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The Food, GI-tract Functionality and Human Health Cluster

PROEUHEALTH

Sitges, Spain

Workshop 3
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The Food, GI-tract Functionality and Human Health Cluster

PROEUHEALTH

Abstracts and posters

3rd Workshop

Sitges, Spain

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Edited by

Raija Ahonen, Maria Saarela & Tiina Mattila-Sandholm

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puh. vaihde (09) 4561, faksi 456 4374

VTT, Bergsmansvägen 5, PB 2000, 02044 VTT
tel. växel (09) 4561, fax 456 4374

VTT Technical Research Centre of Finland
Vuorimiehentie 5, P.O.Box 2000, FIN–02044 VTT, Finland
phone internat. + 358 9 4561, fax + 358 9 456 4374

VTT Biotekniikka, Tietotie 2, PL 1500, 02044 VTT
puh. vaihde (09) 4561, faksi (09) 455 2103

VTT Bioteknik, Datavägen 2, PB 1501, 02044 VTT
tel. växel (09) 4561, fax (09) 455 2103

VTT Biotechnology, Tietotie 2, P.O.Box 1501, FIN–02044 VTT, Finland
phone internat. + 358 9 4561, fax + 358 9 455 2103

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Preface

The scope "Probiotiscs and prebiotics aimed at improving intestinal health" currently represent the largest segment of the functional foods market in Europe and Japan. European Commission through its 5th framework programme has provided a substantial opportunity for scientists, industry and consumer organisations to invest in precompetitive research. This has enabled to create multidisciplinary networks, platforms and finally dialogues between different communities. Evidence continues to emerge demonstrating that probiotics and prebiotics have great potential to improve human health in specific intestinal disorders and the knowledge about the intestinal microbiota, its interaction with the host and methods of manipulating its composition and activity for the improvement of human health is growing.

The Food, GI-tract Functionality and Human Health Cluster (PROEUHEALTH) from 2001 to 2005 brings together eight complementary, multicentre inter-disciplinary research projects. All have the common aim of improving the health and quality of life of European consumers. The collaboration involves 64 different research groups from 16 different European countries. The research results from the cluster are disseminated through annual workshops and the event in Sitges is the third one in the series of workshops and involves the activities of three different platforms: a science, an industry and a consumer platform. The Sitges event provides highlights from research activities in the cluster and gives an excellent opportunity for young talented scientists to bring out their exciting results. The second day will establish an exciting discussion and dialogue between the industry representatives and consumer organisations. Moreover the event brings together interesting representatives from Associated Candidate countries, as well as examples of running CRAFT projects on probiotics.

Welcome to the 3rd PROEUHEALTH Workshop in Sitges, Spain, and ENJOY the networks!

Tiina Mattila-Sandholm
PROEUHEALTH Cluster Coordinator

Programme

SUNDAY, 14 March

9.00–18.00	Closed EU-project meetings for PROEUHEALTH Cluster project partners
20.00	Get-together-party of the workshop at Meliá Gran Sitges

MONDAY, 15 March

8.00–9.00	Registration

PROEUHEALTH cluster achievements, innovations and highlights 2001–2004
Chair: Prof. Tiina Mattila-Sandholm, VTT, Finland

9.00–9.45	MICROBE DIAGNOSTICS: *Prof. Michael Blaut et al., DiFE, Germany*
9.45–10.30	EU AND MICROFUNCTION: Substrates for members of the gut microbiota and probiotics, and probiotic safety *Dr. Muriel Derrien et al., Wageningen University, the Netherlands*
10.30–11.00	Coffee
11.00–11.45	CROWNALIFE: The elderly gut microflora *Dr. Katiana Saunier et al., INRA, France*
11.45–12.30	PROGID: Functional microbes and inflammation – curiouser and curiouser *Prof. Fergus Shanahan et al., University College Cork, Ireland*
12.30–14.00	Lunch and posters at PROEUHEALTH market

Chair: Prof. Willem de Vos, Wageningen University, the Netherlands

14.00–14.45	DEPROHEALTH: Second generation probiotics *Dr. Annick Mercenier et al., Nestlé Research Center, Switzerland*
14.45–15.30	PROPATH: Fight against *Helicobacter* *Prof. Luc deVuyst et al., Vrije Universiteit Brussels, Belgium*

15.30–16.00 Coffee

16.00–16.45 PROSAFE: Highlights from the PROSAFE project: Safety from medical perspectives
Prof. Herman Goossens et al., University of Antwerp, Belgium

16.45–17.30 PROTECH: Technology of probiotics and prebiotics
Prof. Dietrich Knorr, Berlin University of Technology, Germany

TUESDAY, 16 March

Cluster achievements, industry and consumer platform
Chairs: Dr. Maria Saarela and Dr. Jaana Mättö, VTT, Finland

9.00–12.00 Poster sessions

Oral presentations: *C. Cinquin, M. Fallani, D. Fayol-Messaoudi, S. Fuentes, P. Heczko, J. Hejnova, R. Hutson, P. Namsolleck, M. Rajilić, K.M. Tuohy, M. Vancanneyt, J. Wells*

12.00–14.00 Lunch, posters at PROEUHEALTH market

Chairs: Prof. Charlie Daly, University Collece Cork, Ireland and Dr. Liisa Lähteenmäki, VTT, Finland

14.00–17.00 Industry statements and Consumer platform

Industry statements and Consumer platform will be based on panel discussion from questions raised by consumer organisations

Industry statements:

Danone, Jean-Michel Antoine, France
Probiotics a challenging opportunity for science industry and consumer

Nestlé, Christoph Cavadini, Switzerland
Challenges to the extension of the probiotic concept to different food product categories – An industry point of view

Chr. Hansen, Espen Laulund, Denmark
Safety in the post genomics era

Unilever, Aat Ledeboer, the Netherlands
Probiotic research: What is needed? A view from industry

Yakult, Charlotte Shortt and B. Degeest, United Kingdom
Towards generic claims for probiotic lactic acid bacteria

Orafti, Jan Van Loo, Belgium
Prebiotics

Consumer statements by:

BEUC, Beate Kettlitz, Belgium
Awareness and expectations of consumers in relation to so-called functional food and gut health

EFCCA, Rod Mitchell, United Kingdom
EFCCA... and Probiotics 2004

Technical Department OCU, Gemma Trigueros, Spain
Consumer concerns related to probiotic and prebiotic products

20.00 Dinner

WEDNESDAY, 17 March

6th framework programme & new member states
Chair: Dr. Jürgen Lucas, European Commission, DG Research, Belgium

9.00–9.30 Food safety and quality
Dr. Jürgen Lucas and Dr. Liam Breslin
European Commission, DG Research, Belgium

9.30–10.00 Food claims directive EU DG Health and Consumer Protection
Dr. Basil Mathioudakis and Dr. Barbara Moretti
European Commission, Health and Consumer Protection Directorate General, Food Law and Biotechnology Unit, Belgium

10.00–10.30 Future prospects for gut health
Prof. Willem de Vos, Wageningen University, the Netherlands

10.30–11.30 New actions from candidate countries

Research activities of LB Bulgaricum on functional dairy starters and foods
Dr. Tsona Stefanova, LB Bulgaricum, Bulgaria

Studies on probiotics, prebiotics and synbiotics as functional food components
Prof. Maria Bielecka, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Poland

New projects concerning pre- and probiotic bacteria in the Agricultural University of Poznan, Poland
Dr. Wlodzimierz Grajek, August Cieszkowski Agricultural University of Poznan, Poland

11.30–12.00 CRAFT project on microencapsulation of probiotic products
Dr. Jerome Panes, Lallemand SAS, France

12.00–14.00 Farewell lunch

Contents

ABSTRACTS

Microbe diagnostics

M. Blaut[1], L. Rigottier-Gois, M. D. Collins, W. de Vos,
K. Holmstrom, M. van der Rest and V. Smolej

[1]Department of Gastrointestinal Microbiology, German Institute of Human Nutrition, Germany

The bacterial community in the human intestinal tract affects the host with respect to gastrointestinal function, health and well-being. In spite of the importance of the gut microbiota for human health, the targeted manipulation of the gut microbiota by dietary means is still difficult. This is due to the complexity of the interactions between gut microorganisms, host and diet, as well as to the variability between human individuals in respect of the composition of the intestinal microbiota. To improve this situation it is necessary to identify the key parameters that influence the composition and the activity of the intestinal microbiota. This information can only be gained with reliable methods.

The project is aimed at developing, refining, validating and automating the most advanced molecular methods for monitoring human gut flora composition and bacterial gene expression in selected human populations in response to diet and life style. Using a variety of growth substrates and growth media progress has been made in improving the knowledge about the bacteria present in the human intestinal tract. More than 200 isolates were obtained with several independent systematic enrichment approaches. Although the majority of these isolates belong to known species a considerable number of the isolates are novel species. The 16S rRNA gene sequences of the new isolates and their phylogenetic position were determined. In parallel, additional sequences were retrieved by culture-independent methodology. All additional sequences have been fed into private and public databases. This highly improved database has been used to develop new oligonucleotide probes for the culture-independent detection of intestinal bacteria. The panel of probes has been extended to subgroups of bacterial groups in the gut. Probes have also been designed and validated to detect the new isolates and to determine their numerical importance and their distribution in human individuals.

Two approaches have been chosen to develop high throughput methods for the detection of faecal bacteria: The two approaches taken are automated image analysis and flow cytometry. The latter method has been demonstrated to be suitable for the rapid detection of fluorescent bacteria. This could be demonstrated for a set of different fluorescently labelled oligonucleotide probes. This panel will of available probes has been extended considerably and covers the majority of the gut microbiota at the sub-group level. The development of a

high throughput approach based on the application of DNA arrays for the rapid detection of specific rRNAs of dominant human faecal bacteria is under way.

To demonstrate the accuracy and applicability of the probe technology faecal samples have been collected at five different locations in Europe and are being analysed. All possible information concerning the sample donors (e.g. origin, age etc) will be compiled and jointly analysed using multiple variable statistical analysis tools. The developed methods are presently also applied to assessing the links between intestinal microflora and ulcerative colitis.

Substrates for members of the gut microbiota and probiotics, and probiotic safety

M. Derrien[1], W. M. de Vos[1], C. Vernazza[2], G. Gibson[2] and A. Ouwehand[3]

[1]Laboratory of Microbiology, Wageningen University, The Netherlands
[2]School of Food Biosciences, University of Reading, U.K.
[3]Department of Biochemistry and Food Chemistry and Functional Foods Forum, University of Turku, Finland

Mucus provides a protective layer on the intestinal wall, but functions also as a habitat and substrate for the intestinal microbiota. Culture dependent and independent methods were used to identify intestinal bacteria that participate in mucin degradation. A new organism, *Akkermansia muciniphila*, was identified that could grow solely on intestinal mucus, and was used as a model to study the interaction between the human host and mucus-utilizing microbes.

Five species of bifidobacteria and 13 carbohydrates were tested by means of growth curves to find suitable synbiotic pairs. *B. adolescentis* plus inulin, *B. longum* plus GOS and *B. longum* plus IMO were selected for further investigation. Batch culture fermentations were performed to investigate the effects of these combinations in mixed faecal culture. Gut model systems were then used to strengthen the results. Synbiotic supplementation was found to modulate the bacterial composition.

The safety of probiotics is of prime importance. The presence of canonical virulence factors was analyzed in a series of probiotic and other bifidobacteria and lactobacilli. Most of the tested strains were found to be resistant to serum mediated killing, which is an intrinsic property of Gram positive bacteria. No other common risk factors were identified on the strains tested.

The elderly gut microflora (CROWNALIFE)

K. Saunier[1], J. Doré[1], F. Zunft[2], M. Blaut[2], K. Tuohy[3], R. Rastall[3], C. Gill[4], I. Rowland[4], S. Silvi[5], A. Cresci[5], L. Alm[6], E. Norin[6], N. Jonkers[7], J. Van Loo[7], C. Picard[8] and O. Goniak[8]

[1]Laboratoire d'Ecologie et de Physiologie du Système digestif, INRA, France
[2]Deutsches Institut Für Ernährungsforschung Potsdam-Rehbrücke (DIfE), Abteilung Gastrointestinale Mikrobiologie, Germany
[3]Food Microbial Sciences Unit, School of Food Biosciences, The University of Reading, U.K.
[4]Northern Ireland Centre for Diet and Health, University of Ulster, Northern Ireland
[5]Dipartimento di Scienze Morfologiche e Biochimiche, ComparateUniversita' degli Studi di Camerino, Italy
[6]Karolinska Institute, Department of Cell and Molecular Biology, Laboratory of Medical Microbial Ecology, Sweden
[7]Tiense Suikerraffinaderij NV (ORAFTI), Belgium
[8]Danone, Centre International de Recherche Daniel Carasso, France

The complex microflora colonizing the human gut changes with age and may play a role in the observed increase in both infectious and chronic disease in the elderly. Within Europe, there appears to be regional differences in incidence of disease as well as differences in life span and degree of independence in later years. The gut microflora is amenable to modulation using probiotics, prebiotics and synbiotics, and the elderly represent a population group for which such strategies may be particularly suitable. CROWNALIFE has confirmed that the gut microflora within elderly people differs from younger adults, showing a much greater degree of species diversity. Through generation of 16S rRNA based phylogenetic inventories of the faecal microflora of healthy elderly individuals (>65 years) the elderly 80% of cloned 16S rDNA sequences corresponded to previously uncultured bacteria while 1/3 of these represented novel phylogenetic lineages. In a pilot study examining microflora composition within different age groups in two different countries, the increased microflora diversity has been confirmed quantitatively using a set of 16 phylogenetic probes. To affect beneficial modulation within the gut microflora CROWNALIFE has employed a synbiotic formulation, and is determining the suitability and efficacy of this approach by monitoring changes within relative bacterial numbers and microbial biomarkers important in gastrointestinal health and disease. Using a holistic approach, microflora modulation has been analysed with respect to regional dietary differences within Europe. In parallel, novel synbiotic products, employing probiotic strains selected for anti-pathogenic activity from the elderly gut and efficacious prebiotics (both existing and novel) have been formulated to constitute second generation functional foods specifically designed to affect beneficial modulation of the elderly gut microflora towards improved gut health.

Functional microbes and inflammation – curiouser and curiouser

B. Sheil, J. McCarthy, L. O'Mahony, M. W. Bennett, P. Ryan, J. J. Fitzgibbon, B. Kiely, J. K. Collins and F. Shanahan

On behalf of the PROGID research group and Alimentary Pharmabiotic Centre, University College Cork and Teagasc, Ireland

Background: The prophylactic efficacy of oral consumption of probiotic lactobacilli has been demonstrated in IL-10/ko mice and other models of inflammatory bowel disease. Some of these probiotic organisms are now being studied in human trials of maintenance therapy of both Crohn's disease and ulcerative colitis that are underway in Europe (PROGID). Although probiotics are usually considered in the context of oral consumption, the possibility that functional microbes or their metabolites might exert a therapeutic benefit when delivered by the paraenteral route has received little attention.

Aims: (i) To determine the effect of parenteral administration (s.c.) of *Lactobacillus salivarius* 118 on colitis of IL-10/ko mice; (ii) to determine if observed responses are disease-specific and whether similar effects occur in a model of arthritis.

Methods: (i) IL-10/ko mice were injected s.c. with *L. salivarius* 118 or saline over 19 weeks. At sacrifice, the bowels were histologically scored. Isolated splenocytes were cultured *in vitro* and cytokine levels measured. (ii) In the collagen-induced arthritis model, DBA/1 mice were injected subcutaneously with the probiotic or saline. At sacrifice, paw thickness was measured and joints were histologically scored.

Results: (i) Colonic inflammatory scores were significantly decreased in IL-10/ko mice injected with *L. salivarius* 118 compared with controls ($p<0.05$). Proinflammatory cytokine production from stimulated splenocytes was significantly lower for the probiotic group, whereas stimulated TGF-β levels were significantly increased ($p<0.05$). (ii) Scoring of arthritis and paw thickness were significantly improved in the group of mice injected with *L. salivarius* 118 compared with controls.

Conclusions: (1) Subcutaneous administration of *L. salivarius* 118 significantly attenuated colitis in the IL-10 KO model and suppressed collagen-induced arthritis, suggesting that the oral route may not be essential for probiotic anti-inflammatory effects and that responses are not disease-specific. (2) The probiotic effect was associated with reduced production of pro-inflammatory (TH1) cytokines and maintained production of anti-inflammatory TGFβ.

Second generation probiotics

A. Mercenier*, Institut Pasteur de Lille, France

On behalf of the DEPROHEALTH Network
* Present address: Nestle Research Center, Lausanne, Switzerland

The DEPROHEALTH project aims at developing probiotic strains to be used as novel anti-inflammatory treatments or oral vaccines against *Helicobacter pylori* and rotavirus. It is also evaluating the predictive value of current probiotic screening methods by evaluating the correlation between *in vitro* tests and relevant murine models.

A range of wild type strains of lactobacilli were analysed for their potential capacity to resist passage through the upper part of the intestinal tract and for their ability to interact with the host immune system. The latter was achieved by looking at cytokine secretion or up-regulation of surface markers from/on epithelial cells and immune cells (mainly peripheral blood mononuclear cells and macrophages) or on co-cultures of these two types of cells. While the studied isolates interacted in a strain-specific way with immune cells, they seem to have little and non discriminative effect on epithelial cells. However, they were shown to facilitate the cross talk between intestinal and immune cells. While certain *Lactobacillus* species seem to be better suited for oral administration, the immunomodulation profile appears to be strain specific. This *in vitro* evaluation has been extended by studies conducted in mouse colitis models, which confirmed that specific strains exert a beneficial effect on intestinal inflammation. In particular, the beneficial effect described by Steidler *et al.* with the recombinant *Lactococcus lactis* strain secreting murine IL10 was totally confirmed in a third colitis mouse model (TNBS induced colitis). Studies in this model are shorter and less heavy to perform and the TNBS model is presently being used to evaluate additional recombinant strains. From the four recombinant lactobacilli secreting fair levels of murine IL-10 that were constructed, the two based on naturally "anti-inflammatory" strains (i.e. *Lactobacillus plantarum* and *Lactobacillus casei*) are being evaluated for their protective effect *in vivo*. Mutants of *L. plantarum* NCIMB8826 were obtained that synthesise altered lipoteïcoic acids and/or peptidoglycan. They were analysed for their phenotypic and biochemical traits, immune modulation properties and capacity to act as improved antigen delivery system. One of them exhibited a significantly increased protective effect in a mouse TNBS colitis model as compared to the wild type strain. Another mutant was successfully used as live carrier to deliver two model antigens, the C subunit of tetanus toxin and the urease B subunit of *Helicobacter pylori.* In view of these results, a same approach was undertaken for delivery of the rotavirus antigens in order to try to ameliorate the low immune

responses induced so far. In the course of these experiments, new methods to evaluate the *Helicobacter* load in infected mice were developed. Two surface proteins of *Lactobacillus crispatus* 247 were subjected to detailed analysis in order to provide food-grade targeting signals for cell surface presentation of therapeutic molecules. Remarkably, a safe biologically contained strain secreting human IL10 was constructed, thus opening the way to a phase 1 clinical trial in patients suffering from IBD.

Fight against *Helicobacter*

L. De Vuyst[1], L. Makras[1], Q. Yi[2], D. A. Brede[2], H. Holo[2], I. Nes[2], B. Martinez-Gonzales[3], E. Panayotopoulou[3], A. Mentis[3] and D. Sgouras[3]

[1]Research group of Industrial Microbiology, Fermentation Technology, and Downstream Processing, Department of Applied Biological Sciences, Vrije Universiteit Brussel, Belgium
[2]Department of Chemistry, Biotechnology, and Food Science, Agricultural University of Norway, Norway
[3]Institut Pasteur Hellenique, Laboratory of Medical Microbiology, Greece

Several lactic acid bacteria (LAB) strains have been shown to inhibit *in vitro* *Helicobacter pylori*, a Gram-negative pathogen causing gastritis, peptic and duodenal ulcers, and relating to the development of gastric cancer. Moreover, cell-free culture supernatant (CFCS) of *Lactobacillus johnsonii* La1, a commercial probiotic strain, and *Lactobacillus acidophilus* LB, a biotherapeutic agent, decreased *H. pylori* viability *in vitro* and bacterial density in animal models and in healthy volunteers. Current eradication regimens of *H. pylori* encounter problems such as increasing rates of eradication failure, mainly due to resistance development against the administered antibiotics. Thus, the growing need to develop and introduce new antimicrobial drugs should be envisaged on the basis of antimicrobial activity observed by LAB, which could be a potential source of new, antibiotic-like molecules.

The exact underlying mechanism of the anti-*H. pylori* activity exhibited by LAB is not clear, although a number of reports point out that organic acids produced and the resulting low pH are responsible for the observed antimicrobial activity. However, even after pH neutralization, CFCS from selected LAB have shown anti-*H. pylori* activity. In our study, CFCS (adjusted to pH 6.5) from *L. johnsonii* La1 but not other LAB strains, inhibited a number of *H. pylori* reference and clinical strains, in an agar overlay assay. This anti-*H. pylori* activity of CFCS (pH 6.5) of *L. johnsonii* La1 was also observed in time kill assays, involving *H. pylori* liquid cultures. Finally, concentrated CFCS, obtained through protein precipitation and an organic solvent extraction, of *L. johnsonii* La1, *L. casei* Shirota (LcS), and *L. amylovorus* DCE 471 inhibited *H. pylori* ATCC 43504 in a killing assay. These data indicate that antibacterial substances other than lactic acid or acetic acid are responsible for such inhibitory activity.

Among all the compounds produced by LAB, such as organic acids, sugar catabolites, oxygen catabolites, and bacteriocins, the latter have attracted most attention in recent years, as it has been shown that many *Lactobacillus* strains produce different kinds of such proteinaceous molecules. During this study it was

shown that *L. johnsonii* La1 produced protease-sensitive compounds inhibitory to both lactobacilli and *H. pylori.* However, the indicator *Lactobacillus delbrueckii* subsp. *bulgaricus* was several hundred times more sensitive than *H. pylori* to the products secreted by strain La1. Two fractions could be obtained upon purification of the anti-*Lactobacillus* and anti-*Helicobacter* activity, a lactacin F-like bacteriocin and another antimicrobial peptide, respectively.

Further, *in vitro* studies involving a 1-h pre-treatment of *H. pylori* strains with live LAB showed that binding of *H. pylori* to gastric adenocarcinoma (AGS) cells was significantly decreased. One-hour pre-treatment of *H. pylori* strains with CFCS of La1, DCE 471, and LcS, decreased their ability to bind to AGS cells and to induce IL-8 secretion after 24 h. However, both effects could be attributed to the inhibitory effect of the pre-treatment on *H. pylori*, as there was a significant decrease in viable counts (6–7 log CFU for the La1-treated and 2–3 log CFU for the DCE471 and LcS-treated). No effect was observed with CFCS of IBB 801. Finally, 12-week continuous administration (10^8 CFU daily dose) of La1, DCE 471 and IBB 801 to mice infected with *H. pylori* SS1 resulted in a marked attenuation of chronic active gastritis, as reflected by a significant reduction of neutrophils invading the lamina propria of the gastric mucosa. Nevertheless, no eradication or significant reduction of the infecting *H. pylori* population was observed. In the case of La1 there was also significant decrease in the levels of chronic inflammation, reflected by a significant reduction in monocytes infiltrating the gastric mucosa. The observed attenuation in the grade and activity of gastritis was also associated with reduced levels of anti-*H. pylori* IgG antibodies in the serum.

Highlights from the PROSAFE project: Safety from medical perspectives

H. Goossens[1,7], C. Vael[1], T. Van Autgaerden[1], V. Vankerckhoven[1], G. Huys[2], J. Swings[2,3], M. Vancanneyt[3], I. Klare[4], G. Werner[4], W. Witte[4], M.-B. Romond[5], P. Moreillon[6], J. Knol[7], A. Vriesema[7], K. Adema[8] and E. Wiertz[8]

[1]University of Antwerp, Belgium
[2]Ghent University, Belgium
[3]BCCM/LMG Bacteria Collection, Belgium
[4]Robert Koch Institute, Germany
[5]University of Lille, France
[6]University of Lausanne, Switzerland
[7]NUMICO, the Netherlands
[8]The University of Leiden, the Netherlands

The final PROSAFE strain collection comprises 907 isolates: 320 nutritional isolates, including 293 probiotic LAB (as defined by the depositor or isolated from product) and 27 food isolates; 580 human isolates, including 284 strains isolated from normal flora (241 of faecal origin), and 260 sterile isolates (201 from the blood); and 7 animal strains.

Characterisation focused on members of the genera *Lactobacillus* and *Bifidobacterium,* which are considered to be taxonomically most complex and for which identification problems were to be expected. For the identification and fingerprinting of lactobacilli, an Amplified Fragment Length Polymorphism (AFLP) method and library were developed; SDS-PAGE profiling of whole-cell proteins was only performed in cases where no identification could be obtained. We were able to successfully identify 99% of the studied lactobacilli at the species level. For the identification and fingerprinting of *Bifidobacterium*, a rep-PCR method using BOX primers was developed; 16S rDNA sequencing was only performed in case where no identification could be obtained. A reference framework of type and reference strains representing all currently described species in this genus was constructed. We were able to successfully identify 86% of the bifidobacteria analysed so far at the species level.

Typing of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* was started because both species largely dominate the PROSAFE collection. A two-step approach was followed: intra-species genotypic analyses using AFLP fingerprinting followed by PFGE on representative strains from the different AFLP clusters. AFLP indicated the presence of 7 and 11 stable intra-species genomic groups in *L. rhamnosus* and *L. paracasei*, respectively. In particular for *L. rhamnosus* PFGE confirmed a very high genomic relationship among strains from probiotic and human (blood) origin within delineated AFLP groups.

In total, 601 LAB strains were tested for their antibiotic susceptibilities to16 antibiotics representing all antibiotic classes, determined as minimal inhibitory concentrations (MICs) in broth microdilution: 119 strains of enterococci, 321 *Lactobacillus* isolates, 41 *Pediococcus* isolates, 10 *Lactococcus* isolates, and 110 *Bifidobacterium* isolates. In parallel to the MIC determinations, PCR for detection of a wide range of acquired antibiotic resistance genes was finalised, including the following genes: *aad*(E)-*aph*(A), aad(E), *aph*(2'')-*aac*(6'), cat_{pC194}, cat_{piP501}, *erm*(A), *erm*(B), *erm*(C2), *sat*(A) [= *vat*(D)], *sat*(G)[= *vat*(E)], *van*(A), *van*(B), *tet*(K), *tet*(L), *tet(*M). Finally, a set of bacterial reference strains for transferability experiments *in vitro* and in a colon model was established. Among the 111 *E. faecium* strains tested, seven were probiotic *E. faecium* strains of which one displayed erythromycin resistance but not mediated by the *erm*(A), *erm*(B) or *erm*(C) genes. The staphylococcal transposon Tn*554* with *erm*(A) was detected for the first time in an *E. faecium* strain (clinical isolate from a blood culture); *erm*(B)-mediated ERY resistance was frequently found in enterococci. In three *E. faecium* isolates, *vat*(D) could be detected. Other resistance genes that were present in the examined *E. faecium* were: *aad*(E)-*aph*(A), *aad*(E), *aph*(2)''-*aac*(6'), cat_{pC194}, cat_{piP501}, *van*(A), *tet*(L), *tet*(M). Of these in the present *E. faecium* strains detected genes, *aad*(E), cat_{pC194}, *erm*(C), *van*(A) and *tet*(K) were not found in the seven *E. faecalis* strains.

Among the 321 examined lactobacilli [233 from humans, 5 from animals, 74 from probiotics, 9 from nutrients (such as starter cultures)] resistance frequencies were low except those for the glycopeptides, fusidic acid and co-trimoxazole; but these are mostly species-depending natural antibiotic resistances. Altogether, the lactobacilli from different origins showed similar resistance patterns. However, until now, in four *Lactobacillus* isolates (one clinical and three probiotic strains) acquired resistance genes [*erm*(B), *tet*(L) and *tet*(M), respectively] could be detected.

We did not detect by PCR any of the described virulence properties in probiotic *E. faecium* strains. We found no significant difference in adhesion between faecal, clinical and probiotic strains for collagen, fibrinogen or mucus. A new human flora mouse model was developed to study lethality, translocation and inflammatory response after intraperitoneal administration was developed; RT-PCR for bacterial detection in internal organs is being optimised and methods to detect cytokine markers TNF-α, IL-6 and IL-4 were validated.

A semi-dynamic batch fermentation model for screening effects of probiotic strains on the stability of the faecal microbiota was evaluated; the model shows stable overall numbers of bacteria but changes of certain subpopulations and therefore the model needs further improvement.

A master database will be constructed in 2004 to combine the data retrieved and encountered by the various efforts of the PROSAFE partners, ensuring that the objectives are complied with, and achieving the expected integration.

Technology of probiotics and prebiotics

D. Knorr

Berlin University of Technology, Germany

The ongoing EU-funded project, PROTECH (Nutritional enhancement of probiotics and prebiotics: Technology aspects on microbial viability, stability, functionality and on prebiotic function, QLK1-CT-2000-30042) is dealing with the optimization and improvement of processing technology related to the production of probiotics and prebiotics as well as the interaction of both in the whole production pipeline, in order to maintain the health-related performance of these products upon consumption. Figure 1 illustrated the specific objects of investigation and how the partners are interlinked in the multitude of tasks within the project. In the fermentation study development of a fermentation medium as well as optimization of harvesting time with respect to post-harvesting stability were pursued. The developed milk-free fermentation medium, contained food-grade components only and was found to support the growth of the probiotic lactobacilli equally to standard broth. Both freeze-drying and spray-drying processes were evaluated, in terms of the identification of suitable processing regimes, performance of different protectants in offering high survival during drying and storage, as well factors governing storage stability during storage. The use of sub-lethal stress – either by identical or by cross-adaptive stress factors – to improve technological behaviours (i.e. heat and oxygen tolerance) as well as to enhance resistance against extreme conditions in the gastro intestinal tract (bile tolerance) was demonstrated. A proteomic approach was applied in order to examine the specific response of the cells towards the imposed environmental stresses. The survival of probiotics in yoghurt was assessed to determine critical factors governing survival in fermented products and how modifications on this system could be made to reduce cell death, especially by incorporating prebiotics. The possibility of enzymatically modifying prebiotics into a more complex structure, thus making them less fermentable, was investigated. Bifidobacterial enzyme with transglycosylation activity was able to be identified and characterized in terms of their physico-chemical properties. Feeding trials of commercial or technologically modified prebiotics in combination with probiotics were performed to demonstrate the possibility of modulating beneficial changes on the degree of fermentation and short chain fatty acid patterns in different parts of rat colon.

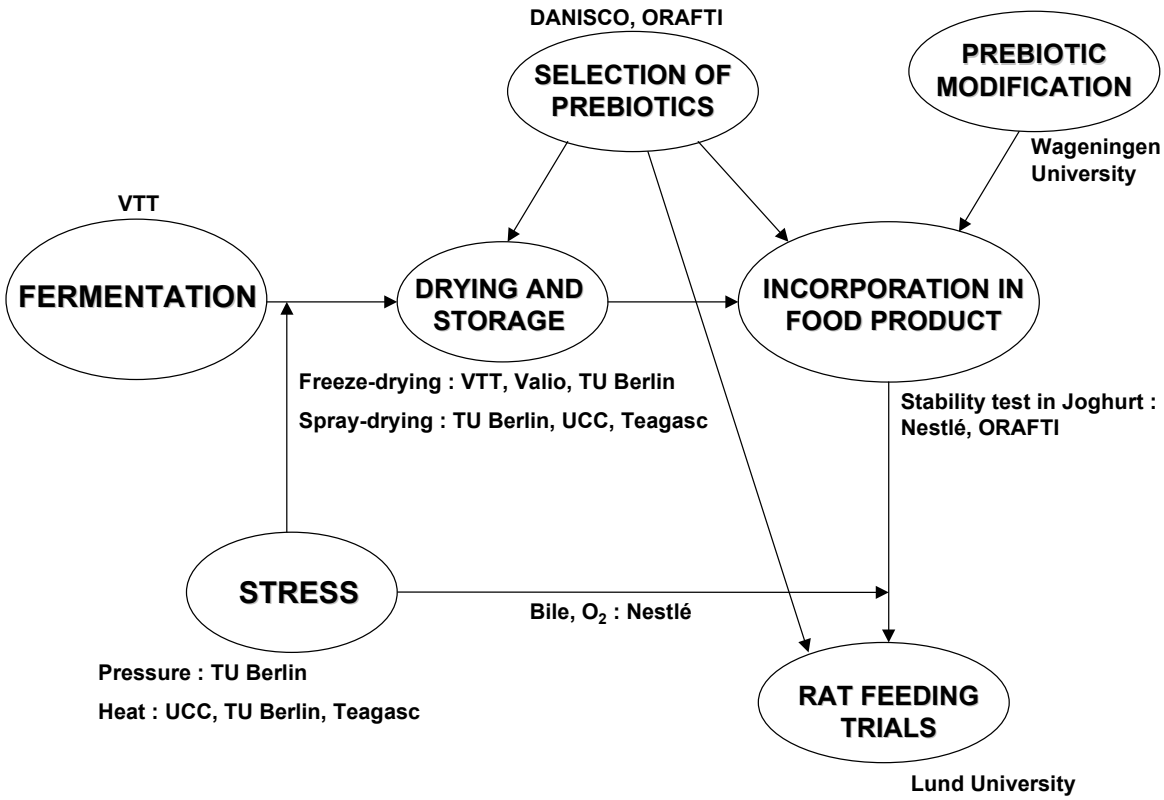

Figure 1. General and specific objects of investigation, which were accomplished by partners participating in PROTECH project along with the network of interaction among partners.

Probiotics a challenging opportunity for science industry and consumer

J.-M. Antoine

Danone, France

Probiotics are very old traditional foods with a double functionality: They are able to improve the storage of a fresh food: milk and to bring some sensory benefits. They are providing some benefits to the host, the two oldest one are lactose digestibility, and coping with diarrhea. These benefits must have been important enough to support and maintain the permanent replication of starters since age ago. This selection process was useful also to identify safe probiotics. By definition Probiotics are living micro-organisms, and they are active in the human gut. They are coming from our environment and they are able to survive in the human gut. Probiotics may act on different mechanisms in the gut: Gut flora, gut digestive wall (mucus and cells), immune system. Most of the mechanisms can be explored in models for ethical (Axenic models, Gut immune system, cancer studies...) and pragmatic reasons (Repeatability, reproducibility...). To get a claim human feeding studies are needed as products will be ingested by humans and global integrative effects shoud be reported. However Probiotics used in food will have a moderate impact on some functions and on the reduction of some risk factor(s) as compared to drugs. Improvement of functions is a new area for food after prevention of nutrient deficiencies, and the effects of probiotics on and through the gut are only at a starting point. Probiotic Associated Characteristics will benefit consumers needs and scientific interests.

Challenges to the extension of the probiotic concept to different food product categories – An industry point of view

C. Cavadini

Nestlé Research Center, Switzerland
e-mail: Christoph.Cavadini@rdls.nestle.com

Probiotics are defined as “living microorganisms which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition” (Guarner and Schaafsma, 1998).

The assumption that probiotics need to reach the gastro-intestinal tract of the consumer alive and probably at high numbers in a physiologically active form to exert their beneficial effects has significant consequences for the selection of candidate strains as well as for the development of food products containing these strains. Today, process and storage stability of probiotics still strongly limits the widespread application of probiotic concept to many product categories.

With respect to shelf live stability good solutions are today available to maintain the viability of probiotic strains in chilled dairy and comparable products (e.g. fruit juices). The same is principally the case for very low moisture products (food and supplements).

Due to the success of these products, interest is increasing to further extend the probiotic concept to new product categories.

However, for many of the potential applications the stability of the probiotic strains becomes a critical issue, especially for shelf stable products with intermediate moisture levels, which may be exposed to high temperatures during distribution and storage.

The development of practical solutions for these product categories is highly challenging and requires an interdisciplinary approach combining competencies of disciplines like molecular biology, microbial physiology, food and material sciences, engineering, and packaging.

Another urgent question that needs to be properly answered when transferring the probiotic concept to new product categories is related to the fact that it is generally agreed that a strong health claim can be deduced from studies in the

target population only. It is furthermore important that the object of the evaluation of efficacy is the food product containing the probiotic microorganism (BgVV, 2000). In consequence the question arises to what extend a probiotic with a (clinically) confirmed functionality in an actually commercialized product require re-confirmation of its functionality in additional studies when it is employed in a newly designed food or product application.

To provide an answer to above question we developed the idea of a *Bio-Equivalence Test* based on the *in vitro* assessment of physiological characteristics potentially associated with positive probiotic activities (Cavadini et al., 1999). The approach can be applied to determine and compare the biological activity of probiotics in different food products with the final objective to demonstrate a general biological equivalence between products tested in human studies and any new application form (demonstration of Bio-Equivalence Test).

BgVV (2000). Arbeitsgruppe "Probiotische Mikroorganismenkulturen in Lebensmitteln" am Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV), Berlin. Abschlussbericht "Probiotische Mikroorganismen kulturen in Lebensmitteln". Ernährungs-Umschau 47(5), 191–195.

Cavadini, Ch., S. Blum, R. Zink, W. P. Hammes and A. Pfeifer (1999). *In vitro* screening assays for biological activity of probiotics. XIII International Symposium on Gnotobiology. Karolinska Institute. Laboratory of Medical Microbial Ecology. Stockholm, Sweden.

Guarner F. and G. J. Schaafsma (1998). "Probiotics," International Journal of Food Microbiology, 39th ed., pp. 237–238.

Safety in the post genomics era

E. Laulund

Chr. Hansen, Denmark

The safety aspect of probiotics and other bacterial microorganisms intended for application in foods has an increasingly awareness from regulatory bodies worldwide.

Preparation of guidelines and methods for the safety assessment of probiotics as well as other bacterial food microorganisms has been initiated within the EU through the SCAN committee and via the EU Frame Work Programmes. Furthermore international organisations like FAO, IDF and EFFCA have initiated related activities.

One of the main criteria discussed in these working groups is the safety question related to the antibiotic resistance pattern of selected bacterial species/strains. Especially the questions related to the nature and origin of the antibiotic resistance mechanism are high on the agenda, e.g if the genes are intrinsic or acquired genes and if the responsible genes are transmissible between species. However, other criteria like virulence factors, immune stimulation, production of toxins and compounds like bacteriocins and biogenic amines are included in the evaluations of some of the groups.

Parallel to this work EFFCA and supported by IDF has established a list of "Microorganisms with a documented history of use in food". This overview has a more empiric approach to the safety aspect - as the list provides an overview of bacterial species/strains which have a long history of use in food production, thereby implying that the mentioned species/strains in the overview have a history of safe use.

With the introduction of the genomics techniques new possibilities concerning the knowledge of the genes present in the bacteria and the interpretation of their function can be established with great accuracy. The genomics techniques therefore provide excellent tools to get improved knowledge of the mode of action of probiotics as well as food cultures.

Today the complete genome of several probiotic and other bacterial microorganisms for food application has been sequenced and are now publicly available. The techniques for acquiring the complete picture of the bacterial genomes have become more sophisticated, and as a follow up the costs have

become lower making the preparation of the gene sequence of individual species and strains economically possible.

The genomics tools can be an important way to secure a holistic approach in the safety assessment and provide a rational risk management of the safety aspects of probiotics and other bacterial food microorganisms. However, the understanding of the genomics must be linked and evaluated together with the history of use and observed performance in practical food applications before decisive conclusions can be drawn on the safety and risk of selected species/strains.

Further understanding of the correlation between the bacterial genomics and the actual performance of a probiotic or a bacterial food microorganism in the environment where they are intended to function is needed to secure a solid safety assessment and provide a proper background for risk management.

Probiotic research: What is needed? A view from industry

A. M. Ledeboer

Unilever, The Netherlands

Functional foods or foods that have a health benefit beyond basic nutrition are widely appearing on the market by now, among others as probiotic foods. Since probiotics are defined as being "living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition" (1), they must be

- Alive during the shelf life of the product and present in a minimal number per serving
- Reach the target organ in numbers high enough and stay long enough to exert the beneficial effects
- Show a measurable health benefit to underpin the product claim.

Although individual consumers still seem to accept the frequently used soft claims, more and more authorities and consumer organizations will require clear and proven claims. In order to fulfil such requirements, to come with even better probiotic strains than are available now, and to extend the range of probiotic applications beyond the dairy range of products, there is a need for new and often fundamental knowledge on mechanisms of action, targeting and survival of probiotic strains under harsh conditions such as dry or semi-dry products or the gut. These are not simple requirements and need a close co-operation of scientists from disciplines as diverse as molecular biology, bioinformatics, microbial physiology, gastro-enterology, food microbiology and food technology either in one team or in cooperating projects. But more than ever, recent information obtained from well designed clinical studies and spectacular advances in the genomics area will make it possible to attain real breakthroughs even in view of the hurdles that will turn up. Any further widespread application of probiotics will require these breakthroughs.

1. Guarner, F. and Schaafsma, G. J. (1998) Probiotics. Int. J. Food Microbiol. 39, 237–238.

Towards generic claims for probiotic lactic acid bacteria

C. Shortt and B. Degeest

Yakult, U.K.

Despite considerable interest in product-specific health claims the jury is still out as to whether scientifically substantiated food-related health claims actively drive demand or ensure commercial success. However, it is acknowledged that generic claims play an important role in increasing consumer awareness in the link between diet and health. Indeed, generic claims can assist consumers in making wise food choices and can impact on consumers' willingness to try new products.

Yoghurts and fermented milks have been consumed for generations and play a strong role particularly in the dietary traditions of Eastern and Northern Europeans. A generic claim is an assertion of something as true or factual that is applicable to a whole class or group. In general, generic claims make a direct connection between a functional or health effect and a component rather than with a specific product. Traditionally the healthiness of food has been associated with nutritional factors and thus most generic claims relate to nutritional rather than microbiological components of foods. One exception to this is that yoghurt and fermented milks, where specific viable lactic acid bacteria (LAB) are considered key health ingredients, have been considered as healthy for centuries. However, despite the long association between these foods and health maintenance generic claims have not yet been established for these components.

The need in Europe for nutritional strategies to improve gastrointestinal health is clear from a perusal of health statistics and the concept of maintaining health by dietary modulation of the gut flora using probiotic LAB is one possible approach. Indeed, interest in the *essentiality* of the gut flora in the maintenance of health is increasing rapidly particularly with the knowledge accumulating due to the sequencing of some of the salient bacterial genomes. Starting with a definition of probiotics for use in human nutrition that is widely accepted in Europe - '*live microbial food ingredients that are beneficial to health'*- the existence and validity of generic claims for probiotic LAB will be explored.

Prebiotics

J. Van Loo

Orafti, Belgium

Prebiotics are food ingredients that cannot be hydrolysed by human digestive enzymes in the upper intestinal tract. Prebiotics are as a consequence completely available to the bacteria that are present in the intestine, and more particularly in the colon where they are a factor 10000 more abundant than upper intestinal tract (mouth, stomach, small intestine). The most important property of prebiotics however is that while they are fermented, they stimulate some groups of bacteria in the colon, and at the same time they suppress other groups of bacteria ("selective" stimulation of colonic bacteria). It happens to be that the stimulated groups of resident bacteria contain many species that are known as probiotics (lactobacillus spp; bifidobacterium spp, etc.) whereas the suppressed groups of bacteria contain many pathogenic bacteria (clostridium perfrigens group, salmonella, veillonellae etc.).

When prebiotics are fermented, they are converted into compounds that are biologically active (acetate, propionate, butyrate and lactic acid). These ingredients are absorbed into the blood and are transported to various organs (intestinal mucosa cells, liver, periferous tissue, etc.).

These particular properties of prebiotics are at the basis of eventual "health claims" in the field of

- improved bowel habit: prebiotics reduce constipation and reduce diarrhoea.

Reduction of constipation has to do with the selective stimulation of colonic fermentation. An increase in bacteria has a bulking effect, and the mild acids they produce regulate peristalsis. Reduction of diarrhoea is attributed by the induced reduction of the pathogenic species. One of the produced acids (butyrate) contributes to the healthy status of the mucosa.

- Modulated lipid metabolism: too high values of cholesterol or serum triglycerides or insulin can be reduced to more 'normal values', and hence reduction of risk for cardiovascular disease.

One of the produced acids (propionate) is transported via the blood to the liver, where it directly interacts with these mechanisms.

- Increased absorption of minerals (calcium, magnesium): reduced risk for osteoporosis.

The selective fermentation of prebiotics increases the intestinal acidity, which solubilises minerals and makes them more available for absorption.

- Modulation of the immune system: reduced risk for allergy, and increased resistance to infections.

The mechanisms would find their origin in the interaction of the bacterial cell walls of the stimulated groups of colonic bacteria with the immune system, which is very developed in the intestine.

- Anticarcinogenic properties: reduced risk for colon cancer.

Here a combination of factors are involved. The immune system, reduced DNA damage in the colon mucosa, improved functioning of DNA repair mechanisms, an increased 'apoptosis' (this 'programmed cell death' which is present in all cells, is switched off in cancer cells; this seems to be reverted partially with prebiotic fermentation), and last but not least reduced cell proliferation (cancer cells have a high proliferation rate; this risk factor is suppressed with prebiotic consumption).

Identified prebiotics to date are: inulin-type fructans (extracted from chicory roots or enzymatically synthesised from sucrose), galacto-oligosaccharides (enzymatically synthesised from dairy lactose), lactulose (isomerisation of dairy lactose). There are several candidate prebiotics: xylo-oligosaccharides, soy bean oligosaccharides, polydextrose, isomaltose, lactosucrose, etc. but they require more scientific evidence to be considered as such. To date by far most evidence related to the potential health claims is available for inulin-type fructans ("ß(2-1) fructans").

Factors that are directly related to the mentioned 5 potential health claims are supported by studies in human volunteers.

The big challenge for nutritional research in the field of prebiotics will be to directly demonstrate the health effects.

Awareness and expectations of consumers in relation to so-called functional food and gut health

B. Kettlitz

BEUC, Belgium
bke@beuc.org

As food production has become more complex, a growing number of consumers are interested in the relationship between the composition of their diet and health. As such consumers have become increasingly reliant on food labels and here in particular on the claims made about a food.

The concept of so-called functional food captures one of the most important trends shaping nutrition science, food industry strategy and product innovation today. On the border between foods and medicines, and traded on a global basis, these products raise difficult issues for consumer awareness – the ability to judge the truthfulness of the information and understanding the link between individual food and overall diet. Moreover it is a challenge for policy makers.

The important role of intestinal flora in the maintenance of health and the prevention of disease is well recognised. Foods or food supplements containing probiotic micro-organisms or prebiotic carbohydrates are those with the greatest market share and are maybe the functional foods best known by consumers.

Test results from our member organisations suggests that improvements are needed in labelling and quality assurance to ensure that the claims are true: that the products contain what they claim to do and that the information given on the label is sufficient to make informed choices. (1); (2)

(1) J. M. T. Hamilton-Miller, S. Shah and J. T. Winkler, Public Health Nutrition: 2(2), 223–229.

(2) "Souvent plus morts que vifs" Test Santé no 54, March-April 2003, 10–14.

EFCCA … and Probiotics 2004

R. Mitchell

European Federation of Crohn's and Ulcerative Colitis Associations - EFCCA, U.K.

Introduction to EFCCA: The European Federation of Crohn's and Ulcerative Colitis Association (EFCCA) is a Europe-wide umbrella organisation comprising of 20 national Crohn's and colitis patients associations and is supported by a team of eminent Medical Patrons. Raising awareness of the diseases and of the patients concerns are two roles among others of cross European patient groups. For EFCCA there is the added "taboo" surrounding bowel disease as Crohn's and colitis, also known as inflammatory bowel diseases or IBD, are often debilitating chronic diseases for which neither a cause or a cure has been found. However there has been much research world-wide and there is now a much greater understanding of IBD.

EFCCA's representative at the Sitges days is it's current Chairman, Rod Mitchell, who is a retired banker and who has been actively involved in the Crohn's and Colitis Patient movement over the last 18 years, firstly in the UK following the diagnosis of CD in his wife and for the past 8 years working also on the European level.

EFCCA also provides an IBD patient perspective at the European level to the authorities, health professionals, and industry and healthcare related organisations. It advocates on IBD patient issues as required and gives assistance to patients and doctors to form Crohn's and colitis patient groups in countries where no inflammatory bowel disease patient group exists. Mobility, cross border treatment and travelling with a long-term illness are other areas where advice is often required by the patient their family and accompanying friends.

In year 2004 we estimate that there are more than 1 million patients throughout Europe (About 1: 400) diagnosed with either Crohn's disease or colitis. Numbers are still rising especially in young people. An EFCCA Data Sheet is available showing a breakdown of the figures for 20 European countries. See also www.efcca.org

EFCCA is also actively providing the patient side perspective in various collaborative multi-centre/multi-country Research Groups in Europe such as the EC-IBD Study Group and through its membership of the European Crohns and Colitis Organisation - ECCO, which Europe-wide organisation is developing IBD related research programmes. Links have also been established with patient organisations across the world, notably in Australia and North America. EFCCA

is also a network member of the cross disease International Alliance of Patient Organisations - IAPO and Rod Mitchell is also a member of their Governing Board.

To summarise EFCCA works actively across a broad area to improve quality of life and care for patients of all ages their partners and families.

Background to interest in Research: Although founded in 1990 it is only in the second stage of its development over the past few years that EFCCA has increasingly taken an active interest in Clinical studies and research connected to the development of treatments for patients with inflammatory bowel disease – IBD, the outcomes of those clinical studies and research programmes, European licensing regulations and treatment availability issues … including the equality of access to new treatments. EFCCA is also currently concerned with the problems associated with compliance/adherence to therapies, the safety and efficacy of the treatments and more easily understood patient information. While not currently providing funding for research EFCCA is keen to encourage research both medical and social. However a number of the IBD national patient associations do make peer reviewed research grants.

Specifically … Probiotics: We are pleased that research is being undertaken in the field of Probiotics, both here in Europe and in other parts of the world. We are aware that certain of the Probiotics or food supplements containing friendly bacteria may be able to positively change the balance of the wide-range of the bacteria found in the intestine, with out being harmful to the patient, or producing the negative side effects found in many drug treatments and this is welcome news for patients. We are though aware that we shall still need to discover how the use of Probiotics may affect the use of other single or combination drug therapies in the treatment of Crohns and Colitis. Will potent drug treatments still be necessary, or will in some cases Probiotics be the sole “treatment” for some patients, especially those who may need help to maintain remission as many patients with IBD face life long undulating disease. In these days of increasingly scarce resources and limited research funding, we are delighted that the collaborative Progid studies have been made possible by the European Union funding. Should we see a positive outcome this should help lead over time to a reduction in long-term IBD healthcare costs ... with similar savings in other disease areas as other research targets different conditions.

However we must acknowledge that the patients will need to be reassured of the benefits of the Probiotic “treatment” and that we will increasingly receive questions from them within our organisations and during patient meetings and other related events which require answers.

Questions: Whilst I am sure that many of the issues surrounding the use of food supplements will be dealt with during the course of the Sitges Workshop it would be helpful if the following topics could be dealt with during the Consumer/Patient Session:

- Are Probiotics safe and what are the side effects if any?
- Will doses be similar to normal drug treatments and are they taken orally?
- When will they be made available to the Crohns/Colitis patient cross Europe other than as a patient in a study programme … how many years will I have to wait?
- Will I have to stop my existing treatments?
- How can I be helped to adhere to the long-term use of Probiotics, as I have been guilty of non-compliance when taking other treatments?
- Will they have to be prescribed by my Hospital Consultant or Primary Care (GP) Doctor or as they are described as a food supplement can I buy them "over the counter" at the Pharmacy?
- Will the same Probiotics be suitable for all patients or will they need individual tailored therapy?
- Given the wide range of Probiotics and as there will be a need for further studies of the coming years how can the patient organisations help to promote this further research?
- Patient Organisations and their patient members can be pivotal in influencing new healthcare policies, so if there are further positive results from Probiotic studies how should they in due course assist in publicising these new "treatments"?
- With the EU being increasingly involved in health policy issues how can the consumer and patient groups work to demonstrate the benefits of EU Research funding and consequently the need for increased resources at the EU level?

Consumer concerns related to probiotic and prebiotic products

G. Trigueros

Technical Department OCU (Organización de Consumidores y Usuarios), Spain

The fermented products have had an evolution are now they are considered by most of the consumers as very healthy products this is due especially to the link between the benefits of the consumption of this kind of products with alive bacteria and the gastrointestinal health. The publicity has strongly influenced this perception of the people, but in the publicity there are contradictory messages and exaggerated claims, so sometimes people are confused about prebiotics, probiotics and conventional pasteurized products all claiming beneficial effects. It is difficult for consumer even understand the differences between them, and even more difficult to assess the different effects.

The list of questions that we would like to be answered in the Industry and Consumer platform are:

- Is there any evaluation of adverse effects of these bacteria prior to the use of them in products? Is this adverse effect possible for all the people or some groups?
- Is there enough LAB in the products ready to be consumed to have the claimed effect? In some analysis made by consumer organizations this is not the case. Is any legislation about this in any EU country?
- How is the health claims legislation going to influence all this products? Are manufacturers prepared to comply with new legislation? (Especially about the demonstration of the claimed effect in the amount present in the product and in a normal diet).
- How are the strains of LAB used in this product selected? Do they use antibiotic resistance in the selection?
- What are the effects well demonstrated of probiotic bacteria (LAB) and of the prebiotics? What are the differences in effects between them?
- What are the benefits of LAB and the differences between them and specific strains like LC1, *Lactobacillus casei*, included in some commercial products and intended to have more specific benefits?
- Are there effects in health of the pasteurized products, pasteurized yogurt have some benefits of the LAB because is fermented or not?

Future prospects for gut health

W. M. De Vos

Wageningen Center for Food Sciences and Laboratory of Microbiology,
Wageningen University, The Netherlands
e-mail: willem.devos@wur.nl

Research and development in the area of gut health is rapidly expanding. This is caused by an unprecedented and unique combination of scientific, industrial and societal developments that all aim to contribute to the understanding of the mechanisms that affect gut health. These include primarily the genomics and systems biology approaches that allow for the discovery and high throughput exploitation of molecular mechanisms and biomarkers. In addition, the industrial needs for functional foods impacting gut health are shifting from a market pull to a technology push – evidently this requires mechanistic insight to strengthen new product development. Finally, in the upcoming EU legislation, claims on functional foods are increasingly based on mechanistic insight. To provide an ultimate benefit to the consumer, scientific collaboration and exchange is required between industries, governments and consumer organizations. An overview of the most salient developments will be provided with specific attention for genomic approaches that advance the understanding and exploitation of host-microbe interactions in the gut. In addition, new avenues will be discussed for increasing the momentum of gut health research within the European Union.

Research activities of LB Bulgaricum on functional dairy starters and foods

T. Stefanova

R&D Center, LB Bulgaricum Plc., Bulgaria

Research activities of LB Bulgaricum R&D Team are concentrated on the technological performance and health benefits of yogurt bacteria, thermophilic lactobacilli and bifidobacteria and consequently the application of these results for development of functional starter cultures and dairy products.

The industrial production of traditional Bulgarian yoghurt and national varieties of cheese in our country required the accumulation of extensive knowledge in the field of dairy starters and the isolation of several hundred lactic acid bacteria from home made milk products which today represent the core of company's LBB collection of LAB.

Research of the technological potential of the strains led to the development of capable symbiotic starters for Bulgarian yoghurt and traditional cheese varieties. Upgrading the assumed health benefits of yoghurt consumption, another part of the research work was performed in collaboration with medical institutions on investigating different aspects of health-promoting properties of yoghurt bacteria, especially concerning lipid metabolism, the human immune system and cancer.

Although some of the results from these clinical and animal model trials remained inconclusive, they encouraged further studies on the probiotic potential of the LBB cultures. The original collection was enriched with lactobacilli and bifidobacteria of human origin, which were identified at species and strain level by molecular methods like SDS-PAGE of total cell proteins, species-specific PCR, PFGE and AFLP.

A complex *in-vitro* and *in-vivo* characterisation of potential probiotic LBB cultures was used, based on several criteria such as survival in artificial gastric and intestinal juices, adhesion to intestinal epithelial cells, induction of cytokine production by macrophages and overall effect on the human intestinal microflora and metabolites. Additionally, screening of cultures with specific probiotic characteristics was performed, resulting in the selection of a yoghurt starter with increased beta-galactosidase activity for products recommended for people with lactose intolerance. A probiotic strain of *Bifidobacterium longum* with favourable technological properties was selected among other human origin isolates. Strains of *L. helveticus* and *L. delbrueckii* ssp. *bulgaricus* producing high levels of

antihypertensive peptides were found. A bacteriocin producing *L. delbrueckii* ssp. *lactis* strain was selected for its antimicrobial activity and applied in a cheese starter. At the final stage of the research process all cultures with proven probiotic properties were included in different forms of ready products – functional starter cultures and foods.

The further development of the process of selection of probiotic cultures and starters production at LB Bulgaricum revealed the necessity for collaboration with partners having similar interests, moreover that profound research in this area has been a priority to many European researchers under FP5 and FP6.

Studies on probiotics, prebiotics and synbiotics as functional food components

M. Bielecka

Department of Food Microbiology, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Poland
e-mail: mabiel@pan.olsztyn.pl

The range of the studies within the Department of Food Microbiology comprises: isolation of *Bifidobacterium* and *Lactobacillus* strains from their natural environments (mainly from gut of animals and humans of different age), examination of their physiological and biochemical properties, phenotypic and genotypic classification into species, capacity to resist passage through the upper part of the intestinal tract, adhesion to Caco-2 and HT29 cell lines and colon epithelium. The selected strains capable of colonising of human gut were examined for interaction with pathogenic bacteria, enhancing the immune system, influence on the ecosystem of gastrointestinal tract (microflora, activity of faecal enzymes of microbial origin, amount and proportion of SCFA in the gut contents and morphological changes of gastrointestinal epithelium). The strains were characterised for their ability to produce phytase enzyme, helpful for mineral absorption in the gut. The studies on their ability to metabolise different oligosaccharides and resistant starch constituted the basis for development of synbiotics. The effectiveness of probiotics, prebiotics and synbiotics were examined *in vivo* on healthy and *Salmonella*-challenged laboratory rats, broiler chickens and trouts. The developed synergistic sets of probiotic strains as well as synbiotics have been applied for development of new probiotic products. The interdisciplinary studies were performed in co-operation with other research groups.

Currently, the new tasks are undertaken, namely interdisciplinary studies on the interaction of gut ecosystem with antioxidants present in food of plant origin as well as on the interaction of bacteria capable of adhesion and the gut epithelial cells.

New projects concerning pre- and probiotic bacteria in the Agricultural University of Poznan

W. Grajek

Department of Biotechnology and Food Microbiology, August Cieszkowski Agricultural University of Poznan, Poland
e-mail: grajek@owl.au.poznan.pl

Our research on the prebiotics includes two projects: (1) isolation of α-oligogalactans from the seeds of pea, lupine and lentils and (2) enzymatic synthesis of oligosaccharides using hydrolases and transferases. Some other projects are focused on the lactic acid bacteria belonging to the specimens, which include probiotic strains. The main problem investigated are: (1) biosynthesis of bacteriocins by lactic and propionic acid bacteria, (2) examination of bactriocin preparations in the protection of food against pathogenic bacteria, (3) adherence of lactic acid bacteria to the solid surfaces, including human intestinal cell line models, (4) elaboration of new model of epithelial cell cultures to study anaerobic, probiotic bacteria, (5) computer image analysis of cell shape changes caused by stress culture conditions, and (6) technological aspects of production of bacteria preparates: encapsulation, membrane filtration and spray drying. We have elaborated the technology to produce α-oligogalactans from plant seeds and an antilisterial bacteriocin produced by *Carnobacterium* sp., in large scale. These preparations were positively examined for their biological activity.

CRAFT project on microencapsulation of probiotic products

J. Panès

Lallemand SAS, France

MEPPHAC QLK1-CT-2002-72376 is a Craft project on "Micro Encapsulation of Probiotics Products for Human and Animal Consumption" for the period 2003–2005. The main objective of the project is to develop a protective technology that maintains probiotics alive in final food and feed products via micro encapsulation.

Stability of probiotics in food and feed is a major challenge because of their high sensitivity to several stresses. In the animal field, probiotics have to be incorporated into pellets which require high compression force and lead to a large increase in temperature thus inducing a high mortality of probiotics.

In the human nutrition field, probiotics forms are currently limited to dairy based food, for which the limit of consumption is short, and food supplements in powder form. Their excessive sensitivity to humidity, heat and most food processing limit their development in the food industry.

This project have several partners, RTD partners as Enitiaa in France, Institut Meurice in Belgium, TNO in Netherlands, Lallemand in France and SME partners as Ordesa and Gironina in Spain, Bifodan in Denmark, Cottes and Baulez in France.

There are four main research part:

- Probiotic pre adaptation before the micro encapsulation process
- Probiotic encapsulation testing of different methods
- Incorporation of micro encapsulated probiotics in food and feed products
- Evaluation of the release of micro encapsulated probiotics in the gastro intestinal tract.

The most promising micro encapsulated products will be evaluated in industrial processing for several applications: feed pellets (Gironina and Baulez), infant milk powder (Ordesa), nutraceutics (Bifodan) and bread (Cottes).

The project is on www.probiotic-mepphac.com

Overview of the EC LABDEL project: oral delivery of vaccine and therapeutic products using non-pathogenic lactic acid bacteria

J. M. Wells[1], J. Delcour[2], P. Hols[2], H. Israelsen[3], M. Kleerebezem[4], A. Mercenier[5], P. Sizer[6], H. Tlaskalova[7] and U. Wiedermann[8]

[1]Institute of Food Research, U.K.
[2]Université Catholique de Louvain, Belgium
[3]Biotechnological Institute, Denmark
[4]Wageningen Centre for Food Sciences, the Netherlands
[5]Institut Pasteur de Lille, France
[6]Provalis plc, U.K.
[7]Institute of Microbiology, Czech Republic
[8]University of Vienna, Austria

Scientific discoveries made in previous EC funded research programs on the lactic acid bacteria (LAB) showed that there was considerable potential to develop products based on the oral delivery of vaccine and therapeutic agents using harmless commensal LAB. The aim of the LABDEL project was to harness this potential by developing prototype LAB-based products for vaccination, prevention and treatment of type I allergy and the mucosal delivery of therapeutic enzymes. This multifaceted strategy was underpinned by research on LAB fermentation and technological innovations to enhance the efficiency of LAB delivery systems.

Lactococcus lactis and *Lactobacillus plantarum* strains expressing antigens and enzymes to be delivered to the GI tract were constructed and evaluated for their vaccine or therapeutic potential in model systems. New data will be presented on the vaccination and protection from infection with *Streptococcus pneumoniae* using a recombinant LAB vaccine. Additionally LAB were shown to be promising vehicles for the prevention and treatment of type I allergy using a murine model of birch pollen allergy. Major improvements in the fermentation protocols for *L. lactis* and *L. plantarum* were achieved giving rise to higher cell densities. This is likely to have a beneficial impact on the industrial use of LAB to produce high value proteins and will ultimately contribute to the efficient production of lactic acid bacteria for product applications in GI tract delivery. New technology was established in *Lactobacillus* to screen for promoters to turn on bacterial gene expression in the gastrointestinal tract and good progress was made in constructing improved genetic systems for mucosal delivery using LAB. In this presentation an overview of the achievements of the Partner laboratories in the LABDEL project (indicated above) will be presented.

POSTERS

Application of microplate scale fluorochrome staining assay for assessment of viability and stability of probiotic *Bifidobacterium* sp.

H.-L. Alakomi, J. Mättö, A. Vaari, I. Virkajärvi and M. Saarela

VTT Biotechnology, Finland

Probiotic cultures encounter harsh conditions during production and in the GI-tract. There is a need for rapid and reliable methods predicting survival and activity of the probiotic cultures in various applications. In this study we describe a fluorescence staining assay for the determination of viability of *Bifidobacterium* cells in microplate scale. LIVE/DEAD *Bac*Light Bacterial Viability Kit (SYTO9 and propidium iodide) was utilized for viability testing of fresh and freeze-dried *Bifidobacterium* cells and the fluorescence intensity was detected by a microplate fluorometer. Validation of the microplate scale assay was performed by comparing results to colony forming units, fluorescence microscopy and spectroscopy. Fresh and freeze-dried *B. animalis* cultures treated in acidic conditions (pH 2.5 and pH 3.0 alone and in combination with pepsin) or stored at different temperatures were used to study the applicability of the microplate assay for viability assessment of stressed cells. To reveal changes in membrane functions during acid treatment, $DIBAC_4$ (a potentiometric fluorochrome) was additionally used for the analysis of the acid treated cells. In general, the results obtained with the microplate assay were comparable with plate count analysis and fluorescence microscopy. Microplate assay with viability stains gave an estimate of the viability of the probiotic preparations (detection level 10^6 cfu ml^{-1}), and the assay was applicable also for acid-treated cells. In the acid tolerance test, *B. animalis* was sensitive to pH 2.5, although pepsin had clearly protective effect in acidic conditions. Potentiometric measurements with $DiBAC_4$ fluorochrome indicate that acidic conditions caused hyperpolarization of the cell membrane in *B. animalis* cells. Benefits from the fluorochromic viability assays are that changes in cell state in probiotic preparations can be estimated earlier compared to the results obtained with traditional cultivation methods.

Crucial aspects of the application of spray drying in the production of probiotics and prebiotics containing preparation

E. Ananta, M. Volkert and D. Knorr

Berlin University of Technology, Department of Food Biotechnology and Food Process Engineering, Germany
e-mail: dietrich.knorr@tu-berlin.de

The objective of this work is to evaluate the applicability of spray drying in the production of skim milk-based preparations in combination with probiotic bacteria *Lactobacillus rhamnosus* GG (ATCC 53103). Furthermore, oligofructose-based or polydextrose-based prebiotic substances were also included in the carrier matrix to assess their protection capacity. When reconstituted skim milk was used as spray drying carrier, a microbial survival rate of 60% was achieved at an outlet temperature of 80°C. Partial substitution of the solid content of the reconstituted skim milk by prebiotic substances resulted in a high level of survivability as well. However, at lower amount of skim milk in the carrier the storage stability of the dried powder was reduced. Each constituent of the carrier medium was further calorimetrically characterized in terms of their glass transition temperatures to evaluate the contribution of glassy state in bacterial stabilization during storage. Although all evaluated carriers were in glassy state, difference was observed in their capacity of conferring protection for probiotics. Furthermore, flow cytometric assessment in combination with functional dyes was applied as a diagnostic tool to evaluate the type of cellular injuries which occurred upon spray-drying. Cell death upon drying was caused mainly by damage on cell membranes and the degree of membrane disintegration was progressively increased at higher outlet temperatures.

Comparative effects of exopolysaccharides and fructo-oligosaccharides in an infant *in vitro* colonic fermentation model with immobilized fecal microbiota

C. Cinquin[1], G. Le Blay[2], I. Fliss[1] and C. Lacroix[2]

[1]Dairy Research Centre STELA, Université Laval, Canada
[2]Laboratory of Food Biotechnology, Institute of Food Science and Nutrition, Swiss Federal Institute of Technology, ETHZ, Switzerland

It has been previously suggested that exopolysaccharides (EPS) from lactic acid bacteria may exert prebiotic effect on intestinal microbiota. The aim of this study was to compare the effects of purified EPS from *Lactobacillus rhamnosus* RW-9595M to a well-known prebiotic (fructo-oligosaccharide; FOS) in an infant continuous colonic fermentation model with immobilized fecal microbiota. Two continuous cultures were carried out to study the effect of EPS and FOS on bacterial populations and activities, for a total period of 4.5 weeks that included an initial colonization-stabilization period of approximately 14 days with a medium formulated to approximate the composition of infant chime. Infant fecal microbiota were immobilized in gel beads (2.5% gellan gum, 0.25% xanthan gum), and inoculated (30%) in a first reactor connected to two free-cell reactors in series, used to simulate proximal, transverse and distal colon conditions. The major bacterial populations were quantified with plate counts and fluorescence *in situ* hybridization (FISH), and bifidobacterial species were also estimated with FISH, using species-specific probes. Short chain fatty acids concentrations were measured by HPLC, and residual EPS concentrations were determined by an ultrafiltration method. In agreement with literature data, FOS increased both bifidobacteria and lactobacilli, and decreased clostridia concentrations. An increase in total organic acid and a decrease in ammoniac concentrations were also measured with FOS. On the other hand, EPS supplementation did not change total organic acid concentrations and was deprived of any prebiotic effect in the infant colonic model. Analyses of EPS residue and batch fermentation experiments confirmed that EPS from *Lactobacillus rhamnosus* RW-9595M was not metabolized by infant microbiota.

Three-stage chemostat with immobilized fecal microbiota used to simulate infant colon fermentation

C. Cinquin[1], G. Le Blay[2], I. Fliss[1]and C. Lacroix[2]

[1]Dairy Research Centre STELA, Université Laval, Canada
[2]Laboratory of Food Biotechnology, Institute of Food Science and Nutrition, Swiss Federal Institute of Technology, ETHZ, Switzerland

In vitro continuous culture systems with free cells have been used to model colonic fermentation. However, when steady-state is reached, the total bacterial number in liquid chemostats (10^9 CFU/ml) is generally lower than observed *in vivo*, and less competitive bacteria may be lost. To solve this problem, we develop a colonic fermentation model with immobilized cells in a three-stage chemostat. The first reactor containing immobilized bacteria is used to inoculate two free-cell reactors in series. Infant faecal microbiota were immobilized in gel beads (2.5% gellan gum, 0.25% xanthan gum), and two continuous cultures fed with a medium formulated to approximate the composition of infant chyme were done to simulate proximal, transversal and distal infant colon conditions. Bacterial composition and metabolic activity were monitored daily during 2 weeks and compared with *in vivo* data. Fluorescence *in situ* hybridization (FISH) was used to quantify effluent populations and to visualize bacterial distribution in beads at pseudo steady state. Microbial plate counts on selective media were also carried out for the main bacterial populations. After an initial colonization-stabilization period of approximately 10 days, the cell counts in beads were high and very close to that in the feces inocula. The proportions from the six bacterial marker groups in beads and effluent medium, and the metabolic activities measured in effluent samples were similar to that in faecal inocula used for immobilization and infant *in vivo* data. Our results showed that bacterial immobilization and continuous *in vitro* colonic fermentation can be used to accurately simulate intestinal fermentation over long time period, with preservation of main gut populations and activities and high stability.

Effect of growth phase and use of prebiotics for spray drying of probiotic lactobacilli

B. M. Corcoran[1,2], R. P. Ross[1,3], G. Fitzgerald[2,3] and C. Stanton[1,3]

[1]Teagasc, Dairy Products Research Centre, Moorepark, Fermoy, Ireland
[2]Department of Microbiology, University College Cork, Ireland
[3]Alimentary Pharmabiotic Centre, Ireland
e-mail: cstanton@moorepark.teagasc.ie

Spray drying of probiotic cultures can be a cost-effective means of culture stabilisation, but suffers from the disadvantage of viability reduction, primarily attributable to the negative effects of heat and dehydration encountered during the process. The aims of this study were a) to examine the effect of growth phase on probiotic culture viability during spray drying and powder storage b) to investigate the effects of inclusion of various combinations of RSM and the prebiotics polydextrose and inulin on probiotic viability during spray drying and storage and c) to investigate the variation in performance of different probiotic *Lactobacillus* strains during spray drying and storage. To examine the effect of growth phase and prebiotics on probiotic viability, *L. rhamnosus* GG was spray dried in lag, early log and stationary phases of growth in reconstituted skim milk (RSM; 20%) or a mixture of RSM (10%) and polydextrose (PD; 10%) at an outlet temperature of 85–90ºC. Stationary phase cultures survived best (31–50%) in both feed media and were the most stable during powder storage at 4–37ºC over eight weeks, with 30–140-fold reductions in cell viability at 37ºC in RSM and RSM/PD powders. Stationary phase *L. rhamnosus* GG was spray dried in the presence of inulin in a feed media consisting of RSM (10%) and inulin (10%) and survival was of the order 7.6–43%, while powders produced using PD (20%) or inulin (20%) alone as the feed media resulted in only 0.011–0.45% survival. Further substantial losses in probiotic viability (20,000- to 90,000-fold) occurred during storage of these powders for 8 weeks at 37ºC. To compare different probiotic lactobacilli during spray drying, stationary phase *L. rhamnosus* E800 and *L. salivarius* UCC 500 were spray dried in either RSM (20%) or RSM (10%) and PD (10%). Compared with *L. rhamnosus* GG, which exhibited 31–50% survival under similar conditions, *L. rhamnosus* E800 experienced approx. 25–41% survival, yielding powders containing ~10^9 $CFUg^{-1}$, while *L. salivarius* UCC 500 performed poorly, experiencing over 99% loss in viability in both feed media. Furthermore, both strains experienced higher losses (570- to 700-fold) of viability during storage at 37ºC over eight weeks compared with *L. rhamnosus* GG. In conclusion, cultures in stationary phase were most suitable for spray drying. The presence of the prebiotics polydextrose and inulin did not enhance viability during spray drying or powder storage.

Validation of a 16S rRNA probe specific for the novel intestinal mucin-degrader *Akkermansia muciniphila*

M. Derrien, K. Ben-Amor, E. E. Vaughan and W. M. de Vos

Laboratory of Microbiology, Wageningen University, the Netherlands
e-mail: muriel.derrien@wur.nl

The human gastrointestinal (GI) tract harbours a complex microbial ecosystem where relationships amongst bacteria, and between these and the host are important. It is well recognised that this microbiota plays an important role in the gut physiology and has a great influence on the health of the host. The GI tract is covered with a mucus layer made up by high molecular weight glycoproteins known as mucins, and serves as a barrier to protect the underlying intestinal epithelium from the attachment of pathogens. On the other hand, the constant availability of these host glycans provides a major growth factor allowing the colonisation of commensal intestinal microorganisms. Recently, we isolated one Gram-negative anaerobic organism from the human intestine, *Akkermansia muciniphila* (CIP 107961^{T}, ATTC BAA-835 ^{T}), which is capable of utilizing mucin as sole carbon and nitrogen source. In order to quantify and to study the spatial distribution of *A. muciniphila* in the GI tract, a specific probe based on the 16S rRNA gene sequence was designed (S-St-Muc-1437-a-A-20). The newly designed oligonucleotide probe Muc1437 was monolabeled at the 5' end with Cy5 and used for *in situ* hybridisation to 40 references strains (non-target organisms). Subsequently, the fluorescence intensity was detected using a flow cytometer and compared with signals obtained with control probes (Eub-338 and NonEub probes). Specific hybridization of the Muc1437 probe was realized at 50°C and an increased concentration of formamide. The validated probe is being used to detect and quantify *A. muciniphila* in faeces and biopsies of healthy individuals and patients with inflammatory bowel disease. Confocal microscopy will be used to unravel the interaction between this newly isolated bacteria and the host.

Screening of lactobacilli and bifidobacteria for their potential to induce TNF alpha production from human monocytic U-937 cells

Z. Dimitrov

LB-Bulgaricum, Bulgaria
e-mail: jechkoelby@yahoo.com

Since Lactic Acid Bacteria (LAB) have been proposed as potential agent that can stimulate the immune system, we used the monocytic cell line U-937, differentiated to its macrophage stage, with the purpose to estimate the cytokine induction by lactobacilli and bifidobacteria. Being one of the factors of the immunoresponse to the presence of bacteria in the medium, TNF alpha was measured by sandwich ELISA method. A total number of 80 strains were estimated in their capability to induce TNF alpha production: 20 bifidobacteria - fecal isolates; 30 *L. bulgaricus* - industrial and plant isolates; 10 *S. thermophilus*; 10 *L. helveticus,* isolated from fecal samples and plants and 10 strains from *L. acidophilus* group - fecal isolates.

For several strains the cytokine induction was twenty times above the level corresponding to added LPS from *Salmonella*. One *Bifidobacterium* and one *L. acidophilus* strain was selected as an active inducer of TNF alpha. The effect of cell viability was evaluated comparing the results between vital and high temperature killed cells. The results were contradictory. For several bifidobacteria and *L. acidophilus* strains the thermal treatment increased the immunoinduction, and the decrease for others wasn't significant. Our results confirm that LAB can stimulate nonspecific immunity.

Design and validation of 16S rRNA probes to enumerate *Clostridium difficile* and *Clostridium perfringens* species in infant faecal microflora

M. Fallani[1], L. Rigottier-Gois[1], C. A. Edwards[2], A. Collignon[3], G. Corthier[1] and J. Doré[1]

[1]Laboratoire d'Ecologie et Physiologie du Système Digestif, Institut National de la Recherche Agronomique, France
[2]Department of Human Nutrition, Glasgow University, Yorkhill Hospitals, U.K.
[3]Service de Microbiologie, Hopital Jean Verdier, France

The prevalence of allergic diseases in infants has increased in Western societies during recent decades. Differences have been suggested to occur in the composition of intestinal microflora from allergic and non-allergic children. *Clostridium difficile* and *Clostridium perfringens* are pathogenic clostridia often associated with gastrointestinal infections and allergy in infants. To enable the molecular detection and quantification of these species in the infant gut, two 16S rRNA oligonucleotide probes were developed: Cdif198 for *C. difficile* and Cperf191 for *C. perfringens*. We defined the probes *in silico* using the RDP sequence database. The probes were then validated using FISH combined with flow cytometry and a collection of target and non-target strains as well as infant's intestinal faecal samples where *C. difficile* and *C. perfringens* infections were observed. The relative probe fluorescence was determined to assess the accessibility of the probe to their target site. Both probes presented a high relative probe fluorescence with their target strains, 90% for Cdif198 and 75% for Cperf191, which showed the accessibility of these probes to their target site. These new probes will be added to a panel of 8 probes targeting the predominant bacterial groups of the infant microflora and defined according to the literature. The composition of the gut microflora will be assessed with this panel to analyse up to 1300 infant faecal samples collected from five European countries (United Kingdom, Sweden, Germany, Italy, Spain). These analyses will be included within the Allergy study and the Cross cultural study of the INFABIO project (QLRT - 2001 02606), which will give in turn a better understanding of the relationship between diet, lifestyle and infant feeding practices in Europe.

Lactobacilli strains examined by PROPATH project produce unknown antibacterial compound(s)

D. Fayol-Messaoudi[1], C. N. Berger[1], M.-H. Coconnier[1], E. Tsakalidou[2], G. Zoumpopoulou[2], G. Kalantzopoulos[2] and A. L. Servin[1]

[1]INSERM U510, Faculté de Pharmacie, Université Paris XI, France
[2]Laboratory of Dairy Research, Department of Food Science and Technology, Agricultural University of Athens, Greece

The selected lactobacilli strains used as probiotics produce antimicrobial compounds: bacteriocins, organic acids or unidentified molecules. The aim of our work was to examine antibacterial activity of lactobacilli strains (reference: GG, La1, Shirota strains or new isolates) against Gram-negative pathogens. We show a dramatic decrease in cultivable *Salmonella enterica* serovar Typhimurium in presence of cell-free culture supernatants (CFCSs) at pH4.5 (4 hours of contact give more than 2 log of decrease compared with MRS controls: MRS-HCl and MRS-lactic acid, 60 mM). When CFCSs activity was examined in presence of Dubelcco Modified Eagle's Medium, the effect was clearly distinct from the lactic acid for which activity is abolished. For all the CFCSs tested, we observe an activity against S*almonella* in exponential phase but also against other Gram-negative pathogens (*P. aeruginosa*, *E. cloacae* and *K. pneumoniae*) in stationary phase. In addition, we examine the internalization capacity of *Salmonella* in cultured human intestinal Caco-2/TC7 cells after a pretreatment by CFCSs for 1 hour (no decrease in cultivable *Salmonella* develops) or in probiotic situation (culture of lactobacilli). We show that the internalization capacity of *Salmonella* is dramatically affected by lactobacilli culture and CFCSs. This suggests that a lactobacilli antibacterial molecule(s) affect(s) the process of *Salmonella* internalization.

Assessing the effect of probiotics on the mouse gastro-intestinal microbiota

S. Fuentes[1,2], W. Akkermans - van Vliet[1], H. G. H. J. Heilig[1], A. Ruiz-Bravo[2], M. Monteoliva-Sanchez[2], H. Smidt[1] and E. E. Vaughan[1]

[1]Laboratory of Microbiology, Wageningen University, the Netherlands
[2]Department of Microbiology, Faculty of Pharmacy, University of Granada, Spain

Colonization of the mucosal surfaces of the gastrointestinal (GI) tract of mice begins at birth with a succession of microorganisms until a relatively stable microbial community is established. The purpose of this study was to use cultivation-independent approaches to characterize the effect on the intestinal microbiota of mice when treated with a probiotic strain of *Lactobacillus plantarum.* Immunocompetent and immunosuppressed female BALB/c mice of 8–10 weeks old were fed with 10^9 CFU of *L. plantarum* for 8 consecutive days. Immunosuppression was achieved by treatment with 100 mg of cyclophosphamide per kg on days 0, 3 and 6. Fecal samples were taken on days 1, 4, 7 and 9, and the last day biopsies were taken from the complete GI tract from stomach to colon. DNA was isolated and used for 16S rDNA-targeted PCR-DGGE (Denaturing Gradient Gel Electrophoresis) fingerprinting. Amplicons were obtained with universal primers for the V6–V8 region of 16S rDNA, and with *Lactobacillus* -specific primers. Fingerprint analysis revealed very diverse profiles, and dominant bands from the *Lactobacillus* community profile were identified by cloning and sequence analysis. In order to assess the biosafety of the strain used in the study, biopsies from mesenteric lymph nodes and spleen were taken to detect bacterial translocation.

Country comparisons of faecal water activity from the Crownalife baseline study

C. Gill[1], J. Doré[2], H. Zunft[3], A. Cresci[4], E. Norin[5] and I. Rowland[6]

[1]Univ. Ulster, U.K.
[2]INRA, France
[3]DifE, Germany
[4]Univ. Camerino, Italy
[5]Karolinska Institute, Sweden
[6]Univ. Coleraine, Italy

One third of the European population will be over 60 by year 2025. Colorectal cancer (CRC) is the second most common cancer within Europe, affecting 6% of men and women by age 75. The crownalife project aims at assessing age-related alterations and exploring strategies to restore and maintain a balanced healthy intestinal environment. Faecal water (FW), the aqueous phase of human faeces is an important source of modulators and inducers for colorectal carcinogenesis. Thus FW risk biomarkers (*in vitro* cell based) represent a range of key stages in the CRC pathway including hyperproliferation via cytotoxicity (MTT assay using HT29 cells) and metastasis (matrigel invasion assay using HT115 cells). The currently blinded baseline study comprises four European countries (A, B, C, D), three groups (n=20 p.g.): two elderly (>65) and one young (25–45). Mean FW cytotoxicity (% cell death) in B (46.8 +/- 38.2) was significantly greater than D (27.6 +/- 28.1) (ANOVA, LSD P=0.013). FW inhibited % invasion rates significantly less in B + C (7.19 +/- 9.31, 8.49 +/- 13.14) than A (2.11 +/- 2.4) (ANOVA, LSD p=0.02). Consistently B is associated with increased risk, this may be associated with diet but such analysis can only occur once the blind is removed.

Country-specific dietary intake plays a major role on short-chain fatty acid production in the gut – results of a cross-sectional study in four European countries

C. Hanisch[1], S. Müller[2], A. Lork[1], S. Silvi[3], C. M. Verdenelli[3], C. Orpianesi[3], A. Cresci[3], K. Saunier[4], J. Doré[4], E. Norin[5], M. Blaut[2] and H.-J. F. Zunft[1]

[1]German Institute of Human Nutrition, Department of Intervention Studies
[2]Department of Gastrointestinal Microbiology, Germany
[3]Department of Comparative Morphology and Biochemistry, University of Camerino, Italy
[4]Unit of Ecology and Physiology of the Digestive System, Institut National de la Recherche Agronomique (INRA), France
[5]Microbiology and Tumor Biology Center, Karolinska Institute, Sweden

Short-chain fatty acids (SCFA) produced in the human large intestine are thought to play an important role in the prevention of colon cancer.

The majority of SCFA is derived from carbohydrate fermentation and protein breakdown by human gut bacteria. The amount of SCFA produced is mainly determined by the composition of the diet, gut transit time and bacterial species being present in the gut microbiota.

In a cross-sectional European human study the relation between diet and faecal SCFA, microbial composition, secondary bile acids and selected markers of tumour risk has been examined. A total of 160 healthy elderly aged > 65 years and 80 adults (25–45 years) were recruited in four different countries across Europe. Volunteers reported their dietary intake in a 3-day food record and provided a fresh faecal sample for the analysis of microbial and enzymatic parameters.

First results show significant differences in the SCFA concentration among the four countries and between age groups. Looking at the dietary intake of study participants significant differences were observed in dietary fibre intake especially in soluble and insoluble fibre intake among the countries. A significant positive correlation between soluble fibre intake and faecal SCFA concentrations could be demonstrated.

Involvement of bacteria in pathogenesis of inflammatory bowel disease (IBD)

P. B. Heczko[1], M. Strus[1], H. Uhlig[2], F. Powrie[2], E. H. Hörnquist[3] and P. Bland[4]

[1]Jagiellonian University Medical College, Poland
[2]Sir William Dunn School of Pathology, University of Oxford, U.K.
[3]Department of Medical Microbiology and Immunology, Göteborg University, Sweden
[4]Division of Molecular and Cellular Biology, University of Bristol, U.K.

We have used two animal models of IBD: SCID mice receiving $CD4^+$, $CD45RB^{hi}$ T cells and showing symptoms of chronic bowel inflammation in about 8 weeks after transfer and Gαi2 mutant mice spontaneously developing colitis. The whole gastointestinal tract of the animals and controls was removed during necropsy, ligated on both ends, immersed in a special transport medium and transported in dry ice for microbiological analysis. After thawing, the tract was opened, content removed and 5 different samples of mucosa taken from stomach, proximal and distal ileum, colon and caecum. Samples of both content and mucosa were cultured on a variety of media used for cultivation of a aerobic and anaerobic bacteria and yeasts. Isolates were identified at species level using both phenotypic methods and species-specific PCR. Localisation of both cultivable and non-cultivable members of the colon flora in relation to mucosa was studied using immunochemical techniques and FISH. The *in vitro* interactions of selected bacteria isolated from mice with antigen presenting cells were also tested using different cytokines assays.

No organisms reported previously as specifically related to experimental IBD in mice such as *Bacteroides vulgatus, Helicobacter hepaticus* or other *Helicobacter* species were found. Moreover, no growth of *Campylobacter* and sulphur-reducing bacteria was recorded. The bacterial flora of the colon content and mucosa samples was composed of Gram- rods, mostly *Escherichia coli, Enterobacter agglomerans, Citrobacter* sp. and *Klebsiella* sp., Gram+ cocci: *Enterococcus faecalis,* different species of *Staphylococcus* and *Aerococcus viridans,* and lactic acid bacteria.

Colon contents of colitic Gαi2 mice but also T cell transfer SCID mice showed significantly higher numbers of Gram- rods than those of control animals. These differences were not found in stomach and proximal ileum samples. The numbers of Gram+ cocci in samples of the IBD mice were, with the exception of colon, and proximal ileum, lower than in controls. On the other hand, populations of lactobacilli adherent to colon mucosa were significantly higher in mice of both models with IBD. Microbial population in control animals were localised almoust exclusively on mucosal layer surfaces while these in colitic mice were displaced to reach a close proximity to colon mucosa.

Genomic characterization of the *Escherichia coli* probiotic strain O83:K24:H31

J. Hejnova[1,2], U. Dobrindt[3], P. Sebo[2], R. Nemcova[4], A. Bomba[4], P. Glaser[1] and C. Buchrieser[1]

[1]Laboratoire de Génomique des Microorganismes Pathogènes, Institut Pasteur, France
[2]Laboratory of Molecular Biology of Bacterial Pathogens, Czech Academy of Sciences, Czech Republic
[3]Institüt für Molekulare Infektionsbiologie, Universität Würzburg, Germnay
[4]Research Institute of Veterinary Medicine, Slovakia
jana@pasteur.fr, hejnova@biomed.cas.cz

Colonization of preterm and newborn infants by the *Escherichia coli* strain A0 34/86 (serotype O83:K24:H31) has proven safe and efficient in prophylaxis and treatment of nosocomial diarrhea in Czech pediatric clinics over the past three decades. In search for traits underlying the probiotic capacity of the A0 34/86 strain, we have started to characterize its genome. 410 ORFs of *E. coli* MG1655 (K12) were undetectable in A0 34/86 by hybridization to DNA arrays, while 72 out of 456 probed pathogenicity island-associated genes of *E. coli* and *Shigella* were present also in the A0 34/86 strain. Further ExPEC-related genes involved in iron uptake, capsule synthesis and adhesion were detected in by multiplex PCR and the presence of uropathogenic *E. coli* 536 pathogenicity island II and of genes for the HlyA and CNF toxins was revealed by end-sequencing of 1152 shotgun and of 593 BAC clones derived from A0 34/86. Gene clusters for adhesion (fim), invasion (ibe), gluconate and mannonate metabolism and restriction/modification (tl) functions were isolated on a BAC clone that reproducibly conferred to a laboratory strain an enhanced capacity to persist in the intestine of newborn piglets. Given the established clinical safety record of the *E. coli* A0 34/86, these results highlight the thin line between 'virulence' and 'fitness' factors.

The detected enterobacterial 'virulence-associated' genes may, indeed, enable this probotic strain to colonize human and animal hosts and to outcompete intestinal pathogens.

Highly specific β-galactosidase from *Bifidobacterium adolescentis* DSM 20083

S. W. A. Hinz, L. A. M. van den Broek, G. Beldman, J.-P. Vincken and A. G. J. Voragen

Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, the Netherlands

Bifidobacteria play an important role in carbohydrate fermentation in the colon. Carbohydrates can be modified to low molecular weight oligosaccharides or monosaccharides by using a wide range of depolymerizing enzymes. An objective of our research is to prepare prebiotics, which could give bifidobacteria a selective advantage. The glycosidases present in bifidobacteria are of interest to us, particularly those capable of elongating oligosaccharides, i.e. those that can catalyze transglycosylation reactions. Using enzymes from bifidobacteria itself for generating prebiotics will ensure that these carbohydrates are indeed degraded. As found in literature[1,2,3] galacto-oligosaccharides seem to have a large prebiotic potential. So far, not much is known about the enzymic machinery for galactose utilization. In this research the galactan and galacto-oligosaccharide degradation mechanisms will be investigated. Knowledge on these mechanisms will help improving prebiotic ingredients.

A galacto-oligosaccharide-degrading enzyme from *Bifidobacterium adolescentis* DSM20083, namely a β-galactosidase, was cloned into a pBluescript SK(-) vector system and transformed into *E. coli* XL1 blue MRF' cells. The enzyme is found intracellularly. The β-galactosidase was purified from the cell extract with anion-exchange chromatography and gel permeation chromatography. The physico-chemical properties and substrate specificity of both enzymes were determined. It appears that the enzyme is more specific to certain substrates then other β-galactosidases.

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Characterisation of β-galactosidase activity in different *Bifidobacterium* spp.

K. Holmstrøm, T. Møller and F. Jørgensen

Biotechnological Institute, Department of Molecular Characterisation, Denmark

Bifidobacteria are considered to constitute a benign and functionally important fraction of the human GI microbiota, and factors that sustain or promote the proliferation and persistence of bifidobacteria are therefore desirable. Non-digestible carbohydrates like galactooligosaccharides have been implicated to present such bifidogenic factors and one of the key-enzymes in bifidobacteria responsible for metabolising these bifidogenic carbohydrates is β-galactosidase. The fully sequenced *Bifidobacterium longum* NCC2705 strain[1] contains four genes that encode putative β-galactosidases (*lacZ, bga, bgaB, yvfO*). *B. bifidum* has been reported to contain at least three different presumed β-galactosidases (BIF1, BIF2, and BIF3)[2], and in *B. infantis* again three genes encoding β-galactosidase in two different strains have been identified; INF1 in *B. infantis* DSM20215[2] and β-*galI* and β-*galIII* in *B. infantis* HL96[3].

In order to decipher regulatory patterns of β-galactosidase expression in different types of bifidobacteria we have employed a number of different assays including single-cell-based and population-level-based monitoring of β-galactosidase activity as well as DNA-micro array-based gene expression analyses. Examples of different regulation of β-galactosidase activity in different bifidobacteria will be presented.

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An infant formula containing prebiotics changes the intestinal microflora of term infants

J. Knol, E. G. M. van der Linde, J. C. K. Wells and H. M. Böckler

Numico Research B.V., the Netherlands

An infant formula with prebiotic fructo- and galactooligosaccharides (FOS/GOS) and hydrolysed whey proteins (PBF) was compared with a standard formula (SF) for the effect on the intestinal flora. The microflora was determined using fluorescence *in situ* hybridisation (FISH) with 11 probes. Previous results showed a significant increase in the percentage of Bifidobacteria in the PBF group (Arch. Dis. Child. 2001, 84(S):A22). The present analysis is focussed on other important groups of bacteria.

Within 0–14 days after birth at term, healthy, bottle-fed infants were enrolled to receive either PBF or SF. Faecal samples were taken at inclusion and at the age of 6 weeks. Using specific probes the following groups where determined; *Bifidobacterium* (Bif164), *Escherichia coli* (Ec1531), *Bacteroides* (Bdis656/Bfra602), *Clostridium* (Chis150/Clit135), *Eubacterium rectale* group (Erec482), *Streptococcus* (Strc493), *Atopobium* (Ato291), *Coriobacterium* (Cor653) and *Lactobacillus/Enterococcus* (Lab158).

Enumeration of the faecal microflora using FISH shows significant changes in the microflora. In parallel to the increase in Bifidobacteria the proportion of Clostridia, *E. coli* and Eubacteria is reduced in the group fed PBF.

Infant formula		Proportion of total bacteria (%)								
		Bif164	Ato291	Cor653	Ec1531	Bdis656/ Bfra602	Chis150/ Clit135	Erec482	Strc493	Lab158
SF	0–2 wks	32.3	5.80	24.5	14.4	2.37	7.67	7.27	17.9	6.04
	6 wks	33.9	12.4*	35.7*	7.00	3.71	8.54	15.3*	10.8*	9.13
PBF	0–2 wks	36.2	2.53	17.8	7.08	3.66	3.75#	0.45#	21.8	6.12
	6 wks	68.7*@	3.85	25.2	1.62*@	2.11	2.30@	3.81*@	14.0*	7.95

*p<0.05 6wks vs. 0–2wks #p<0.05 PBF vs. SF @p<0.05 PBF vs. SF

In the PBF group the ratio of Bifidobacteria to Clostridia, which has been reported to be related to atopic diseases, is shifted into the direction of non-atopic infants (9.7 vs. 29.9, $p<0.05$).

An infant formula containing prebiotic oligosaccharides (FOS/GOS) alters the microflora of bottle fed infants by reducing the levels of potential pathogenic micro-organisms.

Prebiotic evaluation in an *in vitro* three-stage model of the human gut using the prebiotic index equation

S. Kolida, D. M. A. Saulnier and G. R. Gibson

Food Microbial Sciences Unit, School of Food Biosciences, The University of Reading, U.K.

The *in vitro* fermentation properties of a range of commercially available prebiotics were compared and their prebiotic performance was ranked using the prebiotic index (PI) equation.

Prebiotics were tested in the gut model, an *in vitro* three-stage continuous culture system, which simulates the pH and transit conditions in the proximal, transverse and distal regions of the human colon. The system was run continuously until steady state was achieved. Initial and steady state populations of bifidobacteria, bacteroides, lactobacilli and clostridia were monitored using fluorescent *in situ* hybridisation (FISH). The PI equation accounting for the changes in the above-mentioned bacterial species was employed to rank the performance of the test prebiotics. The PI equation was based on the assumption that an increase in bifidobacteria and/or lactobacilli was positive while an increase in bacteroides and/or clostridia was negative for host health. High PI scores signified good prebiotic performances as opposed to low or negative scores.

In vessels one and two the top PI ranking prebiotics were oligofructose and polydextrose. In vessel three the top ranking prebiotics were inulin and polydextrose. Amongst the worst performing prebiotics under all fermentation conditions were lactitol, maltodextrin and the blend of lactitol + maltodextrin (1:1).

Inulin persisted in vessel 3 indicating potential *in vivo* persistence in distal regions of the colon as opposed to oligofructose, which was mainly fermented in vessels two and three.

Intestinal health: Prevention of genotoxicity induced by human intestinal microbiota in cultured enterocytes

C. Krul, M. van Nuenen, J. van de Sandt and K. Venema

TNO Nutrition and Food Research, the Netherlands
e-mail: venema@voeding.tno.nl

The large intestine contains numerous mutagenic and carcinogenic metabolites, and research has revealed that the human colonic microbiota probably plays a major role in colon cancer. It has for instance been demonstrated that the microbiota contains enzymatic activities that convert procarcinogens into carcinogens. However, the genotoxic activity of the human microbiota and the putative preventive effects of food components on colon cancer are largely unknown.

An *in vitro* method was used to investigate the genotoxicity of the large intestinal content and the effects of food components and its metabolites on this activity. A microbiota of human origin, with a physiological density of microorganisms, was maintained in the TNO *in vitro* model of the large intestine (TIM-2), which closely simulates the colonic environment.

The genotoxicity of the large intestinal content was evaluated in the Comet assay. Caco-2 and HT29 cells were exposed for 24 hours to samples taken from the lumen and dialysis fluid of TIM-2. After exposure, cells were harvested and cytotoxicity was determined by trypan blue exclusion. Comets were quantified with an image analysis system and DNA damage was expressed as mean tail moment of at least 300 cells.

This poster will report the results of the effect of quercetin and its microbial metabolites on the genotoxicity in these cultured intestinal cell lines.

A new genus *Anaerofustis stercorihominis* from human faeces

P. A. Lawson[1], R. Hutson[1], Y. Song[2], C. Liu[2] and S. M. Finegold[3]

[1]School of Food Biosciences, University of Reading, Reading, U.K.
[2]Infectious Diseases Section, VA Medical Center West Los Angeles, USA
[3]Department of Medicine, UCLA School of Medicine, USA

During the course of a systematic search for new species diversity within the human gut, we have characterised an unidentified gram-positive, strictly anaerobic, non-spore-forming, rod-shaped bacterium isolated from human faeces which does not correspond to any currently defined taxon. The organism was catalase-negative, resistant to 20% bile, produced acetic and butyric acids as end-products of glucose metabolism, and possessed a G +C content of approximately 72 mol%. Comparative 16S rRNA gene sequencing demonstrated that the unidentified bacterium was a member of the *Clostridium* sub-phylum of the gram-positive bacteria, and formed a loose association with *Clostridium* rRNA cluster XV. Sequence divergence values of 12% or greater were observed between the unidentified bacterium and all other recognized species within this and related rRNA clusters. Treeing analysis showed the unknown anaerobe formed a deep line branching near to the base of rRNA cluster XV and phylogenetically represents a hitherto unknown taxon, distinct from *Acetobacterium*, *Eubacterium sensu stricto*, *Pseudoramibacter* and other related organisms. Based on both phylogenetic and phenotypic evidence, it is proposed that the unknown bacterium from faeces be classified in a new genus *Anaerofustis,* as *Anaerofustis stercorihominis* sp. nov.

Assessment of human faecal microbiota composition using FISH combined with flow cytometry, Pan-European comparison

C. Lay[1], L. Rigottier-Gois, K. Holmstrøm, M. Rajilić, E. Vaughan, M. D. Collins, R. Thiel, P. Namsolleck, M. Blaut and J. Doré

[1]INRA, UEPSD, France

The capacity to enumerate the microbiota represents a first step in understanding molecular contributions of the gut ecosystem to human physiology and health. For the first time, a large scale analysis has been carried out. Faecal samples were obtained from 91 healthy humans (7 to 52 years old) from five European countries (France, Denmark, Germany, United Kingdom, Netherlands) to provide a Pan-European geographic description of the composition of the faecal microbiota. Fluorescent *in situ* hybridization (FISH) combined with flow cytometry, a high throughput method, was applied to analyse the bacterial composition of the faecal microbiota using a panel of 18 phylogenetic probes. Furthermore, all information concerning the samples (e.g. geographic origin, age, sex) were subject to comparative analysis by principal component analyses (PCA). The results showed that on average more than 75% (75.7%±18.6%) of the bacterial cells were detected with the set of group and species-specific probes. In spite of large inter-individual variations, *Clostridium coccoides* group and *Clostridium leptum* subgroup were the main dominant groups (28.0%±11.3% and 25.2%±7.2%, respectively), followed by the *Bacteroides* group (8.5%±7.1%). According to PCA analyses, no significant grouping with respect to age or gender was observed for each country considered individually and no grouping with respect to geographic origin was observed when five countries were considered. On the basis of the distribution of major dominant groups, the gut microbiota of healthy adults is comparable throughout the European countries investigated.

Screening of probiotic strains isolated from the elderly for antimicrobial activity against gastrointestinal pathogens

E. Likotrafiti, K. Manderson, K. M. Tuohy, G. R. Gibson and R. A. Rastall

Food Microbial Sciences Unit, School of Food Biosciences, The University of Reading, U.K.

The human gut microflora changes in old age both in species diversity and relative bacterial numbers. This may lead to a reduction in the colonization resistance afforded by the gut microflora towards ingested pathogens and may account for the increased gastrointestinal infection and disease severity in the elderly. 109 lactic acid bacterial strains (56 bifidobacteria and 53 lactobacilli) were isolated from the faeces of healthy elderly (> 65 years) individuals. Isolates were identified to species level by phenotypic analysis (by API) and by 16S rDNA sequencing. The most frequently isolated lactobacillus was *L. fermentum* and the most frequently isolated bifidobacteria were closely related to *B. infantis* by 16S rDNA sequence alignment. Isolates were characterised for their antimicrobial activity against *Clostridium dificile*, enteropathogenic *Escherichia coli* (EPEC), verotoxgenic *E. coli* (VTEC) and *Campylobacter jejuni* using a deferred diffusion assay. The lactobacilli displayed variations in their antimicrobial activity with few strains showing inhibitory activity against all pathogens. The bifidobacteria displayed higher levels of inhibitory activity against *C. jejuni* and *C. difficile* than against the *E. coli* strains. Importantly, certain strains showed a broad spectrum of anti-pathogen activity, inhibiting all four pathogens. Conversely, different strains of the same probiotic species showed different spectra of anti-pathogenicity.

Antimicrobial potential of probiotic or potentially probiotic lactic acid bacteria

L. Makras, I. Nes, H. Holo, Q. Yi, A. Servin, D. Fayol-Messaoudi, C. Berger, G. Zoumpopoulou, E. Tsakalidou, D. Sgouras, B. Martinez-Gonzales, E. Panayotopoulou, A. Mentis, D. Smarandache, L. Savu, P. Thonart and L. De Vuyst

PROPATH, c/o Research Group of Industrial Microbiology, Fermentation Technology and Downstream Processing (IMDO), Department of Applied Biological Sciences, Vrije Universiteit Brussel (VUB), Belgium
e-mail: ldvuyst@vub.ac.be. http://proeuhealth.vtt.fi

The EU-funded PROPATH project addresses the important health issue of prevention of gastrointestinal disorders through pro- and prebiotics. Seven European laboratories are co-operating in this project, which aims to isolate and characterise the responsible antimicrobials to combat Gram-negative bacteria including *Helicobacter pylori* and *Salmonella enterica* serovar Typhimurium. The first results on the screening for probiotic or potentially probiotic lactobacilli that exhibit antimicrobial activity towards these Gram-negative pathogenic bacteria are presented. Spot-on-lawn assays, well-diffusion assays and time-kill studies were performed among the lactic acid bacterium strains that were either collected from fermented foods and faeces (breast-feed babies, infants, and animals) or isolated from commercial products to investigate if and which of the collected strains were inhibiting growth or were killing certain indicator bacteria. Strains inhibiting the gastrointestinal pathogens mentioned above were found. Evidence has been shown that compounds different from organic acids are produced.

The potential of seven probiotics isolated from elderly individuals for use as an antipathogenic agent

K. Manderson, K. Tuohy, R. Rastall and G. Gibson

Food Microbial Sciences Unit, School of Food Biosciences, The University of Reading, U.K.

Three lactobacilli and four bifidobacteria isolated from healthy elderly Europeans were screened for their suitability as probiotics specifically targeting the elderly, which have an increased risk of *Clostridium difficile* infection. All seven strains of bacteria were capable of inhibiting the growth of *C. difficile* in a deferred cross streak inhibition assay. The mechanism of pathogen inhibition was further investigated using a liquid inhibition test and in mixed culture, to determine inhibitory activity within a competitive environment. The probiotics were determined to be bile resistant up to a level of 0.4% oxgall for 11 hours and were stable in a simulated stomach acid environment at pH 3 for 20 minutes. However at pH 2, their survival was limited. The probiotics all showed a certain level of antibiotic resistance, including resistance to vancomycin and metronidazole, two of the commonly used antibiotics in the treatment of *C. difficile* gastroenteritis. Further, the ability of the probiotic strains to attach to cultured human cell lines (HT29 and CACO-2) was investigated. There was great variation in ability of the probiotic strains to attach to the cell lines with the lactobacilli showing greater attachment, and bifidobacteria being less likely to adhere to both cell lines.

Qualitative analysis of *Bifidobacterium* strains originating from probiotic products following a culture-dependent and culture-independent approach

L. Masco[1], G. Huys[1], R. Temmerman[1] and J. Swings[1,2]

[1]Laboratory of Microbiology and
[2] BCCM™/LMG Bacteria Collection, Ghent University, Belgium.
e-mail: Liesbeth.Masco@UGent.be, http://lmg.UGent.be

From the point of view of consumers and consumer organizations, clarity is lacking regarding the quality and the label correctness of commercial probiotic products. Previous analyses have demonstrated that the recovery of the incorporated probiotic organisms is often poor and that more attention should be paid to the identity, safety and functionality of these strains. Next to the genus *Lactobacillus*, bifidobacteria are the second most applied group of lactic acid bacteria as human probiotics including the species *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum*.

The aim of the present study was to analyse probiotic products claiming to contain bifidobacteria through the use of a culture dependent as well as a culture independent methodology. For the culture dependent approach, three selective isolation media were evaluated for their ability to sustain growth of all probiotic *Bifidobacterium* species as well as to inhibit growth of yoghurt starter cultures and probiotic non-bifidobacteria. Subsequently, isolations were performed on 58 probiotic 'bifidus' products using the most suitable medium. A total number of 679 isolates were collected of which 442 were identified as *Bifidobacterium.* The resulting bifidobacterial isolates were then characterized at the species level using BOX-PCR fingerprinting and additionally characterized at the strain level by means of Pulsed-Field Gel Electrophoresis (PFGE). The most frequently found species was *B. animalis* subsp. *lactis* (74%), but a number of isolates were also identified as *B. bifidum*, *B. longum*, *B. infantis* and *B. breve*. In parallel, the same set of probiotic products was also subjected to culture-independent analysis using (DGGE) analysis of 16S rDNA amplicons. This approach allows an accurate qualitative analysis of any probiotic sample up to the species level provided that an identification database is available. In addition it is a fast, reliable and reproducible approach that compensates some shortcomings associated with conventional culture dependent analysis.

In conclusion, both approaches seem to confirm that a rather high percentage of probiotic products suffer from incorrect labelling and low bacterial numbers, which are likely to affect the claimed health benefits.

Diversity and abundance of *Desulfovibrionaceae* related populations in feces and saliva as detected by group-specific PCR-DGGE and FISH

J. Maukonen, J. Mättö and M. Saarela

VTT Biotechnology, Finland

Keywords: *Desulfovibrionaceae*, PCR-DGGE, FISH

Desulfovibrio species belong to sulfate-reducing bacteria (SRB), which constitute a diverse group of prokaryotes that contribute to a variety of essential functions in many anaerobic environments. However, some *Desulfovibrio* spp. cause infrequently a variety of human infections - for example they have been postulated to have a role in ulcerative colitis. Detailed analysis of occurrence and abundance of SRB within complex microbial communities has formerly been restricted to information obtained by conventional culture techniques and has been biased by the inability to cultivate most of the organisms. However, the application of molecular methods enables a direct visualization of bacterial diversity, monitoring and enumeration of the dominant population, and the opportunity for subsequent identification of community members by sequence analysis. The aim of the present study was to investigate the diversity of *Desulfovibrionaceae* populations in feces of IBS (irritable bowel syndrome) and control subjects. Moreover, the changes in stability of fecal *Desulfovibrionaceae* populations of 5 control subjects were followed over a period of 2 years including a period of probiotic ingestion. Furthermore, the similarity and stability of the fecal and salivary *Desulfovibrionaceae* populations in these subjects was monitored and compared. Fecal samples of 21 IBS patients and 18 control subjects in addition to fecal and salivary samples (3 samples from each subject before and during the feeding trial) of 5 control subjects were analyzed with PCR-DGGE (denaturing gradient gel electrophoresis). According to the DGGE results there were more bands and more variability in the DGGE profiles of IBS patients than in the DGGE profiles of control subjects. Furthermore, differences in intensities as detected by gel electrophoresis after group-specific PCR were considerably large. Therefore, FISH (fluorescent *in situ* hybridization) was used to quantify the number of sulfate-reducing bacteria and the number of *Desulfovibrio* spp. in fecal and salivary samples.

Acacia gum based mixes modify cardiovascular disease biomarkers: a randomised double blind study

S. Meance[1], M. Kondakova[2] and D. Pogogeva[2]

[1]Nutrition and Regulatory Affairs, France
[2]Institute of Nutrition RAMS, Russia

Soluble fibre and especially viscous ones may help preventing cardiovascular disease (CVD). First objective was to test the efficacy of the highly fermentable water-soluble fibre with a low viscosity, Acacia Gum (AG) for modulating validated biomarkers of CVD. Second objective was to test if a mix with increased viscosity (AGvisc) had better results. A randomised double-blind study with 3 parallel groups involved 48 adults with high cholesterol level. After a baseline period, the subjects consumed during 7 weeks either a placebo, AG or AGvisc at the dose of 15g/d added to a low calory diet. Blood pressure, fasting total cholesterol (TC), LDL, HDL, VLDL, triglycerides (TG), glycemia and anthropometric measures were analysed after 7 weeks. In the three groups there was a decrease in the TC level and blood pressure compared to initial values. Compared to the placebo, AG slightly but not significantly reduced TC whereas the effect of AGvisc was statistically significant. Under an energy restricted diet, AGvisc could help preventing CVD. The effect is probably due to the viscosity of the fibre associated with the end products of the fermentation.

Assessment of the gut microflora composition in adults and elderly people for Germany and Sweden

S. Müller[1], C. Hanisch[2], K. Saunier[3], L. Rigottier-Gois[3], J. Doré[3], L. Norin[4], H. J. F. Zunft[2] and M. Blaut[1]

[1]Department of Gastrointestinal Microbiology and
[2]Intervention Studies, German Institut of Human Nutrition, Germany
[3]Unité d'Ecologie et de Physiologie du Système Digestif, Institut National de la Recherche Agronomique, France
[4]Microbiology and Tumor Biology Center, Karolinska Institute, Sweden

Within the EU Project CROWNALIFE a Baseline human study was undertaken in Germany and Sweden to assess differences in fecal microflora composition in elderly people (> 65 years) in comparison with young adults (aged 20–45 years). Two groups of 20 elderly people were included in comparison with one group of 20 adults for each country. The composition of the fecal microbiota was analysed by fluorescence *in situ* hybridisation (FISH) coupled with flow cytometry using 15 phylogenetic probes. Relative proportions and prevalence were compared for each phylogenetic group. The additivity of probe signals was also assessed.

Results confirmed the presence of *Clostridium coccoides, Clostridium leptum* and *Bacteroides* as predominant groups. These groups further showed a high prevalence in all age groups for the two European countries. The data also revealed a wide range of additivities. This observation was indicative of large interindividual variations and suggested a high percentage of so far uncultured microorganisms in some individuals. The additivity was higher for country I [age group A (n=20): 66.9 ± 20.0; age group B (n=19): 71.1 ± 28.0; age group C (n=20): 66.8 ± 28.7] whereas it showed large variations between age groups for country III [age group A (n=18): 58.9 ± 17.8; age group B (n=19): 60.2 ± 23.5; age group C (n=22): 42.4 ± 23.8].

Age-related effects will be further investigated after unblinding of the study. Statistical analyses will give us explicit information on the influence of dietary habits in the four participating countries of the EU Project CROWNALIFE: France, Germany, Italy and Sweden.

Composition of bifidobacterial population in irritable bowel syndrome and control subjects

J. Mättö and M. Saarela

VTT Biotechnology, Finland

Irritable bowel syndrome (IBS) is a chronic intestinal disorder that may involve an altered intestinal microbiota. Although in some studies lower numbers of bifidobacteria have been detected in IBS subjects compared to healthy controls, the composition of bifidobacterial population in IBS subjects is poorly known. The aim of the present study was to investigate the diversity and stability of faecal bifidobacterial population in IBS and control subjects. Faecal samples were obtained from 19 IBS and 16 control subjects at three sampling points (0, 3 and 6 months) and analysed by PCR-DGGE with *Bifidobacterium*-specific primers Bif164-f and Bif662-GC-r. In addition fresh samples from two sampling occasions were cultured on Beerens to determine the bifidobacterial counts. PCR-DGGE analysis revealed 1–7 fragments per sample. No difference in the bifidobacterial count or in the diversity or stability of bifidobacterial population in general was observed between IBS and control groups. However, a single fragment, identified by 16S rDNA sequencing tentatively as uncultured *Bifidobacterium*, was more commonly detected in IBS than in control subjects. The identity and role of this fragment in IBS warrants further studies.

Percoll™ gradient DGGE – a novel, two dimensional approach to study microbial community

P. Namsolleck and M. Blaut

Department of Gastrointestinal Microbiology, German Institute of Human Nutrition, Germany

Denaturing Gradient Gel Electrophoresis (DGGE) is a molecular tool used to analyse the diversity of complex bacterial ecosystems such as the human gastrointestinal tract. A major limitation of this method is related to the fact that only dominant components of the gut microbiota result in a band, while a large proportion of gut micro organisms not result in a band (e.g. up to 38 bands can be visualised for the approximately 400 species found in average faecal sample). Therefore, we developed a two dimensional, high resolution method to overcome this limitation. In the first dimension the bacterial cells are separated by centrifugation in a density gradient (Percoll™). Different density fractions are collected. In the second dimension fractions are analysed by PCR-DGGE. The usefulness of this method was demonstrated by analysing the microbial profiles of faecal samples. Patterns obtained after density separation were more diverse than those obtained with standard DGGE. Many bands were present in only one or two fractions, whereas some bands were found in all fractions. Using this strategy, we were able to detect several species (such as members of the sulphate reducing bacteria) in faecal samples, which could not be detected with the standard DGGE approach.

This method can be used for a more detailed analysis of diverse microbial ecosystems and improve the understanding of their complexity.

Probiotic potencial of breast milk strains in humans volunteers

M. Olivares[1], M. P. Díaz-Ropero[1], N. Gómez-Bastidas[1], R. Martin[1,2], J. M. Rodríguez[2], and J. Xaus[1]

[1]Dept. of Immunology, Puleva Biotech S.A., Spain
[2]Dept. of Nutrition, Bromatology and Food Technology Complutense University, Spain
e-mail: jxaus@pulevabiotech.es

Keywords: probiotic, gut microflora, human milk, infant nutrition.

Breast milk is a complete food that protects the newborn against infectious diseases. In a previous work, we showed that human milk contains lactic acid bacteria (LAB) strains. A number of LAB were isolated from this biological fluid and from other sources but with the ability to be transferred to the breast milk after oral intake. In this work, we show that a fermented dairy product that exclusively contained two of these strains (*L. coryniformis* CECT5711 and *L. gasseri* CECT5714) exerted beneficial effects on healthy human volunteers and compare the results with those obtained with a regular yoghurt. The ingestion of the probiotic product led to an increase in the number of faecal lactobacilli and to a reduction in the enterobacteriae counts during the treatment period. Such effects were not observed in the yoghurt group. In addition, significant differences were detected between both groups, regarding parameters such as faecal water content, faecal enzymatic activities, butyrate production, phagocytic activity of blood cells or NK cell counts. Thus, our results clearly indicate that the combination of two of these "breast milk" strains possess probiotic beneficial effects in healthy humans and could be a suitable and interesting alternative to the "intestinal"-derived strains currently available in the probiotic market.

Different probiotic strains of *Lactobacillus* and *Bifidobacterium* affect differently the severity of D-galactosamine-induced liver injury in rats

N. Osman[1], D. Adawi[2], S. Ahrne[1], B. Jeppsson[2] and G. Molin[1]

[1]Dept of Food Technology, Engineering and Nutrition,
[2]Dept of Surgery University Hospital Malmö, Lund University, Sweden

The intestinal microflora composition is important in physiological and pathophysiological processes in the human tract. Septic complications represent frequent causes of morbidity in liver diseases and following hepatic operations. We therefore studied the effect of *Lactobacillus* and *Bifidobacterium* strains on bacterial translocation, the extent of liver injury and intestinal microflora in an acute liver injury model.

Sprague-Dawley rats were used and divided into six groups, liver injury control and five groups of liver injury with *Lactobacillus* and *Bifidobacterial* strain administration (*L. plantarum* 299v, *L. paracasei* 8700:2, *L. gasseri* 5B3, *Bifidobacterium* 3B1, *B. infantis* DSM 15158). The bacteria were administered orally twice daily for 8 days. Liver injury was induced on the 8th day by intraperitoneal injection of D-galactosamine (1.1 g/kg BW). Samples were collected 24 hours after the liver injury. Liver enzymes and bilirubin serum levels, bacterial translocation (to arterial and portal blood, liver and mesenteric lymph nodes), and intestinal microflora were evaluated.

L. plantarum 299v, *L. gasseri* 5B3, *B. infantis* DSM 15158 decreased bacterial translocation to the liver compared to the liver injury control group. *L. paracasei* 8700:2 translocated to the liver. The Enterobacteriaceae count in the cecum decreased in the *L. plantarum* 299v, *L. gasseri* 5B3, *Bifidobacterium* 3B1 and *B. infantis* DSM 15158 groups, while all the administered probiotics decreased Enterobacteriaceae in the colon. The levels of alanine aminotransferase and bilirubin were significantly lower in the *L. plantarum* 299v and *B. infantis* DSM 15158 groups compared to the liver injury control group. The *B. infantis* DSM 15158 decreased significantly AST compared to the control group.

Administration of different *Lactobacillus* and *Bifidobacterium* strains in an acute liver injury model has shown different effects on bacterial translocation and hepatocellular damage. *L. plantarum* 299v and *B. infantis* DSM 15158 reduced bacterial translocation and hepatocellular damage. *L. gasseri* 5B3 reduced bacterial translocation but they did not show significant effect on hepatocellular damage. *L. paracasei* did not reduce bacterial translocation and hepatocellular damage, but translocated to the extraintestinal sites.

Effect of probiotics on *H. pylori* binding to gastric adenocarcinoma cells and subsequent IL-8 secretion. An *in vitro* study

E. G. Panayotopoulou, B. Martinez-Gonzales, D. Sgouras and A. Mentis

Laboratory of Medical Microbiology, Institut Pasteur Hellenique, Greece

We aimed to ascertain the potential effect of lactic acid bacteria (LAB) and their respective cell free spent culture supernatants (SCS), on the ability of different *H. pylori* strains to adhere to gastric adenocarcinoma (AGS) cells and induce IL-8 secretion. The LAB used (*L. johnsonii* La1, *L. casei* strain Shirota LcS, *L. amylovorus* DCE 471, *L. acidophilus* IBB 801 and *L. macedonicus* ACAD-DC 198) belonged to the PROPATH culture collection. Following 1 hour pretreatment with LAB or LAB-SCS, the adherence of CFSE-labeled *H. pylori* on AGS was evaluated by FACS. *H. pylori* viability was assessed by viable counts and levels of secreted IL-8 in the supernatant were measured by ELISA. Pretreatment of *H. pylori* strains with live LAB significantly decreased *H. pylori* binding to AGS. La1-SCS pretreatment resulted in dramatic inhibition of *H. pylori* viability and IL-8 secretion at 24 hours post-infection, whereas a less pronounced effect was observed for DCE 471 and LcS supernatants. However, no effect was observed for IBB 801 and ACA-DC 198. In conclusion, the observed reduction in the *H. pylori* binding efficiency and AGS IL-8 secretion could be attributed to the inhibitory effect of lactobacilli on *H. pylori* viable counts.

Fermented sausages as source of potentially probiotic *Lactobacillus* strains

C. Pennacchia[1,2], F. Villani[1] and E. E. Vaughan[2]

[1]Università degli Studi di Napoli "Federico II", Dipartimento di Scienza degli Alimenti, Italy. e-mail: capennac@unina.it
[2]University of Wageningen – Laboratory of Microbiology, the Netherlands.
e-mail: carmela.pennacchia@wur.nl

The most common mode of consumption of probiotic microorganisms is in fermented dairy products, but other fermented products such as vegetables and meat may also be used as a vehicle for these functional microbes. So far the available studies describe the behaviour of previously selected probiotic strains during sausage production or the potential probiotic use of some selected strains present in commercial meat starter cultures. The aim of the present study was to isolate *Lactobacillus* species from fermented sausages and to investigate their potential for production of novel probiotic sausage. Furthermore, the effect of the potential intestinal conditions on the lactobacilli is studied by a proteomics approach. The strains were selected using conditions typical for the human intestinal tract. Applying a procedure for a rapid screening at pH 2.5 and in presence of 1% bile salts, a variety of resistant lactobacilli were isolated directly from 10 traditional dry fermented Italian sausages (salame "Tipo Napoli", salame "Tipo Milano", soppressata)[1]. API 50CHL test strips and 16S rDNA sequence analysis of the 25 strains showing good potential as probiotics, indicated that the majority belonged to the *Lactobacillus plantarum*-group. Comparison of the strains was achieved by pulsed field gel electrophoresis (PFGE) using the restriction enzyme *Not*I, and a representative of each different strain identified was tested for adherence properties. Adhesion to the *in vitro* human intestinal epithelial-Caco-2 cell lines of the 11 selected *Lactobacillus* strains ranged from 10^5 to 10^8 CFU/well. The development of a proteomics approach to monitor the differential protein expression of the selected *Lactobacillus* strains due to different intestinal conditions such as acid stress and bile salts will be presented.

[1]Pennacchia, C., Ercolini, D., Blaiotta, G., Pepe, O., Mauriello, G. and Villani, F. (2004). Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. *Meat Science* (In press).

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Inhibition of adhesion of *Salmonella typhimurium* SL1344 to human epithelial cells by *Lactobacillus rhamnosus* GG

M. Perea-Vélez, T. Verhoeven, J. Vanderleyden, S. Dekeersmaecker and I. Nagy

Centrum voor Microbiële en Plantengenetica (CMPG), Katholieke Universiteit Leuven, Belgium

Lactic acid bacteria as members of the normal microbial flora in the intestine are considered to have beneficial, health–promoting effects on their host. To decipher the molecular mechanisms underlying the antagonistic effect of probiotic strains exerted on intestinal pathogens, an *in vitro* system was developed consisting of three partners, i.e. *Salmonella typhimurium*, *Lactobacillus rhamnosus* GG and the human intestine epithelial Caco-2 and/or HT-29 cell lines. *In vitro* adhesion inhibition experiments showed that LGG strain and the control conditions did not inhibit completely the adhesion of *Salmonella* to the cell lines Caco-2 and/or HT-29. To evaluate the effect of mucus on the adhesion of *Salmonella thyphimurium* SL1344 and LGG to HT-29 cells, pig-mucus was used at different concentrations. Nonetheless, no significant adhesion inhibition was observed.

The proteomes of the growth phases of two *Lactobacillus plantarum* strains

C. Plumed-Ferrer[1], K. Koistinen[1], E. Kilpi[2], S. Auriola[3],
S. Kärenlampi[1] and A. von Wright[1]

[1]Institute of Applied Biotechnology, University of Kuopio, Finland
[2]MTT Agrifood Research Finland, Finland
[3]Department of Pharmaceutical Chemistry, University of Kuopio, Finland

Lactobacillus plantarum is a facultative heterofermentative lactic acid bacterium widely used in food and feed preservation for its capacity to reduce the pH of the matrix and, therefore, prevent the growth of spoilage bacteria. Thus, the understanding of the growth properties of these bacteria is an attractive area of research. In the present study, the growth phases of two strains of *L. plantarum*, which are being examined for their properties as vegetable and feed starters, were analysed and compared at the protein level. The proteomes from lag, early-exponential, late-exponential and stationary phases of both strains were obtained by two-dimensional gel electrophoresis using pH 4–7 gradients, followed by mass spectrometric identification of the most differentially expressed proteins. Statistical analysis showed proteins up/down regulated as well as proteins that seemed to be expressed just in some specific phases. Most of the proteins that were successfully identified belong to the group of glycolytic enzymes, as well as stress related proteins. The results also indicated differences in the growth behaviour of the strains analysed, showing specially differences in the length of the lag phases, where the bacteria are acclimatizing to the environment.

Diversity of sulphate-reducing bacteria in the human gastrointestinal tract in relation to ulcerative colitis assessed by 16S rDNA analysis

M. Rajilić, E. E. Vaughan, L. Pereira and W. M. de Vos

Laboratory of Microbiology, Wageningen University, the Netherlands

Sulphate-reducing bacteria (SRB) are a phylogenetically diverse group of bacteria with the common ability to reduce sulphate to sulphide. SRB have been isolated from the human gastrointestinal tract in numbers varying from 9.5×10^4 to 1.65×10^{10} cfu/g of wet faeces. The end product of their metabolism, due to the pH conditions of the colon, is hydrogen-sulphide (H_2S), which is highly toxic for humans. Thus, although SRB form a fraction of the normal human gut microbiota it is suggested that they have been implicated in playing a role in either the aetiology or maintenance of ulcerative colitis (UC). In the present study we have analysed the diversity of the SRB community in the human gut in healthy subjects and UC patients by molecular techniques using 16S rDNA directed genera-specific PCRs and denaturing gradient gel electrophoresis (DGGE). All the analysis were done on faecal samples from 12 UC patients and 12 corresponding controls originated from two locations – Spain and Ireland. Stool samples from UC patients were collected at defined time points during a one-year trial. Similar to culturing studies, results showed that species belonging to the genus *Desulfovibrio* were the most dominant and diverse of all SRB, and thus were the focus of further analysis. The DGGE profiles indicated that the *Desulfovibrio* community was significantly more diverse in UC patients then in healthy subjects. The diversity of *Desulfovibrio* DGGE profiles in UC patients that remained in remission during one year of trial tended to become simpler over time. This was not observed in cases of relapsed UC patients. DNA sequence information was obtained from six 16S rDNA libraries constructed from joined *Desulfovibrio* specific PCR products from healthy and diseased subjects from both locations and from two UC patients. Results of comparative sequence analysis indicated that a proportion of *Desulfovibrio* species inhabiting the human intestine have not yet been cultured or described.

Culture independent molecular analysis of the elderly faecal microflora reveals an extreme complexity

K. Saunier[1], K. Tuohy[2], E. Likotrafiti[2], M. Sutren[1], R. Rastall[2], S. Silvi[3], A. Cresci[3] and J. Doré[1]

[1]Laboratoire d'Ecologie et de Physiologie du Système digestif, INRA, France
[2]Unit of Food Microbiological Studies, University of Reading, U.K.
[3]Dipartimento di Scienze Morfologiche e Biochimiche Comparate, Università degli Studi di Camerino, Italy

Understanding age-related alterations in the gastrointestinal tract is important in the design of preventive nutrition strategies. The elderly fraction of the population is currently rising in Western societies, and yet specificities of its gut microbiota, involved in health, remain largely unknown.

The present study was conducted to evaluate the bacterial diversity of the dominant faecal microbiota of elderly.

We used comparative sequencing of 2000 cloned 16S rDNA from faecal DNA of 11 healthy elderly persons (6 women - 5 men, aged 69–87, mean 77).

In total, 420 phylotypes were identified using 98% similarity criterion and affiliated with the 6 phylogenetic groups commonly found as dominant in adults: *Clostridium leptum*, *Clostridium coccoides*, *Bacteroides*, enteric, *Lactobacillus-Streptococcus* and *Bifidobacterium*. We further observed other dominant groups: *Sporomusa*, *Acholeplasma-Anaeroplasma*, uncultivable bacteria from clusters B and E described by Leser (2002) and *Atopobium*. Other remaining groups were less represented with a weaker prevalence. Among the 2000 sequences analysed, 80% corresponded to uncultured bacteria and 1/3 were novel sequences. Each faecal microbiota appeared unique: only one phylotype was common to 11/11 faecal samples (*Faecalibacterium prausnitzii*) and one common to 10/11 (*Bacteroides vulgatus*).

In term of dominant species composition, the elderly faecal microbiota is extremely complex and specific of the individual.

Effect of probiotics on *H. pylori* infection and associated gastritis. An *in vivo* study utilizing the SS1 *Helicobacter pylori* infection model

D. Sgouras, B. Martinez-Gonzales, E. G. Panayotopoulou and A. Mentis

Laboratory of Medical Microbiology, Institut Pasteur Hellenique, Greece

We aimed to study the effect of the continuous administration of probiotics through the animal water supply, on *H. pylori* infection and associated gastritis in the *H. pylori* SS1 infection mouse model. The probiotics administered belonged to the PROPATH culture collection (*L. johnsonii* La1, *L. amylovorus* DCE 471, *L. acidophilus* IBB 801 and *L. macedonicus* ACAD-DC 198). Administration of live lactobacilli at a daily dose of 10^8 cfu/animal did not eradicate or reduce *H. pylori* colonization at 6 and 12 weeks post-infection, as assessed by viable cultures, PCR and histopathology of gastric samples. However, there was a significant reduction in the numbers of invading neutrophils in the lamina propria, in all probiotic-treated groups over the course of 12 weeks. Except in the case of La1-treated animals, no simultaneous reduction in the numbers of infiltrating monocytes in the epithelium was observed. Anti-*H. pylori* IgG antibody titers in the serum collected from the probiotic-treated *H. pylori* infected animals were significantly depressed in the case of La1 and ACA-DC 198. Collectively, the results point out that factors either secreted by or expressed on live lactic acid bacteria can influence both anti-*H. pylori* antibody response and neutrophilic infiltration in the lamina propria, a hallmark of *H. pylori* infection.

Bile acids concentrations in faecal water samples of different European populations

S. Silvi[1], M. C. Verdenelli[1], C. Orpianesi[1], G. Morozzi[2], A. De Bartolomeo[2], R. Fabiani[2], B. Sebastiani[2], C. Hanisch[3], S. Müller[3], H.-J. F. Zunft[3], M. Blaut[3], E. Norin[4], I. R. Rowland[5], K. Saunier[6] J. Doré[6] and A. Cresci[1]

[1]University of Camerino, Italy
[2]University of Perugia, Italy
[3]German Institute of Human Nutrition, Germany
[4]Karolinska Institute, Sweden
[5]University of Ulster, Northern Ireland
[6]Institut National de la Recherche Agronomique (INRA), France

The primary bile acids are the major end products of cholesterol metabolism and they are extensively metabolised by the intestinal microflora, predominantly 7-α-dehydroxylation, which converts cholic (CA) to deoxycholic acid (DCA) and chenodeoxycholic (CDA) to lithocholic acid (LCA).

Secondary faecal bile acids (DCA and LCA) can be considered indicators of the status of intestinal microbiota and they have demonstrated co-carcinogenic and co-mutagenic activity.

Bile acids concentrations have been evaluated in 220 samples of faecal water from healthy subjects from different European countries (Italy, Sweden, Germany and France) and of different age groups.

The primary and secondary bile acids concentrations have been evaluated by gas-chromatography and confirmed by gas-chromatography- mass-spectrometry.

Generally the results show high concentrations of secondary bile acids, and as expected, low concentrations of primary ones, although, in few cases, high concentrations of primary bile acids were observed together with a low concentration of secondary, so indicating a low value of dehydroxylating enzymatic activity (7α – dehydroxylase).

The preliminary statistical analysis carried out by the Principal Component Analysis (PCA) considering all the variables (single bile acids and their sum) didn't show differences among the groups of subjects, although some outliers are present.

Moreover in 30 subjects the presence of oxo-bile acids has been found. This finding is, at the moment, difficult to explain, but it could be related to the variation of redox potential in the complex large bowel ecosystem.

Bringing probiotic ME-3 to the market according to guidelines FAO/WHO, 2002

E. Songisepp[1], H. Annuk[1], T. Kullisaar[2], J. Shchepetova[1], P. Hütt[1], R. Mändar[1], K. Truusalu[1], P. Elias[3], M. Zilmer[2] and M. Mikelsaar[1]

[1]Department of Microbiology, Tartu University, Estonia
[2]Department of Biochemistry, Tartu University, Estonia
[3]Institute of Food Technology, Estonian Agricultural University, Estonia

Evaluating *Lactobacillus fermentum* ME-3 as a probiotic based on the recommended criteria outlined in Guidelines for Evaluation of Probiotics in Food by Joint FAO/WHO Working Group, 2002.

L. fermentum ME-3 was previously isolated from a gastrointestinal tract of a healthy child. The strain has been identified by API CHL 50 and by ITS-PCR using the reference strain *L. fermentum* ATCC 14931. ME-3 has been tested for production of different metabolites (lactic, acetic, succinic acids, ethanol, biogenic amines, H_2O_2), antibiotic resistance and resistance to acid and bile ME-3 is deposited in a culture collection under the number DSM 14241. The patent applications have been submitted to the Estonian Patent Agency and to International Bureau of WIPO.

ME-3 has high antimicrobial activity against various entero- and uropathogens. The cells and cell lysate of ME-3 have a strong antioxidative potency. Safety of the strain has been assessed in mouse model.

Three different double blind, randomised, placebo-controlled clinical trials showed, that the consumption of ME-3 containing capsules or fermented milk improves some antioxidative indices of healthy volunteers and patients with atopic dermatitis.

L. fermentum ME-3 has been successfully incorporated into two different cheese varieties and various fermented dairy products (yoghurt, cream, sour cream, kefir). The strain sustained its probiotic properties (antimicrobial and antioxidative activity) in all fermented dairy products.

Localisation of bacteria in relation to mucus barrier in colon of mice with inflammatory bowel disease (IBD)

M. Strus[1], H. Uhlig[2], F. Powrie[2] and P. B. Heczko[1]

[1]Jagiellonian University Medical College, Krakow, Poland
[2]Sir William Dunn School of Pathology, University of Oxford, U.K.

In this study were used SCID mice receiving $CD4^+$, $CD45RB^{hi}$ T cells and showing symptoms of chronic bowel inflammation in about 8 weeks after transfer, and unreconstituted SCID mice without symptoms of the inflammation. The whole GI tract of colitic animals and controls was removed, ligated on both ends, and transported in dry ice for microbiological and immuno/histochemical and FISH analysis. After thawing, the tract was opened, content removed and 5 different samples of mucosa taken from stomach, proximal and distal ileum, colon and caecum for microbiology. Other samples from the same sites were used for localisation studies. Paraffin-embedded tissues were stained with haematoxylin and eosin. Colon sections were assessed for epithelial hyperplasia, loss of goblet cells, and lamina propria infiltrate. Alcian blue and PAS staining was performed with haematoxylin counterstaining. Probes used to stain different groups of bacteria in FISH were fluorescein-labelled EUB-338 (all bacteria), Cy3 labelled MIB-661 (mouse intestinal bacteria), Cy3 labelled LAB-158 (LAB) and *Enterococcus* and Cy3 labelled SFB-1008 (segmented filamentous bacteria). To demonstrate the border between epithelial cells and colon content, the unspecific tissue staining with Evans blue was performed.

Intestinal inflammation in the T cell transfer model mice was associated with a reduction of goblet cells. It was therefore possible that changes in the mucus layer explained the spatial separation between epithelial surface and bacteria in the non-colitic colon compared to the more intimate association in the inflammed colon. Under non-inflammatory conditions, large numbers of goblet cells were related to a continuous mucus layer. Consistent with this, wide areas of the intermediate colon lumen were completely filled with mucus. These areas were predominantly free of intestinal planktonic bacteria. In areas with intestinal luminar content, the mucus layer separated the epithelial surface from bacteria in the colon content. In colitic animals, reduced mucus production together with epithelial hyperplasia and epithelial ulcerations resulted in a discontinuous mucus layer. Our data suggest, that under physiological conditions the mucus barrier plays an important role in keeping intestinal bacteria distant from the epithelium. In the chronically inflamed colon, the mucus production becomes affected and the integrity of the mucus barrier is destroyed and this is associated with increased adherence of various bacteria to the epithelium.

Generation of a novel synbiotic specifically designed for the elderly population

K. M. Tuohy, E. Likotrafiti, K. Manderson, F. Fava, G. R. Gibson and R. A. Rastall

Food Microbial Sciences Unit, School of Food Biosciences, The University of Reading, U.K.

Gastrointesintal infections are a serious cause of morbidity and mortality within the elderly population. Probiotic microorganisms and prebiotics have the potential to combat this situation both in the form of preventatives and in complementary therapy. Synbiotics, which combine efficacious probiotic strains with prebiotic oligosaccharides, may augment the microflora modulatory capabilities of both approaches while alleviating some of their short comings e.g. probiotic survival or competitiveness within the gut microflora. Here, we describe the validation of a synbiotic product combining a previously uncultured probiotic strain, closely related to *Bifidobacterium infantis* with a probiotic oligosaccharide specifically designed to augment its activity within the gut microflora. The probiotic strain was chosen for its ability to inhibit important gastrointestinal pathogens of the elderly. Using an enzymatic synthesis reaction, an oligosaccharide mixture was synthesized using β-galactosidase produced by the probiotic strain from lactose. This novel galacto-oligosaccharide preparation was assessed for its prebiotic potential using model systems of the human gut microflora. The ability of the prebiotic to augment the activity of the *B. infantis*-like probiotic strain within the gut microflora was also determined.

Czech research activities on bactocereal synbiotics

K. Vaculova and V. Erban

Agricultural Research Institute Kromeriz, Ltd., Czech Republic

Projects concerning development and *in-vivo* verification of effects of bactocereal food additives exhibiting perspective hypocholesterolemic effect aims at design of synbiotics, made on the basis of domestic raw materials (bacterial starter cultures and scratch bran fibre extract with high beta-glucan content from hulless barley grain). Growth, sorption and resistance characteristics of selected probiotics in different pH and cholic acid concentration have been assessed and the preventive effect of prebiotic extracts demonstrated on surviving of probiotic. It was concluded during *"in vivo"* experiments with Prague Hereditary Hypercholesterolemic Rats characterised by lower and higher cholesterolemia and with human volunteers (moderate hypercholesterolemia, 6–8 mmol/l) that new bactocereal food additive could decrease the total and LDL blood cholesterol concentration.

The following research would be targeted on the optimalisation of raw material processing methods, on the study of effectiveness of extraction and conservation of prebiotics, on the further selection and application of probiotics and on the food technologies of synbiotics production. From the future partnership we will expect verification and comparison influence of the new bactocereal food additive with another synbiotics on others chronic and diet-related diseases (diabetes, gut disorders, immunity, etc.).

Intraspecific genotypic characterization of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* strains used as probiotics or isolated from human faecal and sterile sites

M. Vancanneyt[1], G. Huys[2], L. Verbrugghen[1], K. Lefebvre[1], H. Goossens[3] and J. Swings[1,2]

[1]BCCM/LMG Bacteria Collection and
[2]Laboratory of Microbiology, Ghent University, Belgium
[3]Department of Medical Microbiology, University Hospital, UIA, Belgium
e-mail: Marc.Vancanneyt@UGent.be, http://lmg.UGent.be

Within the framework of the European-funded project (PROSAFE; QLK1-2001-1273), about 900 lactic acid bacteria have been collected and identified at the species level. The species *Lactobacillus rhamnosus* and *Lactobacillus paracasei* largely dominated this collection being represented by approx. 120 and 80 isolates, respectively. Both species are used as probiotics for human consumption and are autochthonous to the human faecal microflora, but they have also been recovered from human sterile sites where these organisms can be involved in e.g. blood infections such as endocarditis. This observation has questioned their safety for human use. The present work aimed to reveal the genotypic relationships between strains of *L. rhamnosus* and *L. paracasei* from diverse geographical and biological origins.

In a first approach, *L. rhamnosus* and *L. paracasei* strains were genotypically typed with AFLP using three different primer combinations. Within these two species, 7 and 11 stable intra-species genomic groups were delineated, respectively. Some of these groups comprise strains used as probiotics as well as isolates originating from human blood samples. In a second approach, PFGE of *Not*I restriction patterns was applied on representative strains from the different AFLP clusters. For *L. rhamnosus* PFGE confirmed a very high genomic relationship among particular strains within delineated AFLP groups and in some instances yielded nearly identical fingerprints for isolates from both probiotic and human (blood) origin. For *L. paracasei*, a much higher heterogeneity was observed among strains clustered in defined AFLP groups and no analogous PFGE patterns between probiotic strains and human blood samples were found.

The reported typing study is still in progress and additional experiments are necessary to allow speculations about possible clonal relatedness between strains used as probiotics and isolates from human blood origin. However, if the latter statement is confirmed for particular intraspecific groups, and if there is a possible link with the presence of virulence factors and/or antibiotic resistances, the use of these LAB strains as human probiotics may need to be reconsidered.

Temporal stability analysis of the human fecal microbiota by denaturing gradient gel electrophoresis (DGGE) using universal and group-specific 16S rDNA primers

T. Vanhoutte[1], G. Huys[1], E. De Brandt[1] and J. Swings[1,2]

[1]Laboratory of Microbiology and
[2]BCCM™/LMG Bacteria Collection, Ghent University, Belgium.
e-mail: T.Vanhoutte@UGent.be, http://lmg.UGent.be

According to the current insights, the predominant bacterial community in human feces is considered to be stable and unique for each individual over a prolonged period of time. Further extension of our current knowledge of the temporal stability and uniqueness of indigenous colon microbiota and their interaction with introduced (e.g. probiotic) bacteria will certainly benefit the development of functional 'health-improving' foods.

In this study, the temporal stability of both the predominant population and a number of specific subpopulations of the fecal microflora of four healthy volunteers was monitored over a six weeks to three month period. For this purpose, a combination of different universal (V_3 and V_6-V_8) and genus- or group-specific (targeting the *Bacteroides fragilis* subgroup, the genera *Bifidobacterium* and *Enterococcus* and the *Lactobacillus* group, which also comprises the genera *Leuconostoc*, *Pediococcus* and *Weisella*) 16S rDNA primers was used. DGGE was used to analyze the 16S rDNA amplicons generating population fingerprints which were compared visually and by numerical analysis. DGGE profiles that resulted from the use of universal primers were relatively stable over a 3 month period and these profiles grouped by numerical analysis in subject-specific clusters. In contrast, the genus- and group-specific primers yielded profiles with varying degrees of temporal stability. The *B. fragilis* subgroup and *Bifidobacterium* populations remained relatively stable which was also reflected by subject-specific profile clustering. The *Lactobacillus* group showed considerable variation even within a two-week period and resulted in complete loss of subject-grouping. The *Enterococcus* population was detectable by DGGE analysis in only half of the samples.

In conclusion, numerical analysis of 16S rDNA-DGGE profiles clearly indicates that the predominant fecal microbiota is host-specific and relatively stable over a prolonged time period. However, some subpopulations tend to show temporal variations (e.g. the *Lactobacillus* group) whereas other autochthonous groups (e.g. the bifidobacteria and the *B. fragilis* subgroup) do not undergo major population shifts in time.

Inventory and identification of lactic acid bacteria used as probiotics for human consumption

V. Vankerckhoven[1], T. Van Autgaerden[1], G. Huys[2], M. Vancanneyt[3], J. Swings[2,3] and H. Goossens[1]

[1]Laboratory of Medical Microbiology, University of Antwerp, Belgium
[2]Laboratory of Microbiology, Ghent University, Belgium
[3]BCCM/LMG Bacteria Collection, Ghent University, Belgium
e-mail: vanessa.vankerckhoven@ua.ac.be

Within the framework of the European project PROSAFE - an inventory of commercial probiotic strains was made, descriptive strain and product information was collected and strain identifications were verified.

A total of 54 companies involved worldwide in the production and/or distribution of probiotics were invited to submit their strains and to complete a questionnaire. Species identification of the strains was verified using AFLP, repetitive DNA element (rep)-PCR fingerprinting, and protein profiling

Of the 54 companies contacted, 27 submitted their strains, 13 companies claimed not to manufacture probiotics and were therefore excluded from the survey, 2 companies did not wish to participate, and 12 companies, most of which located in the US, did not respond. All 27 participating companies returned the questionnaire. In total, 202 industrial strains were submitted to the project and were received as belonging to *Lactobacillus* (54.0%), *Bifidobacterium* (26.7%), *Enterococcus* (5.9%), *Propionibacterium* (5.9%), *Lactococcus* (2.5%), *Pediococcus* (2.0%), *Streptococcus* (2.0%), *Bacillus* (0.5%) and *Oenococcus* (0.5%). According to the questionnaire, the most frequently used identification techniques included biochemical characterisation (34.9%), DNA fingerprinting (21.8%) and 16S/23S rDNA sequencing (20.8%). Comparison with our identification results obtained so far for 174 commercial strains, the identity of 17.2% of these strains did not correspond to the identification received by the company. Identification discrepancies between *L. casei* and *L. paracasei* (4 strains) and *B. lactis* and *B. animalis* (5 strains) were not considered as misidentifications because the taxonomic status of these respective species is still under debate. Out of 202 strains with descriptive information, 53.5% are of human origin and 44.5% of non-human origin, whereas for 2.0% the source of isolation is unknown. Two strains were submitted as genetically engineered. 46.0% of the strains were isolated in the 1990s, whereas for 28.2% the year of isolation is unknown. 46.5% of the strains are used for human consumption, 5.4% for animal use, 7.0% for both human and animal use. For the remaining part, 7.4% of the strains were simply categorised as probiotic, 5.0% are industrial starters and 28.7% are still under investigation.

Biomarkers for intestinal health: Effect of inulin and its microbial fermentation products on the metabolic profile of cultured enterocytes

K. Venema, J. van Nesselrooij, R.-J. Lamers and J. van de Sandt

TNO Nutrition and Food Research, The Netherlands
e-mail: venema@voeding.tno.nl

There is a great need for *in vitro* biomarkers and *in vitro* bioassays for intestinal health that allow the possibility to develop and screen new (functional) food components. At the moment there are no proper biomarkers available. We have used the unique combination of TIM-2 (TNO's dynamic *in vitro* model of the large intestine), *in vitro* cell based assays and pattern-recognition to allow for the development of these biomarkers and to use these for claim-support. In the present project, we assessed the metabolic fingerprinting of Caco-2 cell cultures after exposure to inulin and its microbial fermentation products.

After 48 hours of exposure, the Caco-2 cells were collected and prepared for NMR spectroscopic analysis. After multivariate analyses of the NMR data, a difference in metabolic fingerprint was observed between Caco-2 cells exposed to control medium and cells exposed to inulin. Similarly, a difference in metabolic profile was observed between cells exposed to control TIM-samples and cells exposed to metabolized inulin. This suggests that both inulin and its metabolites have an additional effect compared to the control sample. The score plot identified which peaks were different between the control and test incubations. Identification of these peaks and the corresponding specific metabolites and their relation to the functionality of the intestinal wall will lead to biomarkers that help to substantiate claims in the field of intestinal health.

Short chain fatty acids in the characterization of human intestinal ecosystem of different European populations

M. C. Verdenelli[1], S. Silvi[1], C. Orpianesi[1], C. Hanisch[2], S. Müller[3], H.-J. F. Zunft[2], M. Blaut[3], E. Norin[4], I. R. Rowland[5], K. Saunier[6], J. Doré[7] and A. Cresci[1]

[1]Department of Comparative Morphology and Biochemistry, University of Camerino, Italy
[2]German Institute of Human Nutrition, Department of Intervention Studies, Germany
[3]German Institute of Human Nutrition, Department of Gastrointestinal Microbiology, Germany
[4]Microbiology and Tumor Biology Center, Karolinska Institute, Sweden
[5]Northern Ireland Centre for Diet and Health, University of Ulster, Northern Ireland
[6]Unit of Ecology and Physiology of the Digestive System, Institut National de la Recherche Agronomique (INRA), France

In a world of rapidly changing food habits and stressful life styles it is more and more recognised that a healthy digestive system is essential for overall quality of life. One of the factors that is being recognised to be of major importance for the maintenance of a healthy digestive system is the colonic flora, especially its bacterial composition and the "nutrients" that it metabolises.

Short chain fatty acids (SCFA) are the main end products of anaerobic microbial metabolism in the human colon and can be considered indicators of the status of intestinal microbiota.

In our study we analysed the distribution of major SCFAs in faecal water samples from 160 elderly subjects and 80 young adults from Italy, Sweden, Germany and France. We observed that the SCFA concentration is significantly different among the four European populations studied, showing also lower values in elderly groups than in the adults. These data reflect a change in microflora composition or activity which could be associated to a higher incidence of intestinal disorders in elderly. Higher levels of colonic SCFA might be beneficial for colon health since they determine a reduction in the colonic pH which promotes glycolytic activities rather than proteolytic activities and inhibits bacterial 7-α-hydroxylase activity, thereby reducing the concentration of secondary bile acids (FBA). This action is supposed to be important because some endproducts of proteolytic activity as well as secondary FBA have demonstrated co-carcinogenic and co-mutagenic activity.

Effect of chitosan and its oligosaccharide on gut microflora populations

C. Vernazza, R. Rastall and G. Gibson

University of Reading, School of Food Biosciences, U.K.

Chitosan and its oligosaccharides are used as preservatives by the food industry and have well-documented antimicrobial properties. Chitosan is also used in many brands of slimming aids with the claim that it blocks the absorption of dietary fat. The effects of these polymers on the gut flora are currently unknown. To determine these effects pH controlled anaerobic batch cultures were performed. Three different molecular weights of chitosan and chitosan oligosaccharide lactate were added to the medium as the sole carbohydrate source and fructooligosaccharides and glucose were used controls. Bacterial groups were enumerated using classical microbiology techniques and fluorescent *in situ* hybridisation (FISH). Species-specific denaturing gradient gel electrophoresis (DGGE) was used to check for any changes to the flora at species level. Short chain fatty acids were also analysed. Gut model investigation of chitosan oligosaccharide lactate was also performed. Chitosan and its oligosaccharide caused changes in some bacterial groups measured indicating a partially-selective fermentation. DGGE also showed that some of these changes were at the species level. Changes in numbers of bacterial groups were less pronounced in the gut model system.

Overview of the EC LABDEL project: oral delivery of vaccine and therapeutic products using non-pathogenic lactic acid bacteria

J. M. Wells[1], J. Delcour[2], P. Hols[2], H. Israelsen[3], M. Kleerebezem[4], A. Mercenier[5], P. Sizer[6], H. Tlaskalova[7] and U. Wiedermann[8]

[1]Institute of Food Research, U.K.
[2]Université Catholique de Louvain, Belgium
[3]Biotechnological Institute, Denmark
[4]Wageningen Centre for Food Sciences, the Netherlands
[5]Institut Pasteur de Lille, France
[6]Provalis plc, U.K.
[7]Institute of Microbiology, Czech Republic
[8]University of Vienna, Austria

Scientific discoveries made in previous EC funded research programs on the lactic acid bacteria (LAB) showed that there was considerable potential to develop products based on the oral delivery of vaccine and therapeutic agents using harmless commensal LAB. The aim of the LABDEL project was to harness this potential by developing prototype LAB-based products for vaccination, prevention and treatment of type I allergy and the mucosal delivery of therapeutic enzymes. This multifaceted strategy was underpinned by research on LAB fermentation and technological innovations to enhance the efficiency of LAB delivery systems.

Lactococcus lactis and *Lactobacillus plantarum* strains expressing antigens and enzymes to be delivered to the GI tract were constructed and evaluated for their vaccine or therapeutic potential in model systems. New data will be presented on the vaccination and protection from infection with *Streptococcus pneumoniae* using a recombinant LAB vaccine. Additionally LAB were shown to be promising vehicles for the prevention and treatment of type I allergy using a murine model of birch pollen allergy. Major improvements in the fermentation protocols for *L. lactis* and *L. plantarum* were achieved giving rise to higher cell densities. This is likely to have a beneficial impact on the industrial use of LAB to produce high value proteins and will ultimately contribute to the efficient production of lactic acid bacteria for product applications in GI tract delivery. New technology was established in *Lactobacillus* to screen for promoters to turn on bacterial gene expression in the gastrointestinal tract and good progress was made in constructing improved genetic systems for mucosal delivery using LAB. In this presentation an overview of the achievements of the Partner laboratories in the LABDEL project (indicated above) will be presented.

Development of a new lyoprotective formulation to enhance the viability and stability of the probiotic *L. rhamnosus* E800 and *L. rhamnosus* GG

J. Wesenfeld and U. Stahl

Berlin University of Technology, Department of Microbiology and Genetics, Germany

Probiotic strains lose their viability due to freeze-drying and subsequent storage. Recognizing the nature and the potential causes of these changes, appropriate methods to prevent such cellular damage and to improve cell survival in dried products can be designed. Little information is available on the site and nature of cellular damage of the strains we study.

This study investigates the development of a new lyoprotective formulation in order to enhance the probiotic viability and stability after freeze-drying.

The new formulation contains polymers (gelatine, maltodextrin) for stable formation of a glassy state with high glass transition temperature Tg. Additionally, excipient stabilizers such as carbohydrates (lactose, sucrose) are used for depressing phase transition temperature Tm of the phospholipid of cell membrane, which undergoes from the liquid crystalline to the gel phase or vice versa during drying and rehydration.

The results show that the new lyoprotective formulation is effective to protect cultures from freeze- drying and subsequent storage.

However, the lyophilisates are hygroscopic and the humidity affects their stability. The on-going research is focused on using emulsion-techniques for coating in order to improve the stability against high humidity.

Anti-Helicobacter activity and bacteriocin production by *Lactobacillus johnsonii* LA-1

Q. Yi[1], D. A. Brede[1], L. Avonts[2], L. De Vuyst[2], I. F. Nes[1] and H. Holo[1]

[1]Department of Chemistry, Biotechnology and Food Science, Agricultural University of Norway, Norway
[2]Department of Applied Biological Sciences, Vrije Universiteit Brussel (VUB), Belgium

Lactobacillus johnsonii LA-1 produces protease sensitive compounds inhibitory to both lactobacilli and *Helicobacter pylori.* However, the indicator *Lactobacillus delbruekii* is several hundred times more sensitive than *Helicobacter pylori* to the products secreted by strain LA-1. A procedure was developed for the purification of the anti-lactobacillus and anti-helicobacter activity. The major fraction of antimicrobial activity was identified as a derivative of the two-peptide bacteriocin lactacin F. A minor fraction obtained during the purification showed low antimicrobial activity against *H. pylori*, and the activity against *L. delbrueckii* was only 8 times higher, indicating the presence of an antimicrobial peptide different from lactacin F-like bacteriocin.

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The Food, GI-tract Functionality and Human Health Cluster PROEUHEALTH 3rd Workshop

Abstract

The Food, GI-tract Functionality and Human Health Cluster PROEUHEALTH brings together 64 research partners from 16 European countries in the quest to obtain greater knowledge of the role of the intestinal microbiota in human health and disease and to develop new functional foods and therapies. The research will run for four years starting February 2001 and is subsidised by the European Commission's 5th Framework Programme, Quality of Life and Management of Living Rescources Key Action 1, "Food, Nutrition and Health", GUTHEALTH MEASURES QLAM-2000-00197 and QLM-2000-00199.

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