influenzae* type b conjugate vaccines. Immunollogy 2004; 113: 163- 174.
5. Kalka-Moll WM, Tzianabos AO, Wang Y, Carey VJ, Finberg RW, Onderdonk AB, Kasper DL Effect of molecular size on the ability of zwitterionic polysaccharides to stimulate cellular immunity. J Immunol. 2000 ;164:719-24.
6. Ruiz-Perez B, Chung DR, Sharpe AH, Yagita H, Kalka-Moll WM, Sayegh MH, Kasper DL, Tzianabos AO. Modulation of surgical fibrosis by microbial zwitterionic polysaccharides. Proc Natl Acad Sci U S A. 2005; 102 :16753-8.
7. Willis-Karp M, Santelliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. Nature Rev Immunol2001; 1: 69-75.
8. Kalliomaki M, Isolauri E. Pandemic of atopic disease- a lack of microbial exposure in early infancy? Curr Drug Targets Infect Disord 2002; 2: 193-199.

Update on research with *Lactobacillus reuteri*

Eamonn Connolly, PhD,
Senior Vice President Research, BioGaia AB, Stockholm, Sweden

True probiotics are live micro-organism which, when given in adequate amounts, confer health benefits on the host (WHO Expert Consultation definition) and extensive clinical study of *L. reuteri* ATCC 55730 over the past 15 years confirms the true probiotic properties of strains of this species. *L. reuteri* effectively colonizes the entire human gastro-intestinal tract and it is one of few probiotics studied showing safety and efficacy, not only in adults but also in children, infants and premature neonates from birth. It is well-established that *L. reuteri* supplementation reduces the severity and extent of diarrhoea in young children (Shornikova et al. 1997a,b).

The mechanism of action of this probiotic is related, not only to a direct interaction with pathogens leading to microfloral balance in the gastrointestinal tract (Casas & Dobrogosz, 2000), but also to interaction and communication with the immune system through the gut mucosal wall. *L. reuteri* has been shown to modulate the activity of CD4+ T-helper cells in the human ileum (Valeur et al 2004) and this may be a key mechanism by which this probiotic leads to immune changes distant from the gut, such as modulation of cytokine expression in breast milk and effects on respiratory infections. Further, expecting mothers who took either *L. reuteri* for 4 weeks prior to giving birth were found had significantly higher levels of anti-inflammatory cytokines in the colostrum compared to those who received placebo (Jakobsson et al. 2005). This provides 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 to other epithelia, in this case the mammary gland.

Daily supplementation of infants and babies with *L. reuteri* protect them from gastro-intestinal infections, which would otherwise have necessitated medical attention. *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 (Weizman et al., 2005). The authors noted that *L. reuteri* was found to be superior to both placebo and supplementation with *B. lactis* in maintaining the gastro-intestinal health of the infants. Similar results have been demonstrated in adults, where daily supplementation of a healthy workforce with *L. reuteri* could half the absenteeism due to short-term gastrointestinal or respiratory illness (Tubelius et al. 2005).

Probiotics are live micro-organisms and must be delivered to the consumer at the documented efficacious dose throughout the shelf-life of the product. Formulations must therefore be shown to deliver the correct bacteria and at the correct dose. Convenience for the consumer is also an important factor, for example in infants where the recently developed *L. reuteri* drops formulation makes administration trouble-free, particularly in the hospital intensive case setting. The ease of use of *L. reuteri* drops formulation has stimulated *L. reuteri* clinical research, particularly in small infants.

A multi-centre trial on the effect of long-term supplementation of high allergy risk newborn infants with *L. reuteri* on the incidence of allergy in the first two years of life was recently completed (Abrahamsson et al. 2007). The cumulative incidence of eczema was similar between the *L. reuteri* – supplemented and placebo infants (36% versus 34% respectively), but *L. reuteri* treated infants had significantly less IgE-associated eczema during the second year of life (8% versus 20%). Skin prick test reactivity was less common in the *L. reuteri* babies, particularly if the mother was allergic (14% versus 31%). Sensitization markers of are known to be strongly correlated with the incidence of later onset respiratory allergy and thus there is good possibility that the *L. reuteri* supplemented infants will develop less asthma and allergic rhinoconjunctivitis when they reach school age and beyond (Abrahamsson et al. 2007). This is currently under investigation.

L. reuteri has a positive effect on gut function, particularly in infants. Savino et al. (2007) recently presented a comparison of *L. reuteri* with simethicone for the alleviation of crying in colicky infants. Crying times were reduced from 197 to 51 minutes per day after 1 month supplementation with *L. reuteri*, with 95% of the infants responding to the treatment. Simethicone-treated infants reduced from similar baseline crying to 145 mins/day after 1 month, with only 7% of the infants responding to treatment (Savino et al., 2007). Recent observations on gut motility in preterm, but healthy infants show that the *L. reuteri* drops induce significantly improved gastric emptying, associated with reduced gastro-intestinal disturbances such as regurgitation. Crying time was again reduced in *L. reuteri* babies, probably as an indicator of improved gut motility, function and food tolerance (Indrio et al., 2007).

There is growing interest among paediatricians to utilise probiotics as supplements in infants at risk of infection, particularly preterm infants. Romeo et al. (Romeo et al., 2006; Betta et al., 2007) presented convincing data to show that *L. reuteri* can reduce the risk of life-threatening infection with *Candida* or bacteria in premature and surgical infants in the neonatal intensive care unit. The finding that supplementation with *L. reuteri* reduced hospital stay from 42 to 21 days (Romeo et al., 2006) is remarkable and is stimulating intense further study in other major clinics around the world.

Ingestion of *L. reuteri* leads to a colonization of the human gastro-intestinal mucosa (Valeur et al. 2004; Glintborg et al., 2006). Colonisation and growth of *L. reuteri* in the gastric antrum, corpus 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, it is of interest to determine the effect of *L. reuteri* in *H. pylori* infected subjects. Francavilla et al. have shown that the use of *L. reuteri* can alleviate the symptoms of *H. pylori* infection as well as the side effects of eradication antibiotics used to treat the infection (Lionetti et al., 2006; Francavilla et al. unpublished). The prospects for using *L. reuteri* as a complement to the present therapeutic armamentarium in the treatment of *H. pylori*-infected patients look very promising. Further, new strains of *L. reuteri,* which have strong and specific anti-inflammatory

effects, are about to enter clinical trials where the main aim will be to reduce detrimental inflammation caused by *H. pylori*.

Advances are being made concerning the genetic basis of the function of the *L. reuteri* probiotic strains. The genomes of *L. reuteri* ATCC 55730 and *L. reuteri* ATCC PTA 6475 have been sequenced and coupling of gene expression to phenotypic characteristics related to the differing probiotic effects of these strains is in progress (Storm et al., 2007). Mapping of the antibiotic resistance patterns of the *L. reuteri* species (Egervärn et al., 2007) together with mapping of the genes carried by specific plasmids in the *L. reuteri* ATCC 55730 parent strain has allowed the natural generation of the daughter strain *L. reuteri* DSM 17938 in which plasmid-bourne TetW and Lnu resistance genes have been removed without affecting the function of the probiotic.

Research on *L. reuteri* at the genome level combined with an extensive clinical program will provide not only a deeper understanding of the mechanisms of action of this probiotic, but also allow the development of new probiotic concepts for specific uses in the future.

References

Abrahamsson T et al. 2007. *J Allergy Clin Immunol* 119:1174-1180.
Betta P et al. 2007. *40th ESPGHAN Annual Meeting,* abstr PG4-13.
Casas IA & Dobrogosz WJ. 2000. *Microbial Ecology in Health and Disease* 12: 247-285.
Egervärn M et al. 2007. *J Food Protection* 70: 412-418.
Glintborg V et al. 2006. *Int J Probiotics Prebiotics.* 1:225-232.
Indrio F et al. 2007. *48th ESPR Annual Meeting*, Prague, October 2007.
Jakobsson T et al. 2005. *38th ESPGHAN Annual Meeting*, abstr. OP4-05.
Lionetti E et al. 2006. *Aliment Pharmocol Ther* 24:1461-1468.
Romeo MG, Betta P, Olivieri S. 2006. *J Perinat Med* 34 (suppl I) A9, abstract MSL24.
Savino F. et al. 2007. *Pediatrics* 119: 124-30.
Shornikova A et al. 1997a. JPGN 24:399-404.
Shornikova A et al. 1997b. *Pediatr. Infect. Dis. J.* 16:1103-7.
Storm M et al. 2007. 107th ASM Meeting Toronto, May 2007, abstr. O-083.
Tubelius et al. 2005. *Environmental Health* 4: 25.
Valeur N et al. 2004. *Appl Environ Microbiol* 70:1176-1181.
Weizman Z & Alsheikh A. 2006. *J Am Coll Nutr* 25: 415-9.
Weizman Z et al. 2005. *Pediatrics* 115: 5-9.

Probiotic bacteria for treatment of gastroenteritis

Jorge Amil Dias
Hospital S. João, Porto, PORTUGAL

Probiotics are living microbial organisms consumed as food supplements that beneficially affect the host by improving its mucosal balance. Its mechanism of action is still unclear although several hypotheses have been formulated. Many different species and strains have been used, but lactic acid bacteria and the yeast *Saccharomyces boulardii* have attracted most attention.

Many clinical trials have addressed the beneficial effect of probiotics in the treatment of acute gastroenteritis. The concept of using live organisms to re-establish the balance of microbiota of the gut and counteract the effect of pathogenic bacteria and virus is indeed very tempting. However the design, preparations used, methods and outcome measures of the various studies create difficulty in the global evaluation of the efficacy of probiotics in acute diarrhoea. Meta-analyses help evaluating and comparing different publications. Four meta-analyses have addressed this issue evaluating randomized controlled trials (RCT's) and will be briefly reviewed.

Szajeswska and Mrukowicz (1) reviewed 10 treatment and 3 prevention trials. Treatment outcomes evaluated consisted in the presence of diarrhoea lasting more than 3 days (8 RCT's, n=731). In this regard the pooled effect of probiotics showed an estimated risk of 0.40 (95% CI, 0.28 to 0.57) and 4 patients would need to be treated to avoid one case of diarrhoea lasting > 3 days. Of the examined strains in the various studies only *Lactobacillus GG* had a consistent effect (3 RCT's). Regarding duration of diarrhoea (8 RCT's, n=773) the analyses revealed that probiotics significantly reduced the duration of diarrhoea by -18.2h (95%CI, -26.9 to -9.5).

Van Niel et al (2) scanned 9 RCT's (n=765) evaluating effects of probiotics versus placebo regarding duration and frequency of diarrhoea. They concluded that there was a reduction of 0.7 days in diarrhoea duration (95% CI, 0.3 to 1.2) in patients receiving *Lactobacillus*. There was also a reduction of frequency of diarrhoea (1.6 fewer stools in treated subjects, 95% CI, 0.7 to 2.6).

Huang et al (3) reviewed 18 RCT's (n=1917) . Results of analyses showed that treated groups had diarrhoea reduced by 0.8 day (95% CI, -1.1 to -0.6). Despite considerable heterogeneity the authors conclude that reviewed studies provide confirmatory evidence of efficacy of probiotic supplementation in reducing duration of symptoms among

children up to 5 years of age with acute non-bacterial diarrhoea. Probiotics and particularly lactobacilli reduced the duration of an acute diarrhoeal episode in an infant or child by approximately one day.

Allen et al (4) reviewed 23 studies (n=1917 of which 1449 children). Analyses revealed that probiotics reduced the risk of diarrhoea at 3 days (RR 0.66, 95% CI, 0.55 to 0.77) and the mean duration of diarrhoea by 30.48h (95% CI, 18.51 to 42.46). The authors concluded that probiotics appear to be a useful adjunct to rehydration therapy in treating acute, infectious diarrhoea in adults and children.

All the previous reviews included trials with different probiotics strains which makes difficult to identify if any is more effective than the others. Reviews of studies using a single strain are therefore important to answer this issue.

Szajewska et al (5) evaluated 8 RCT's (n=988) that used *Lactobacillus GG* as a single probiotic. There was no effect in the total stool volume but there was a reduction of -1.1 day (95% CI, -1.9 to -0.3) in the duration of diarrhoea particularly of *Rotavirus* aetiology and duration of hospitalization (-0.58 days, 95% CI, -0.8 to -0.4).

Szajewska et al (6) reviewed 5 RCT's (n=619) that used *Saccharomyces boulardii* and showed that there was a reduced duration of diarrhoea in treated groups (-1.1 day, 95% CI -1.3 to 0.8) and there also was a reduced risk of diarrhoea on days 3, 6 and 7.

One may speculate that different strains might actually perform better than a single one and indeed this hypothesis was addressed in a recent paper (7) that showed better results with *Lactobacillus GG* and a mixture of several strains (*L. delbrueckii* var *bulgaricus*, *Streptococcus thermophilus*, *L. acidophilus* and *Bifidobacterium bifidum*) than other species.

It seems therefore that there is a consistent evidence of moderate clinical benefit of some probiotics strains in the treatment of acute diarrhoea in children. This effect is more evident in cases of *Rotavirus* infection. It remains to be demonstrated clearly which species and strains provide best results. In several trials probiotics were used in the rehydration solution and this method provides easier intake although it does not guarantee a fixed dose of the probiotic. On the other hand there are some unanswered concerns regarding the safety of these organisms. The risk of infection seems to be negligible except for very rare cases of disseminated fungemia with *S. boulardii* in patients with co-morbidities. However there is a theoretical risk of antibiotic resistance transfer among species (8).

In conclusion, probiotics seem to provide some added benefit in the treatment of acute gastroenteritis, especially due to *Rotavirus*, but do not replace the main treatment consisting of rehydration, and to be effective should be used early in the course of the disease. Further research will provide more answers regarding the mechanism of action and the choice of the best strain or combinations according to each disease entity.

References

(1) Szajewska H, Mrukowicz JZ. Probiotics in the treatment and prevention of acute infectious diarrhea in infants and children: a systematic review of published randomized, double-blind, placebo-controlled trials. J Pediatr Gastroenterol Nutr 2001 Oct;33 Suppl 2:S17-25.:S17-S25.

(2) Van Niel CW, Feudtner C, Garrison MM, Christakis DA. Lactobacillus therapy for acute infectious diarrhea in children: a meta-analysis. Pediatrics 2002 Apr;109(4):678-84.

(3) Huang JS, Bousvaros A, Lee JW, Diaz A, Davidson EJ. Efficacy of probiotic use in acute diarrhea in children: a meta-analysis. Dig Dis Sci 2002 Nov;47(11):2625-34.

(4) Allen SJ, Okoko B, Martinez E, Gregorio G, Dans LF. Probiotics for treating infectious diarrhoea. Cochrane Database Syst Rev 2004;(2):CD003048.

(5) Szajewska H, Skorka A, Ruszczynski M, Gieruszczak-Bialek D. Meta-analysis: Lactobacillus GG for treating acute diarrhoea in children. Aliment Pharmacol Ther 2007 Apr 15;25(8):871-81.

(6) Szajewska H, Skorka A, Dylag M. Meta-analysis: Saccharomyces boulardii for treating acute diarrhoea in children. Aliment Pharmacol Ther 2007 Feb 1;25(3):257-64.

(7) Canani RB, Cirillo P, Terrin G, Cesarano L, Spagnuolo MI, Vincenzo AD, et al. Probiotics for treatment of acute diarrhoea in children: randomised clinical trial of five different preparations. BMJ 2007 Aug 9;.

(8) Courvalin P. Antibiotic resistance: the pros and cons of probiotics. Dig Liver Dis 2006 Dec;38 Suppl 2:S261-5.:S261-S265.

Probiotics and Irritable Bowel Syndrome: Rationale and Clinical Evidence for their Use

Giovanni Barbara, *MD**, Vincenzo Stanghellini, *MD*, Cesare Cremon, *MD*, Roberto De Giorgio, *MD*, *PhD*, Luciana Gargano, *CLT*, Francesca Pallotti, *MD*, Roberto Corinaldesi, *MD*

Department of Internal Medicine and Gastroenterology
University of Bologna, Bologna, Italy

Introduction

The irritable bowel syndrome (IBS) is a functional bowel disorder characterized by abdominal pain and changes in bowel habit that occur in the absence of identifiable structural or biochemical abnormalities. The concept that changes in the intestinal milieu, and particularly microbiota, could participate to symptom generation in IBS, has been suspected for a long time although not supported, until recently, by substantive evidence (1). Now, studies showing quantitative and qualitative modifications of intestinal microbiota are beginning to come forward, although these data are still largely debated and far to be conclusive. This evidence is timely paralleled with a rapidly growing knowledge demonstrating that bidirectional signaling between bacteria and enteric tissues is required not only for mucosal and immune system homeostasis but also for neuro-muscular function (2). Taken together, these novel findings provide implications for intestinal microbiota in IBS pathophysiology and symptom generation. They also offer a rationale for a microbiota-directed intervention, including selective dietary manipulation, the use of antibiotics, prebiotics, probiotics and synbiotics.

Evidence of altered microbiota in IBS

Earlier studies have suggested the presence of qualitative changes in intestinal microbiota in patients with IBS. In general, these studies showed decreased fecal *Lactobacilli* and *Bifidobacteria*, and increased facultative bacteria dominated by *Streptococci*, and *Escherichia coli* as well as increased anaerobic organisms such as *Clostridium* (3-5). However, these studies have been subject of controversy since they were carried out in a limited number of subjects and have not been subsequently confirmed. In addition, classical microbiological studies could be underpowered to detect micobiota changes since the majority of bacterial microflora cannot be cultured with conventional methods. This limitation has been recently circumvented with the introduction of molecular techniques. Using real time polymerase chain reaction, Malinen et al. found significant lower amounts of *Lacobacillus* spp. in fecal samples of diarrhea-predominant IBS patients and increased amounts of the anaerobic bacteria *Veillonella* spp. in patients with predominant constipation (6). In a subsequent study, the same group has applied an interesting molecular strategy in which the applied guanine-plus-cytosine profiling of colonic bacteria and extensive 16s rRNA gene cloning and sequencing to compare the fecal microbiota between IBS patients and controls (7). This study provided evidence that the microbial community is significantly altered in IBS and that

the composition varies with the main symptoms reported by patients (i.e. higher numbers of *Lactobacilli* and *Colinsella* sequences in healthy controls, higher numbers of *Bacteroides* and *Allisonella* sequences in IBS patients with mixed bowel habit, *Ruminococci* and *Streptococci* in constipation predominant IBS) (7). Clearly, although these studies are far from being exhaustive and conclusive they provide promising results that deserve further investigation.

There has also been certain interest around the latest data showing quantitative changes in intestinal microflora in IBS, an particularly in the presence of increased amounts of bacteria in the upper small intestine, a condition know as small intestinal bacterial overgrowth (SIBO). Based on the wide availability and non-invasive nature of hydrogen breath tests (e.g., lactulose and glucose breath tests) recently they have been broadly applied to detect SIBO in IBS patients. The results of these studies have provided evidence of a previously unexpected high prevalence of 38–84% of SIBO in IBS (8-12). These data are corroborated by initial evidence that bacterial overgrowth eradication, by means of the non-absorbable antibiotic rifaximin, improved IBS symptoms in the short term along with a reduction in breath hydrogen excretion in those patients who symptomatically improved (13).

Although limited by its invasive nature and inability to detect all the bacteria, culture of jejunal aspirates is still considered the gold standard to detect SIBO (generally defined as a total growth of $\geq 10^5$ cfu/ml of intestinal aspirate). In a recent large study, Posserud et al. have demonstrated that culture-confirmed SIBO could be detected in only 4% of IBS patients, a percentage not different from controls without gastrointestinal symptoms (14), rising some concern on the data obtained with hydrogen breath tests. Of interest however, the authors found that a significant subset of patients with IBS had mildly increased amounts of bacteria (detected as total growth $\geq 5 \times 10^3$ cfu/ml) in jejunal aspirates (14). However, this finding is still of unknown relevance for IBS symptom perception. Thus, although promising, the studies on intestinal bacterial overgrowth in IBS patients have provided contradictory results (15) suggesting that this area requires further work.

Putative consequences of altered intestinal microflora

The putative consequences of changes in intestinal microbiota span a wide range including increased fermentation of food with gas production (16), bile acid malabsorption (17) changes in intestinal motor and sensory function (2) and mucosal immune activation and minimal inflammation (18, 19), all of which may bear some relevance for symptom generation in IBS. Among these factors, probably the aspect that has received most attention relates to mucosal immune activation, occurring both in patients who develop IBS following enteric infection (i.e. post-infective IBS) and in unspecific IBS (1). Although endoscopically and histologically “normal”, the intestinal mucosa of a significant subsets of IBS patients contains an increased number of immunocytes (*e.g.*, T lymphocytes, mast cells) (1). In addition, recent evidence has also shown an increase mucosal release of cytokines (20), histamine, proteases, and prostaglandins (19). These mediators are likely candidates of disturbed sensory and motor function of IBS patients. Our own studies have demonstrated an increased density of mast cells in the colonic mucosa of IBS patients. Furthermore, a close vicinity of mast cells to mucosal nerves correlated with patients’ pain severity and frequency (19). In a more recent study, we have extended these findings demonstrating that mediators released by the intestinal mucosa of IBS patients (i.e. mast cell histamine and proteases), determined a hyper-activation of sensory pathways, providing a functional link between immune activation and pain experience in IBS patients (21, 22). In this context, increased intestinal permeability described in subsets of IBS patients may play an important by exposing the mucosa to

an abnormal challenge of luminal antigens of dietary, and more importantly bacterial origin promoting and maintaining mucosal immune activation (23).

Probiotic trials in IBS

Studies in IBS patients have attempted to target changes in intestinal microflora with different therapeutic approaches, including the use of prebiotics, probiotics, synbiotics, non-absorbable and systemic antibiotics (2). In most probiotic trials single strains of *Lactobacilli* or *Bifidobacteria* have been used (24-32). Two studies used a composite probiotic (VSL#3), containing multiple strains of *Bifidobacteria*, and *Lactobacilli* and one strain of *Streptococci* (33, 34)). Another recent study used a multispecies probiotic including, *L. rhamnosus GG*, *L. Rhamnosus Lc705*, *P. freudenreichii ssp. shermanii JS*, *B. breve Bb99* (35*)*. Although there are several trials assessing the role of probiotic treatment in IBS the majority of them are small sample size and only a few were well-designed randomized and placebo controlled. Overall, the results obtained in these studies suggest some beneficial effect of probiotics over placebo in the relief of IBS symptoms (24, 25, 27, 30-35), but negative trials have also been reported (26, 28, 29) (Table 1). The positive studies included, improvement in cumulative symptom scores (24, 27, 30, 31, 35) or single symptoms such as abdominal pain(25, 27), flatulence (25, 34), bloating (32, 33) and stool frequency (32). A recent well designed study, in which *B. Infantis* 35624 was used, demonstrated also a significant improvement of global assessment with a 20% gain over placebo (31). There is also initial evidence that *B. animalis* DN-173 010 could improve health-related quality of life in IBS patients (32). It is interesting to note that some studies have also evaluated the impact of probiotics on certain pathophysiological features of IBS. In their study, O'Mahony et al. have shown that *B. infantis* 35624, but not *L. Salivarius,* was able to reduce systemic pro-inflammatory cytokine profile (30), thus providing a possible mechanism through which this probiotic could be beneficial in IBS. Kim et al. have demonstrated that VSL#3 delays colonic transit in IBS patients with bloating (34). Interestingly, there is also initial evidence indicating the beneficial effect of a multispecies probiotic formulation on certain metabolic aspects of intestinal microbiota in IBS patients (36).

Conclusions and future perspectives

There is growing promising research suggesting a potential role of microbiota in the pathophysiology. Clearly more work needs to be done to reveal microbiota changes and the complex mechanisms through which these changes may lead to symptom generation. Although encouraging, results obtaind in probiotic trials should be confirmed in larger well designed placebo controlled studies. A number of open questions remain to be addressed, including the dose, type and time of administration of probiotics.

Table 1. Controlled randomized trials assessing the role of probiotics in irritable bowel syndrome.

Probiotic	No. of patients	Criteria	Treatment duration (weeks)	Outcome	Reference
L. acidophilus	29	Unspecified	6	Improved cumulative scores	Halpern *et al.* [24]
L. casei GG	25	Rome	20	Negative	O'Sullivan and O'Morain [26]
L. plantarum	60	Rome	4	↓ Abdominal pain; ↓ flatulence	Nobaek *et al.* [25]
L. plantarum	40	Unspecified	4	↓ Abdominal pain; improved all IBS symptoms	Niedzielin *et al.* [27]
L. plantarum	12	Unspecified	4	Negative	Sen *et al.* [28]
VSL#3	25	Rome II	8	↓ Bloating	Kim *et al.* [33]
*L. salivarius, B. infantis 35624**	77	Rome II	8	Improved all IBS symptoms	O'Mahony *et al.* [30]
VSL#3	48	Rome II	4-8	↓ Flatulence	Kim *et al.* [34]
Multispecies probiotic	103	Rome I-II	24	Improved IBS symptoms	Kajander *et al.* [35]
L. reuteri	54	Rome II	24	Negative	Niv *et al.* [29]
B. infantis 35624	362	Rome II	4	Improved all IBS symptoms; global assessment > 20% gain over placebo	Whorwell *et al.* [31]
B. animalis	274	Rome II	6	Improved HRQoL and stool frequency, ↓ bloating	Guyonnet *et al.* [32]

**L. salivarius* had no significant effect over placebo; HRQoL, health related quality of life

References

1) Barbara G, De Giorgio R, Stanghellini V, Cremon C, Salvioli B, Corinaldesi R. New pathophysiological mechanisms in irritable bowel syndrome. Aliment Pharmacol Ther 2004;20 Suppl 2:1-9.

2) Barbara G, Stanghellini V, Brandi G, Cremon C, Di Nardo G, De Giorgio R, Corinaldesi R. Interactions between commensal bacteria and gut sensorimotor function in health and disease. Am J Gastroenterol 2005;100:2560-8.

3) Balsari A, Ceccarelli A, Dubini F, Fesce E, Poli G. The fecal microbial population in the irritable bowel syndrome. Microbiologica 1982;5:185–94.

4) Wyatt GM, Bayliss CE, Lakey AF, Bradley HK, Hunter JO, Jones VA. The faecal flora of two patients with food-related irritable bowel syndrome during challenge with symptom-provoking foods. J Med Microbiol 1988;26:295–9.

5) Madden JAJ, Plummer S, Sen S, Dear K, Tarry S, Hunter JO. Comparison of the caecal and faecal microflora of healthy subjects and patients with irritable bowel syndrome. Gut 2001;48(Suppl 1):A58.

6) Malinen E, Rinttila T, Kajander K, Matto J, Kassinen A, Krogius L, Saarela M, Korpela R, Palva A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. Am J Gastroenterol 2005;100(2):373-82.

7) Kassinen A, Krogius-Kurikka L, Makivuokko H, Rinttila T, Paulin L, Corander J, Malinen E, Apajalahti J, Palva A. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. Gastroenterology 2007;133:24-33.

8) Pimentel M, Chow EJ, Lin HC. Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. Am J Gastroenterol 2000;95:3503–6.

9) Pimentel M, Chow EJ, Lin HC. Normalization of lactulose breath testing correlates with symptom improvement in irritable bowel syndrome. a double-blind, randomized, placebo-controlled study. Am J Gastroenterol 2003;98:412–19.

10) Pimentel M, Wallace D, Hallegua D, Chow E, Kong Y, Park S, Lin HC. A link between irritable bowel syndrome and fibromyalgia may be related to findings on lactulose breath testing. Ann Rheum Dis 2004;63:450–2.

11) McCallum R, Schultz C, Sostarich S. Evaluating the role of small intestinal bacterial overgrowth (SIBO) in diarrhea predominant IBS (IBS-D) patients utilizing the glucose breath test. Gastroenterology 2005;128:T1118.

12) Nucera G, Gabrielli M, Lupascu A, Lauritano EC, Santoliquido A, Cremonini F, Cammarota G, Tondi P, Pola P, Gasbarrini G, Gasbarrini A. Abnormal breath tests to lactose, fructose and sorbitol in irritable bowel syndrome may be explained by small intestinal bacterial overgrowth. Aliment Pharmacol Ther 2005;21:1391–5.

13) Pimentel M, Park S, Mirocha J, Kane SV, Kong Y. The effect of a nonabsorbed oral antibiotic (rifaximin) on the symptoms of the irritable bowel syndrome: a randomized trial. Ann Intern Med 2006;145:557-63.

14) Posserud I, Stotzer PO, Bjornsson ES, Abrahamsson H, Simren M. Small intestinal bacterial overgrowth in patients with irritable bowel syndrome. Gut 2007;56:802-8.

15) Simren M, Stotzer PO. Use and abuse of hydrogen breath tests. Gut. 2006;55:297-303.

16) King TS, Elia M, Hunter JO. Abnormal colonic fermentation in irritable bowel syndrome. Lancet 1998;352:1187–9.

17) Floch MH. Bile salts, intestinal microflora and enterohepatic circulation. Dig Liver Dis 2002;34(Suppl 2):S54–7.

18) Barbara G, De Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? Gut 2002;51(Suppl 1):i41–4.

19) Barbara G, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. Gastroenterology 2004;126:693–702.

20) Collins SM. Dysregulation of peripheral cytokine production in irritable bowel syndrome. Am J Gastroenterol 2005;100:2517–8.

21) Barbara G, Wang B, Stanghellini V, de Giorgio R, Cremon C, Di Nardo G, Trevisani M, Campi B, Geppetti P, Tonini M, Bunnett NW, Grundy D, Corinaldesi R. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterology 2007;132:26-37.

22) Cenac N, Andrews CN, Holzhausen M, Chapman K, Cottrell G, Andrade-Gordon P, Steinhoff M, Barbara G, Beck P, Bunnett NW, Sharkey KA, Ferraz JG, Shaffer E, Vergnolle N. Role for protease activity in visceral pain in irritable bowel syndrome. J Clin Invest 2007;117:636-47.

23) Barbara G. Mucosal barrier defects in irritable bowel syndrome. Who left the door open? Am J Gastroenterol 2006;101:1295-8.

24) Halpern GM, Prindiville T, Blankenburg M, Hsia T, Gershwin ME. Treatment of irritable bowel syndrome with Lacteol Fort: A randomized, double-blind, cross-over trial. AmJ Gastroenterol 1996;91:1579–85.

25) Nobaek S, Johansson ML, Molin G, Ahrne S, Jeppsson B. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. Am J Gastroenterol 2000;95:1231–8.

26) O'Sullivan MA, O'Morain CA. Bacterial supplementation in the irritable bowel syndrome. A randomised double blind placebo-controlled crossover study. Dig Liver Dis 2000;32:294–301.

27) Niedzielin K, Kordecki H, Birkenfeld B. A controlled, double-blind, randomized study on the efficacy of Lactobacillus plantarum 299V in patients with irritable bowel syndrome. Eur J Gastroenterol Hepatol 2001;13:1143–7.

28) Sen S, Mullan MM, Parker TJ, Woolner JT, Tarry SA, Hunter JO. Effect of Lactobacillus plantarum 299v on colonic fermentation and symptoms of irritable bowel syndrome. Dig Dis Sci 2002;47:2615–20.

29) Niv E, Naftali T, Hallak R, Vaisman N. The efficacy of Lactobacillus reuteri ATCC 55730 in the treatment of patients with irritable bowel syndrome--a double blind, placebo-controlled, randomized study. Clin Nutr 2005;24:925-31.

30) O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: Symptom responses and relationship to cytokine profiles. Gastroenterology 2005;128:541–51.

31) Whorwell PJ, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahony L, Kiely B, Shanahan F, Quigley EM. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. Am J Gastroenterol 2006;101:1581-90.

32) Guyonnet D, Chassany O, Ducrotte P, Picard C, Mouret M, Mercier CH, Matuchansky C. Effect of a fermented milk containing Bifidobacterium animalis DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: a

multicentre, randomized, double-blind, controlled trial. Aliment Pharmacol Ther 2007;26:475-86.

33) Kim HJ, Camilleri M, McKinzie S, Lempke MB, Burton DD, Thomforde GM, Zinsmeister AR. A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther 2003;17:895–904.

34) Kim HJ, Vazquez Roque MI, Camilleri M, Stephens D, Burton DD, Baxter K, Thomforde G, Zinsmeister AR. A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. Neurogastroenterol Motil 2005;17:687-96.

35) Kajander K, Hatakka K, Poussa T, Farkkila M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. Aliment Pharmacol Ther 2005;22:387-94.

36) Kajander K, Krogius-Kurikka L, Rinttila T, Karjalainen H, Palva A, Korpela R. Effects of multispecies probiotic supplementation on intestinal microbiota in irritable bowel syndrome. Aliment Pharmacol Ther 2007;26:463-73.

Probiotics protect mice against experimental infections

Leda Quercia Vieira*, Liliane Martins dos Santos*, Elisabeth Neumann*, Anderson Paulo da Silva*, Lilian Nobre Moura**, Jacques Robert Nicoli**

*Departamento de Bioquímica e Imunologia and **Departamento de Microbiologia, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, MG, Brazil.

Introduction

Lactobacillus delbrueckii var. *bulgaricus* UFV-H_2b_{20} was isolated at the Universidade Federal de Viçosa, Minas Gerais, Brazil, from feces from a human newborn child. Our group has been investigating the possible probiotic properties of this strain. The work described here was designed to investigate the capacity of this strain to survive and establish in the intestinal tract and affect the host phagocytic capacity. We have also investigated the capacity of this train to protect mice against bacterial infections.

Methods

Mice were maintained at the Gnotobiology Laboratory of the Universidade Federal de Minas Gerais, MG, Brazil, according to the procedures described by Pleasants[1]. Conventional Swiss/NIH mice with same age and of both sexes were obtained from our colony. C57BL/6 and BALB/c conventional mice were obtained from the UFMG animal colony. *Lactobacillus delbrueckii* UFV-H2b20 was isolated at the Federal University of Viçosa, Minas Gerais, Brazil, and maintained at -70^{o}C in non-fat reconstituted dry milk containing 20% glycerol. The strain was grown in MRS broth (De Man, Rogosa & Sharpe, Merck, São Paulo, Brazil) for 18 hours at 37^{o}C just before use. *Escherichia coli* B_{41}, *Listeria monocytogenes* and *Salmonella tiphymurium* were maintained as previously described[2-4]. Monoassociation of germfree mice was achieved

by inoculating mice with 10^9 viable cells. Treatment of conventional mice was obtained by adding 10^9 viable cells in drinking saline daily. Clearance of *E. coli* and infection with *S. enterica* were previously described[2,3]. *L. monocytogenes* was administered i.p.

Results and Discussion

L. delbrueckii successfully installed in the gut of germ free mice (figure 1).

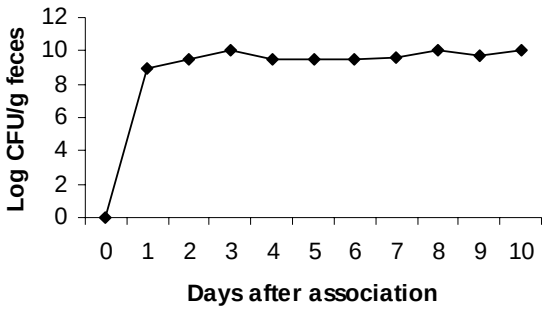


Figure 1: Kinetics of colonization of germfree mice with *L. delbrueckii*

Monoassociated mice cleared *E. coli* from the circulation more efficiently than germ-free mice. This clearance was dependent on Küpffer cells and correlated with higher levels of inflammatory cytokines in sera (data not shown). Oral treatment of conventional mice with *L. delbrueckii* was protective against infection with *S. enterica* (data not shown). Monoassociation also protected germ free mice against infection with *L. monocytogenes* (figure 2). This protection was confirmed by the fact that monoassociated mice showed lower numbers of bacteria in livers and spleens (data not shown). Again, monoassociation triggered higher production of inflammatory cytokines (interferon-gamma and tumor necrosis factor-alpha) and nitric oxide. Interestingly, IL-10 levels were not altered by monoassociation or infection.

We postulate that *L. delbrueckii* confers protection against bacterial infections by upregulating inflammatory cytokines that trigger killing mechanisms that are effective against infection, such as nitric oxide production. Hence, it would be a probiotic

candidate since it successfully resists the gut environment and modulates the immune system to protect the host against infection.

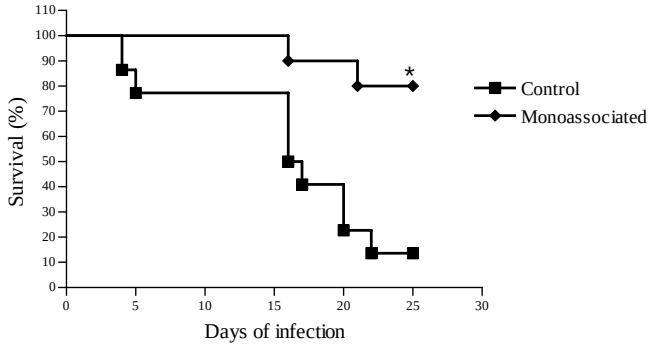


Figure 2: mortality of germ-free and *L. delbrueckii* -monoassotiated mice infected with *L. monocytogenes.* * indicates statistical significance ($p<0.05$).

References

1. J. R. Pleasants. Gnotobiotics. In: *Handbook of Laboratory Animal Science*, edited by E. C. Melby Jr. and N. H. Altman, Cleveland:CRC Press, 1974, p. 119-174.

2. E. Neumann *et al.*. Monoassociation with Lactobacillus acidophilus UFV-H2b20 stimulates the immune defense mechanisms of germfree mice. *Braz.J.Med.Biol.Res.* 31 (12):1565-1573, 1998.

3. L. N. Moura *et al.* Protection by Lactobacillus UFV-H2b20 against experimental oral infection with Salmonella enterica subp. Enterica ser typhimurium in gnotobiotic and conventional mice. *Braz. J.Microbiol.* 32:66-69, 2001.

3. S. T. Oliveira Mendes *et al.* In vitro evaluation of the cytotoxicity of two root canal sealers on macrophage activity. *J.Endod.* 29 (2):95-99, 2003.

Acknowlegments: the authors are indebted to Mr. Antonio Vaz Mesquita for expert animal care. This work was supported by CAPES, CNPq and FAPEMIG.

Mechanisms involved in the intestinal interaction between host and bifidobacteria

Patrizia Brigidi

Department of Pharmaceutical Sciences, CIRB-center for Biotechnology, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

The quantity of living bacteria which compose the human intestinal microbiota can range from 10^{12} to 10^{14} CFU/g of luminal content and contains up to 1000 different species. During evolution the association of microbes with tissues of the human gastrointestinal tract (GIT) resulted in the development of a balanced symbiotic relationship, where the microorganisms profit by the acquisition of nutrients and a stable temperature and in turn provide important health benefits to the host. *Bifidobacterium* genus represents one of the most important health-promoting groups of human microbiota. However, the knowledge of the mechanisms involved in the bifidobacterial beneficial activities is very limited and, in particular, no information about their specific mechanisms for the interaction with the host is available.

Several pathogenic bacterial species intervene with the plasminogen (Plg)-plasmin system of the human host. In particular, within the human gastrointestinal niche, enteropathogens such as *Salmonella enterica, Listeria monocytogenes, Helicobacter pylori* and *Escherichia coli,* as well as *Bacteroides fragilis* have been shown to capture human Plg on their cell surface. Plg is a single-chain glycoprotein with a molecular mass of 92 kDa and represents the monomeric proenzyme of the serine protease plasmin. It is abundant in human plasma and extracellular fluids, and its active form plasmin plays a crucial role in fibrinolysis, homeostasis and degradation of the extracellular matrix proteins. With the recruitment of human Plg on the bacterial cell surface and its subsequent conversion to plasmin, microorganisms acquire a surface-associated proteolytic activity useful to facilitate the migration across physical and molecular barriers, and to respond to the nutritional demands during colonization process. Since pathogens and symbionts of the human GIT colonize the same ecological niche, the hypothesis that they share common molecular mechanisms to initiate and maintain their relationship with the host has been addressed. In this study we investigated whether *Bifidobacterium* possesses human Plg-binding activity.

In this perspective, to assess the capability of whole *Bifidobacterium* cells to bind human Plg, four strains belonging to the species *B. longum*, *B. bifidum* and *B. lactis*, were incubated with different amounts of human Plg and bound Plg was detected using an anti-Plg antibody followed by secondary FITC-labelled antibody. The increase in fluorescence intensity due to the captured Plg

was evaluated by flow cytometry analysis, showing that all strains tested possessed a dose-dependent Plg-binding activity. It is known that binding of Plg to mammalian and some bacterial species is mediated by lysine binding sites (LBS) of Plg. To investigate the role of LBS for Plg recruitment on the bifidobacterial cell surface, we evaluated the binding of human Plg to *Bifidobacterium* in presence of the lysine analog EACA. Our results demonstrated that EACA inhibited the Plg-binding to the surface of *Bifidobacterium* in a concentration-dependent manner, suggesting that the LBS of Plg are involved in Plg recruitment on the cell surface.

Many of the identified plasminogen-receptor proteins have a carboxyterminal lysine residue that is involved in binding of Plg. To evaluate the impact of the C-terminal lysine(s) in Plg-binding to *Bifidobacterium*, *B. lactis* and *B. longum* cells were treated with increasing concentrations of carboxypeptidase B, which is a C-terminal lysine-specific endopeptidase. In flow cytometry analysis, both strains showed a significantly reduction of Plg-binding after pretreatment with higher doses of carboxypeptidase B. However, treatment with carboxypeptidase B did not completely abolish Plg-binding to bifidobacteria, suggesting that Plg recruitment to the surface of bifidobacteria partially depends on C-terminal lysine residues present in Plg receptors.

For the visualization of human Plg on the bifidobacterial cell surface, immunoelectron microscopy experiments were performed with *B. lactis* cells. Bacteria were incubated with human Plg, washed, and then incubated in pre-embedding conditions with anti-human Plg antibody followed by incubation with the secondary antibody labelled with 10 nm gold particles. Ultra-thin sections were examined at magnification of x 22000 and x 13000. Plg aggregates were clearly visible on *B. lactis* cell surface by aggregates of gold particles. In control experiments, bifidobacterial cells incubated with anti-human Plg antibody resulted in no labeling, demonstrating the specificity of the Plg labeling.

In order to detect possible cell surface protein candidates for the interaction with human Plg, we focused our study on *B. lactis* BI07 as a model strain. The cell wall fraction from *B. lactis* BI07 was purified, and a Plg overlay assay was carried out. The cell wall proteins were resolved in 2D-gel, immobilized to a nitrocellulose membrane, incubated with human Plg, and probed with anti-Plg antibody to identify bound Plg. Binding of Plg to eight *B. lactis* BI07 cell wall proteins was observed and the co-ordinates of the major putative Plg-binding proteins detected could be matched to a protein spot on the replique gel stained for protein. Spots identified as putative Plg-binding proteins were excised from the gel, subjected to trypsin digestion and MALDI-TOF mass spectrometry. The obtained peptide fingerprints allowed an unambiguous identification for six of the eight putative Plg-binding proteins. The protein spots have been identified as DnaK, glutamine synthetase, enolase, bile salt hydrolyse, and phosphoglycerate mutase. With the exception of

glutamine synthetase, all the putative Plg-binding proteins identified are predicted to have a C-terminal lysine.

Enolase is a glycolytic enzyme catalysing the formation of PEP from 2-PGE. The enzyme is essential for the dehydration of carbohydrates as well as sugar phosphate synthesis via gluconeogenesis. Several reports demonstrated that the surface displayed enolase binds human Plg with high affinity in both eukaryotes and prokaryotes. In this perspective, the localization of enolase protein on *B. lactis* BI07 cell surface was studied by immunoelectron microscopy with cross-reactive polyclonal anti-pneumococcal enolase antiserum. Analysis of ultrathin sections at magnification of x 22000 and x 13000 allowed to detect enolase directly on the bacterial cell wall.

In order to characterize the Plg-binding activity of *B. lactis* BI07 enolase, the gene was sequenced, cloned, and the recombinant His-tagged enolase protein purified by affinity chromatography. The nucleotide sequence of *B. lactis* BI07 enolase revealed a 98% of identity with the enolase gene of *B. longum* NCC2705. The deduced amino acid sequence of *B. lactis* BI07 enolase shared 57% sequence identity with the pneumococcal enolase, and showed a C-terminal lysine residue. The enzymatic activity for the recombinant His-tagged *B. lactis* BI07 enolase was dose-dependent. Furthermore, to test the presence of an internal Plg-binding motif, the codons for the C-terminal lysine were deleted by inverse PCR. The truncated enolase protein (enodel) was purified, and the Plg-binding activity of *B. lactis* enolase and enodel was compared under reducing and non-denaturing conditions. In a Plg overlay assay, enodel showed a substantial decrease of Plg-binding activity compared to wild-type enolase, suggesting that under reducing conditions the interaction between *B. lactis* enolase and Plg involves the C-terminal lysine of enolase. In contrast, blot overlay assays carried out under non-denaturing conditions demonstrated a dose-dependent Plg-binding activity of wild-type enolase and, more important, a dose-dependent Plg-binding activity of enodel. These data suggest the presence of an internal additional Plg-binding epitope besides the C-terminal lysine, whose function is dependent on the protein structure.

Our findings provide insights in understanding the mechanisms involved in *Bifidobacterium*-host interaction, and open the perspective to study the role of Plg in the colonization process of the human host by the commensal bacterial genus *Bifidobacterium*.

Use of VSL #3 probiotic mixture for prevention and therapy of allergic diseases: studies in mouse model of allergic sensitization to inhalant and food allergens

Gabriella Di Felice, M Boirivant
Dept. of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità, Rome, Italy.

The immunological mechanisms responsible for the immune-modulating and in particular the anti-allergic effects of probiotic bacteria are still poorly defined. Recent studies indicate that they are associated with the induction of Th1 or T regulatory responses. In this context, the combined effects of mixtures of different species of probiotic bacteria have been only in part explored in suitable animal models to better understand the *in vivo* processes that inhibit allergy responses.
The present study describes the immunomodulatory activity of a mixture of different probiotic strains on mouse models of allergic sensitization to inhalant and food allergens. The probiotic preparation VSL#3 (VSL#3 Pharmaceuticals, Fort Lauderdale, FL, USA), which contains eight different bacterial strains (four lactobacilli, three bifidobacteria, and one *Streptococcus thermophilus*) was used throughout the study.

Inhalant allergy. The potential of a sonicate preparation of VSL#3 for the prevention of allergic sensitization was studied in a mouse model of sensitization to the major allergen of *Parietaria judaica* pollen (recombinant Par j 1, rPar j 1), previously developed and characterized in our laboratory. Two groups of Balb/c mice were administered intranasally the sonicate preparation of VSL#3 or PBS for eight days. Then, mice were intraperitoneally immunized with rPar j 1 in alum. Serum total and specific IgE antibodies and specific IgG subclasses were measured. Cytokine production by splenocytes was evaluated by ELISA. At the same time, bronchoalveolar lavage fluids (BALF) were also collected for the evaluation of cytokine production in the lung.
The prophylactic treatment with VSL#3 induced a significant reduction of serum total and specific IgE as well as of specific IgG1, accompanied by a significant increase in the production of IFN-γ and a significant reduction of IL-4 secretion by splenocytes stimulated with rPar j 1. At a local level, VSL#3 pre-treatment induced a significant increase in IL-10 but does not affect either levels of inflammatory cytokines (IFN-γ and IL-5) found in the BALF or the expression of the T-bet transcription factor, as compared with PBS-treated controls.
Intranasally delivered probiotic bacteria have the capacity to prevent at the systemic level the development of an allergic response induced *in vivo* against rPar j 1, by down-regulating allergen-

specific Th2 responses. Different effects are obtained at the systemic and local level (mainly regarding IL-10 and IFN-γ production). Probiotic prophylactic treatment does not induce at the local level a switch to Th1-like responses (absence of upregulation of IFN-γ production and T-bet expression), but rather could induce IL-10 producing CD4+/CD25+ regulatory T cells. Combined together, results obtained at local and systemic level underline the central role of IL-10 and IFN-γ in the tolerance induction against allergens, suggesting that both systemic Th1 skewing and local T regulatory activation are involved in the immune modulation induced in our system by probiotic bacteria.

Food allergy.

The therapeutic activity of VSL#3 mixture was assessed in a mouse model of oral sensitization and anaphylaxis to the food allergen Shrimp Tropomyosin (ST), which is the only major allergen of this species, and it is recognized by above 80% of shrimp-allergic subjects.

C3H/HeJ mice were orally sensitized with purified ST from *Metapenaeus ensis*. ST-specific serum IgE, IgG1 and IgG2a responses were evaluated by ELISA. To induce *in vivo* anaphylaxis, mice received an oral challenge with ST. Local and systemic anaphylactic reactions were scored according to symptoms observed. Faecal samples were collected to evaluate local IgA production and histamine levels. At the end of the sensitization period and after the first challenge, different groups of mice were orally treated for four weeks with live VSL#3 preparation. The effects of the probiotic treatment were evaluated by assessing ST-specific serum antibodies, and anaphylactic responses induced by the oral challenge with ST (in terms of symptom score and histamine levels in faecal extracts).

Oral therapeutic treatment with live VSL#3 was able to significantly reduce symptoms of anaphylaxis, as well as histamine levels in faecal extracts. Serum antibody levels were not affected by probiotic treatment. These results, obtained in a mouse model mimicking human anaphylaxis to a food allergen, support the therapeutic potential of the oral (mucosal) administration of a probiotic mixture on an established food allergen sensitization

The induction of protective immune responses at the sites of direct allergen exposure linked to counterregulatory local and systemic immune responses, as it can be achieved by mucosal delivery of safe probiotic bacteria balanced mixtures, might become an effective strategy in the prevention and therapy of type I allergy. Further studies are needed to fully elucidate at the preclinical level the mechanisms of their immunomodulatory action.

Health, probiotics and inflammation

Elena Mengheri
Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (INRAN), Via Ardeatina 546, 00178 Roma, Italy

The intestine is continuously exposed to a myriad of antigens and thus, other than exerting the primary role of nutrients absorption, should protect the mucosa against pathogens and dangerous substances. To establish an efficient barrier, intercellular spaces are strictly sealed by the tight junctions (TJs), composed by a complex of proteins in close apposition to the cytoskeletal actin and myosin ring, that opens and closes in response to various external signals in a strictly regulated manner. Disruption of mucosal barrier may be caused by several pathogen induced changes to TJ proteins or actin and myosin organization, favouring indiscriminate passage of pathogens and other external antigens, dysregulation of epithelial cell signalling and impairment of mucosal immune function, with consequent development of inflammatory reactions including production of cytokines than can affect the TJ proteins, thus promoting further leakiness.
Some studies have shown an ability of probiotics to protect against pathogen induced membrane barrier disruption (1, 2, 3). In a recent study conducted *in vitro* on intestinal cells, we have found that a new *Lactobacillus* species*, L. sobrius* strain DSM 16698^{T}, isolated from unweaned piglets (4), was able to prevent the enterotoxigenic *Escherichia coli* (ETEC) K88 induced membrane damage by inhibiting the delocalization of ZO-1, loss of occludin, rearrangement of F-actin caused by the pathogen. In addition *L. sobrius* was able to counteract the ETEC induced dephosphorylation of occludin, thus allowing the interaction of this protein with the other junctional and cytoskeleton proteins and consequently the correct TJ assembly and function. We have found that the maintenance of barrier integrity was achieved by IL-10 mediated signalling involving down-regulation of IL-8 (5).

The gut immune system has to be able to protect the mucosa against pathogens, but also to avoid hypersensitivity reactions to food proteins, normal bacterial flora and other environmental macromolecules. Oral tolerance is the active non-response of the immune system to an antigen administered through the oral route (6). It has been postulated that food allergy results from a failure in the establishment or maintenance of oral tolerance in infancy (7). However, the association of defective oral tolerance with food allergy has not yet been demonstrated in allergic patients. Induction of allergen-specific T regulatory (Treg) cells has been shown to be essential for the maintenance of a healthy immune response to allergens. The Treg are characterized by high IL-10 and TGF-β production, and by the expression of the transcriptional regulator Foxp3 (8). A recent study conducted on healthy neonates from atopic and non-atopic mothers has reported that a microbial stimulation of cord blood mononuclear cells with an agonist of the probiotic receptor, the Toll-like receptor-2, induced higher IL-10 level in cells from non-atopic than atopic mothers. In addition, the IL-10 production correlated with the increased expression of Foxp3 in T cells (9). Other studies have shown that *L. reuteri* and *L. casei* induced the Treg cells through modulation of dendritic cells (10). Recently, we have found that long-term feeding of *B. animalis* and *L. rhamnosus* GG to rats immunized with ovalbumin (OVA), may regulate intestinal immune response through an involvement of Treg cells. Our findings indicated that, after restimulation in vitro with OVA, the proliferation of mesenteric lymph-nodes (MLN) lymphocytes was lower in probiotic treated than untreated rats, whereas it remained high in splenic lymphocytes of both probiotic treated and untreated rats, indicating that probiotics may induce a different OVA specific immune response at intestinal and peripheral sites. The mechanisms underlying this hyporesponsiveness was different for the two probiotics. In fact, while *B. animalis* induced apoptosis, *L. rhamnosus* GG supplementation resulted in an increase of IL-10 secreting Treg in MLN lymphocytes (11). Thus, these studies support a role of these probiotics in prevention of allergic disease and inflammatory

associated reactions. In previous studies conducted in intestinal cells we have also shown that the same probiotic strains exerted an anti-inflammatory activity by reducing the pathogen-induced migration of neutrophils through regulation of chemokine and cytokine expression (12).

Evidence is accumulating that breakdown in tolerance toward intestinal bacteria is a primary cause of inflammatory bowel disease (IBD; 13). A recent accredited theory is that a reduced consumption of fermented foods results in defective maturation of the Treg cells and thus to an imbalance between them and the Th cells, leading to the development of IBD characterized by a Th1 response (14). Based on this assumption, the modification of the intestinal microflora towards a healthier probiotics enriched microflora appears a good strategy to cure or prevent IBD. Some studies support this concept, showing that supplementation of *L. rhamnosus* to patients suffering for Crohn disease modulated dendritic cell function to induce a novel form of T cell hyporesponsiveness (15), while administration of VSL#3 mixture to patients with ulcerative colitis alleviated disease severity (16). Recent studies have shown that probiotics ameliorated recurrent colitis in mice by inducing IL-10 and IL-10 dependent TGF-bearing regulatory T cells (17). By using a mouse model of experimentally induced colitis, we have found that feeding a mixture of *B. longum* and *L. acidophilus* prevented the inflammation and mucosal ulcerations induced by trinitrobenzene sulfonic acid (TNBS). This protection was associated with a decrease of the $CD4^+$ population, that was increased in TNBS-treated mice, and with an up-regulation of IL-10 and down-regulation of IL-12. A different pattern of $Foxp3^+CD4^+CD25^+$ cells in the intraepithelial and lamina propria lymphocytes were also found (unpublished results). At this regard it is important to consider that recent studies have reported an accumulation of $Foxp3^+CD4^+CD25^+$ cells in colon samples from patients with ulcerative colitis or Crohn's disease, and that subsets of IL-10-producing $CD4^+CD25^+$ T cells were present mainly within the intestinal lamina propria suggesting compartmentalization of the Treg response at effector sites (18).
All these results suggest that probiotics may offer an alternative approach to conventional therapy in IBD by altering the intestinal microflora and modulating the host immune system, and encourage to further explore the role of these bacteria in preventing human inflammatory diseases.

REFERENCES

1. Lievin-Le Moal V., R Amsellem, AL Servin, and MH Coconnier. 2002. *Lactobacillus acidophilus* (strain LB) from the resident adult human gastrointestinal microflora exerts activity against brush border damage promoted by a diarrhoeagenic *Escherichia coli* in human enterocyte-like cells. Gut 50:803-811.
2. Madsen KL, A Cornish, P Soper, C McKaigney, H Jijon, C Yachimec, J Doyle, L Jewell, and C De Simone. 2001. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 121:580-591.
3. Resta-Lenert S, and KE Barrett. 2002. Enteroinvasive bacteria alter barrier and transport properties of human intestinal epithelium: role of iNOS and COX-2. Gastroenterology. 122:1070-1087.
4. Konstantinov, SR, E. Poznanski, S Fuentes, ADL Akkermans, H Smidt, and WM de Vos. 2006. *Lactobacillus sobrius* sp. nov., abundant in the intestine of weaning piglets. Int J Syst Evol Microbiol. 56:29-32
5. Roselli M, A Finamore, MS Britti, SR Konstantinov, H Smidt, WM de Vos, and E Mengheri. *Lactobacillus sobrius* DSM 16698^T protects intestinal cells from enterotoxigenic *Escherichia coli* K88 through inhibition of pathogen adhesion and maintenance of IL-10 mediated membrane barrier integrity. Submitted
6. Ko J, and L. Mayer L. 2005. Oral tolerance: lessons on treatment of food allergy. Eur J Gastroenterol Hepatol;17:1299-1303.

7. Rautava S,and E. Isolauri. 2004. Cow's milk allergy in infants with atopic eczema is associated with aberrant production of interleukin-4 during oral cow's milk challenge. J Pediatr Gastroenterol Nutr. 39:529-533.
8. Akdis M, and CA Akdis. 2007 Mechanisms of allergen-specific immunotherapy. J Allergy Clin Immunol. 119: 780-791
9. Schaub B, M Campo, H He, D Perkins, MW Gillman, DR Gold, S Weiss, E Lieberman, and PW Finn. 2006 Neonatal immune responses to TLR2 stimulation: influence of maternal atopy on Foxp3 and IL-10 expression. Respir Res. 21:7:40.
10. Smits HH, A Engering, D van der Kleij, EC de Jong, K Schipper, TM van Capel, BA Zaat, M Yazdanbakhsh, EA Wierenga, Y van Kooyk, and ML Kapsenberg. 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol. 115:1260-1267.
11. Britti MS, N Merendino', A Finamore, M Roselli and E Mengheri. Different modulation of intestinal immune response by probiotics *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* MB5 in rats. Submitted.
12. Roselli M, A Finamore, MS Britti, and E Mengheri. 2006. Probiotic bacteria *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG protect intestinal Caco-2 cells from the inflammation-associated response induced by enterotoxigenic Escherichia coli K88. Br J Nutr. 95:1177-1184.
13. Sheil B, F Shanahan, and L O'Mahony. 2007. Probiotic effects on inflammatory bowel disease. J Nutr. 137:819S-24S.
14. Yu QT, M Saruta, A Avanesyan, PR Fleshner, AH Banham, and KA Papadakis. 2007. Expression and functional characterization of FOXP3+ CD4+ regulatory T cells in ulcerative colitis. Inflamm Bowel Dis. 13:191-199.
15. Braat H, J van den Brande, D van Tol E, Hommes, M Peppelenbosch, and S van Deventer. 2004. *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. Am J Clin Nutr. 80:1618-1625.
16. Bibiloni R., RN Fedorak,., GW Tannock, , KL Madsen, , P Gionchetti, , M Campirei, C De Simone, and RB Sartor. 2005. Am. J. Gastroenterol. 100: 1539–1546.
17. Di Giacinto C, M Marinaro, M Sanchez, W Strober, and M Boirivant. 2005. Probiotics ameliorate recurrent Th1-mediated murine colitis by inducing IL-10 and IL-10-dependent TGF-beta-bearing regulatory cells. J Immunol. 174:3237-3246.
18. Uhlig HH, J Coombes, C Mottet, A Izcue, C Thompson, A Fanger, A Tannapfel, JD Fontenot, F Ramsdell, and F Powrie. 2006. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. J Immunol. 177:5852-5860.

This work was partly supported by European Community grant, project Feed for Pig Health, contract FOOD-CT-2004-506144.

Response of resident gut bacteria to the application of probiotics or prebiotics

Karen P. Scott, Jenny C. Martin, and P. Louis.
Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, Scotland, UK

Probiotics and prebiotics are promoted as being beneficial for health and are included in an increasing number and variety of foodstuffs. There are many claims associated with their use in alleviating the symptoms of specific gut disorders, some of which are backed by scientific evidence. However, the impact that they have in modifying the microbiota in healthy individuals has not been thoroughly investigated.

We have shown that a specific probiotic bacterium is able to establish against a background faecal microbiota in vitro and in vivo in healthy volunteers, and can persist for at least 3 weeks after probiotic intake ceases.

The addition of dietary prebiotics, specifically based on fructo-oligosaccharides (FOS) and inulin, has been shown in several studies to enhance the numbers of Bifidobacteria species. We have found that isolates of several other predominant gut anaerobic bacteria are also able to grow efficiently on FOS and inulin in pure culture. A gene cluster involved in sucrose utilisation has been identified in one of these bacteria. We have investigated whether these other groups of bacteria are also stimulated by the addition of FOS in a mixed ecosystem.

Effect of the probiotic agent *Clostridium butyricum* M588 strain on enteric pathogens

Kamiya S[1], Taguchi H[1, 2], Osaki T[1], Oka K[1, 3] ,Tanaka M[3] and Takahashi M[3]

[1]Department of Infectious Diseases, School of Medicine, [2]Department of Immunology, School of Health Science, Kyorin University, [3]Miyarisan Pharm Co., Tokyo, Japan

Aim

The inhibitory effects of probiotics such as *Lactobacillus, Bifidobacterium, Saccharomyces* etc on various enteric pathogens have been reported in vitro/in vivo studies and clinical trial studies. In the present study, the effects of probiotic agent *Clostridium butyricum* M588 strain on enteric pathogens including *Clostridium difficile,* enterohaemorrhagic *Escherichia coli* O157:H7 and *Salmonella* serotype Enteritidis were examined.

Method

C. butyricum M588 strain was obtained from Miyarisan Pharmaceutical Co., Ltd., Tokyo, Japan. GAM agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) was used for routine anaerobic culture of *C. butyricum* M588 strain. Toxigenic *C. diffcile* VPI10463 strain was used, and anaerobically cultured in brain heart infusion broth. Toxin production by *C. difficile* was evalulated by cytotoxicity assay using Vero cells. Clinically isolated EHEC O157:H7 strain 6 (stx1+, stx2+, eaeA+) was used, and aerobically cultured on sorbitol MacConkey agar. Quantification of shiga toxins, Stx1 and Stx2, produced was done by using reversed passive latex agglutination test kit (VTEC-RPLA “SEIKEN”, Denka Seiken Co. Ltd., Niigata, Japan). Three strains (88, 144 and 204) of *Salmonella* serotype Enteritidis were used, and aerobically cultured in GAM broth.

Specific pathogen-free (SPF) (C57BL/6, male, 5 weeks old) mice and germ-free (GF) (IQI/jic, female, 8 weeks old) mice were obtained from Japan Clear Inc., Tokyo, Japan, and maintained in a sterilized vinyl isolator and fed a sterilized diet and water *ad libitum.*

An adhesion assay was performed by flow cytometric analysis. Bacterial culture labeled with PKH-2 lipophylic dye (Zynaxis Cell Science, Phoenixville, USA) were

added to human intestinal Caco-2 cells. A flowcytometry (FACS Vantage, Becton Dickinson Immunocytometry System, San Jose, USA) was used for the measurement of fluorescence intensity.

Results

Eighty-six percent (6/7) of the gnotobiotic mice died due to lethal caecitis within 2 days of infection with *C. difficile.* In contrast, only 20% (2/10) of the gnotobiotic mice pre-infected with *C. butyricum* M588 strain died. Both the number of *C. difficile* and cytotoxic activity in the caecal content in the dead mice were significantly higher than those of surviving mice. In dead mice, the number of *C. difficile* recovered from mice infected with only *C. difficile* was significantly higher than that in mice co-infected with *C. difficile* and *C. butyricum* M588 strain

The growth of EHEC O157:H7 strain 6 in BHI broth was strongly inhibited by co-incubation with *C. butyricum* M588 strain. The ability to produce Stx1 and Stx2 of EHEC was also inhibited by *C. butyricum* M588 strain. The bactericidal effect of butyric acid on EHEC O157:H7 was demonstrated not only at low pH, but also at neutral pH adjusted to 7.0. Flowcytometric analysis showed that that pre-incubation of intestinal Caco-2 cells with *C. butyricum* M588 strain inhibited the adhesion of EHEC O157:H7. Inhibitory effect of *C. butyricum* M588 strain on EHEC O157:H7 was also shown in the animal experiments using GF mice. All of the gnotobiotic mice mono-associated with EHEC O157:H7 died within 7 days post infection. In contrast, the survival rate of the therapeutically treated mice with *C. butyricum* M588 strain was 50% at 14 days after the infection with EHEC. The survival rate of the mice prophylactically treated with *C. butyricum* M588 strain was 100% at 14 days after the infection with EHEC. In the gnotobiotic mice prophylactically treated with *C. butyricum* M588 strain, the number of EHEC O157:H7 in the feces was detected at the level of 10^7 cfu/g, which was nearly 100 times less compared to that in the feces of the EHEC mono-associated mice. The level of 10^8 cfu/g of EHEC was detected in the gnotobiotic mice therapeutically treated with *C. butyricum* M588 strain. A statically significant difference was observed in the counts of EHEC between non-treated and therapeutically treated mice. Similarly, the titers of Stx1 and Stx2 in the gnotobiotic mice prophylactically or therapeutically treated with *C. butyricum* M588 strain were significantly lower than those in untreated mono-associated gnotobiotic mice with

EHEC O157:H7.

The growth of *S.* Enteritidis strains in GAM broth was strongly inhibited by co-incubation with *C. butyricum* M588 strain. After pre-incubation of the Caco-2 cells with *C. butyricum* M588 strain, the mean fluorescence intensity of the Caco-2 cells incubated with *S.* Enteritidis strains decreased significantly. The survival rate of the C57BL/6 mice treated with *C. butyricum* M588 strain was 60% (9/15) at 14 days after infection with *S.* Enteritidis 144 strain, which was significantly higher than that (26.7%, 4/15) in *S.* Enteritidis infected mice. In the mice treated with *C. butyricum* M588 strain, the number of lactobacilli and total anaerobes significantly increased.

Discussion and Conclusion

The inhibitory effects of *C. butyricum* M588 strain on *C. difficile,* EHEC O157:H7 and *S.* Enteritidis were demonstrated. Probiotic treatement with *C. butyricum* M588 strain decreased both the number of enteric pathogens colonized and toxin production in *C. difficile* and EHEC O157:H7 infection models. These results indicate that the probiotic *C. butyricum* M588 strain has preventive and therapeutic effects on various enteric pathogens.