

Gut microbiota in health and disease

Report of the 2nd International Workshop in Amsterdam, April 26-28, 2007

Gut flora science is making rapid progress. New technologies such as 16S rDNA screens, metagenomics and metaproteomics are shedding light on the wide diversity of the gut bacteria. Cell and animal experiments point to the intimate relationship between the immune system and the gut bacteria. And increasing evidence suggests that gut and immune related diseases - such as Crohn's disease, autism, allergy and infections - are associated, maybe even causally related to a disturbed gut flora.

So it's not surprising that a growing number of human studies with carefully selected probiotic strains are showing positive results. These preliminary studies seem to hold out a promise that at least some patients will find relief from probiotics. However, it is still too early to draw conclusions about the effect of particular strains or doses, working in specific clinical situations. These are some of the themes that were presented during the workshop on Gut microbiota in health and disease, held in Amsterdam in April 2007.

1. 16S rDNA screens shed light on the diversity in our gut

For years, the 10-metre-long human intestinal tract was like a dark tunnel. Some light had been shed on it by culturing bacteria from the faeces, but the darkness was overwhelming, because about 70 to 90 percent of the bacteria cannot be cultivated in laboratory dishes. These uncultured bacteria remained completely unknown. Microbiologists knew that trillions of microbes live in the gut, but they had no idea which ones.

This situation is changing rapidly, however, thanks to new high throughput technologies and matching software, explained Willem de Vos from Wageningen University and Helsinki University. For the last two or three years, it has become possible to find out which 'species' or 'phylotypes'¹ of bacteria live in our body, by analyzing the genes for 16S rRNA's with a phylogenetic microarray. These 16S rDNA screens enable scientists to distinguish the different phylotypes in an environment directly without having to make cultures. And by comparing the 16sRNA's with the 16SRNA's of known phylotypes, the phylotypes of the gut microbes can be determined. Several institutes and companies are producing micro array chips, with more and more probes for different phylotypes. An example of a recently made chip is the Human Intestinal Tract Chip (HIT-Chip), developed by Wageningen University and based on a platform of Agilent Technologies. This chip has 5000 tilling probes for about 1250 intestinal phylotypes.

Microbiologists have already found more than 1250 phylotypes or (sub)species in the human intestine and gut.² "We think there are about 5000 human phylotypes," said De Vos. "The diversity in gut bacteria is much greater than we expected."

¹Definition of a phylotype: all bacteria with more than x (for example 98 %) similarity in the 16S rRNA.

² See for example: Eckburg et al, Science 308 (2005) 1635 – 13,355 (16S rDNA sequences of bacteria in 3 individuals yielded about 350 new phylotypes)

In fact, each person seems to have their own composition of faecal microbiota. Joël Doré from INRA in Montpellier (France) presented several 16S rDNA screens³, revealing that each individual has his or her own bacterial profile. The French group cloned and sequenced 1580 DNA pieces for 16S rRNA, obtained from ten healthy elderly people. Within these DNA pieces, they detected 342 different species – a ‘species’ was defined as all phylotypes with more than 98 percent similarity in the 16S rRNA gene. Two-thirds of these species were totally host-specific, and only one specie (*Faecalibacterium prausnitzii*), was common to all individuals. Nine percent (30 species) were remarkably prevalent: 40 percent of all cloned rDNA (646 pieces) were from these 30 species. So most species occurred only occasionally, represented by just a few cloned rDNA’s.

Newly born babies already show great individual differences. Wageningen University has studied the microbiota in faecal samples of breast-fed babies in the first twenty days of their life⁴. The researchers observed rapid and dynamic colonization. For example, in one baby, Bifidobacteria were detected from day 2; Enterococci from day 3 and Ruminococci from day 13. But there were large individual differences in colonization rate, probably related to the mother’s milk and the genotype of the baby. Most remarkable was that three of the six babies already had Bacteroidetes species at a certain age, while three other baby’s were still free of this phylum. However, the babies without the Bacteroidetes species had more Actinobacteria species. It appears therefore that there is an inverse relation between the abundance of Actinobacteria and Bacteroidetes in the early life.

The composition of the microbiota in healthy people is not only unique, but also appears to be quite stable over time. There seems to be a vast ‘core’ of approximately 300 phylotypes that are likely to be found at any moment of sampling, explained Doré and De Vos. This core changes only slightly when people age. It is resistant to modification and resilient to antibiotics.⁵ In addition to the core group, there also are ‘passengers’ or transients, sometimes in great numbers, sometimes undetectable. The size of the transitory population depends on external or coincidental influences such as diet, travelling or infections. That genetics influences the composition of the core was shown in a study carried out at Wageningen University comparing twins with unrelated individuals.⁶ The microbiota of monozygotic twins is notably more similar than the microbiota of unrelated individuals. While 16S rDNA screens are bringing more light into the tunnel, these chips cannot provide answers to all the questions.

2. Metagenomics is necessary to understand the functions of the phylotypes

The following question concerns the role or function of the individual phylotypes. We know that the gut bacteria as a whole fulfil many functions that are crucial for our health, such as development of the neonatal gut and the immune system, defence against pathogens, support in digesting food components and production of vitamins. But why do we have so many different phylotypes, families and phyla in our gut? Does each play a specific role? And is the core of 300 phylotypes also a functional core? These questions are as yet unanswered. To gain more insight, explained Doré, we need data on several levels: species diversity, the

³ Suau 1999, Bonnet 2001, Mangin 2004, Wang 2003, Hold, Hayashi 2002-03, Eckburg 2005, Manichanh 2006, Lepage

⁴ Christine Favier et al AEM 68 (2002) 219, collaboration with Lorenzo Morelli (AAT)

⁵ Seksik et al. Gut 2003, de la Cochetiere et al, J Clin Mic 2005

⁶ Zoetendal et al 2003

microbiome (total number of bacterial genes), the mRNA level (which genes are active?), proteins and metabolites.

Fortunately, the technologies and software available for gathering data on these levels are becoming increasingly sophisticated. This is the field of functional genomics and metagenomics, involving sequencing and detecting all the DNA, mRNA's and/or microRNA's in a sample. This systematic mining of all nucleotides in the gut will yield a huge amount of data, for example about the microbiome (all bacterial genes in the gut which amount to bacterial genome - in total over a hundred times bigger than the human genome).

De Vos presented the possibilities of the *metaproteomics* approach, comprising two-dimensional gel electrophoresis and mass spectrometry. This approach was applied to the largely uncultured faecal microbiota of a baby. The Dutch group observed how the faecal microbial proteins (the 'metaproteome profiles') changed over time. For example, one protein spot contained a peptide sequence that showed a high degree of similarity to those of bifidobacterial transaldolases. Transaldolase is involved in the production of NADPH. So the bifidobacteria in this baby may have had a function in protection against oxidative stress.⁷ One of the shortcomings of this approach, concluded De Vos, is the still limited amount of bacterial protein sequences in the databank. Most bacterial proteins are still unknown. INRA used the proteomics approach on the faecal microbiota of two healthy subjects. The bacterial protein combinations in individuals seem to be less unique than the phylotypes, Dore concluded: 397 proteins were specific to subject 1, while 376 were specific to subject 2, but 1092 proteins were common to both subjects.

3. Many diseases are characterized by a disturbed gut microbiota (Crohn's disease, diabetes, autism)

With 16S rDNA screens, scientists can also compare the gut flora of healthy people with that of patients with a given disease. The first studies in this field now have been completed. And indeed, gut flora related diseases such as Crohn's disease are characterized by a disturbed microbiota. INRA compared the faecal pool of 6 patients with quiescent Crohn's disease.⁸ The scientist detected 88 different species in the faeces of the healthy people, but only 54 species in the faeces of the patients. The Firmicute diversity in particular was reduced: in the faeces of the healthy people they were able to detect 43 different Firmicute species, whereas in the patients they found only 13 species. Within this phylum, the Clostridia were out of balance: the relative proportion of *C. leptum* among the faecal Firmicutes was higher in healthy subjects than in Crohn's patients. But an important question remains, said Doré. "Are these changes inducing the disease or are they an effect of the disease? To answer this question, we have to know more about the relationship with human cells."

In Ulcerative Colitis (UC) – one of the Inflammatory Bowel Diseases (IBD) - scientists from the University Hospital Vall d'Hebron in Barcelona have also found a reduction in the diversity of faecal microbiota.⁹ Moreover, the microbiota in these patients was less stable than in healthy persons. In the healthy subjects the similarity index after two years was 80 percent; in patients the similarity index after one year was only 40 percent. "So the expression of IBD may depend not only on genetic susceptibility and a dysbiosis between aggressive and

⁷ Klaassens et al AEM 73 (2007) 1388

⁸ Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. C. Manichanh et al. Gut 2006

⁹ Martinez et al. 2006

protective bacteria,” concluded Francisco Guarner. “But also on a reduced diversity and instability of the intestinal ecosystem.”

Scientists at the Alimentary Pharmabiotic Centre in Cork (Ireland) have studied the gut flora in patients with Irritable Bowel Syndrome (IBS), a syndrome characterized by ‘recurrent abdominal pain or discomfort’. They detected in general fewer Bifidobacteria and more Enterobacteriaceae from the faecal samples of 25 IBS patients, compared with the faecal samples of healthy persons.

Anne McCartney from the University of Reading (UK) presented three studies that confirmed an altered gut flora in children with autistic spectrum disorders. In a first study, the stool samples of 8 healthy children were compared with those of 13 children with late-onset autism.¹⁰ In an analysis of Clostridia it was found that faeces of autistic children not only contained higher levels of Clostridia, but also an altered combination of species: nine of the 25 cultivated Clostridia species were only found in autistic children; 3 were only found in control children. These differences were confirmed by other groups that used Real-time PCR and Fluorescence in situ hybridization (FISH).^{11 12} In the FISH study, the scientists compared 58 autistic children with 12 healthy siblings and 10 unrelated healthy children. The numbers of *Clostridium histolyticum*, were significantly higher in the autistic children than in the unrelated healthy children. Interestingly, the healthy siblings harboured intermediate levels, so there may be a genetic component involved. And, not coincidentally, the autistic children had more gut and intestinal problems than the healthy children, and they had also received multiple courses of antibiotics.

The emerging picture may be that specific diseases are characterized by a specific gut flora imbalance. Knowledge of these imbalances can help in selecting strains of probiotics to restore the flora. Diagnostic micro array chips could be designed that would allow scientists to differentiate between these sometimes overlapping clinical syndromes. These easy-to-use screening methods may help to predict, for example, which probiotics or other therapies are likely to be most suitable for the patient. Alternatively, they may help to predict whether a person will develop a specific disease. Nico Bos from the University of Groningen raised the question whether it is possible to predict Type 1 diabetes. His research group has studied BB-DP (diabetes-prone) rats. They have found that the composition of the microbiota is already different in the BB-DP rats that will become diabetic long before onset of diabetes.¹³

4. Gut flora influences obesity

The most pronounced influence of the gut flora has been found to be on obesity. The gut flora does not influence body weight at the evolutionary level of species, but at the deeper level of divisions or ‘phyla’. Normally, two bacterial phyla of beneficial bacteria are dominant in the human gut, the Bacteroidetes and the Firmicutes. In the past two years, the collaborating laboratories of Fredrik Bäckhed from Sahlgrenska University Hospital, Gothenburg (Sweden) and Jeffrey Gordon from the Washington University in St. Louis (USA) have shown that the relative proportion of the Bacteroidetes is remarkably lower in obese mice and humans than in lean mice and humans. For example, one study showed that lean mice had 40 percent

¹⁰ Finegold et al, 2002 (Clin. Infect. Dis)

¹¹ Song et al, 2004 (Appl Environ Microbiol)

¹² Parracho et al, 2005 (J Med Micro)

¹³ Brugman, 2006, Diabetologia, 49:2105

Bacteroidetes, while obese mice had only 20 percent Bacteroidetes (i.e. 20 percent of the analysed 16S rRNA sequences in the stool samples were from a Bacteroidetes phylotype).¹⁴ This important role of bacteria in regulation of body weight is understandable, explained Bäckhed: “Bacteria metabolize polysaccharides into short chain fatty acids (SCFA) and lactic acid. And 5 to 10 percent of our daily energy comes from these acids.” The crucial role of bacteria in food digestion is confirmed by the fact that germ-free mice are very lean, until they get gut bacteria. From that moment, they rapidly become fatter.¹⁵ The scientists have also confirmed the increased efficiency of the gut microbiota in obese mice through metagenomic and biochemical analyses.¹⁶ They demonstrated that changes in the relative abundance of the Bacteroidetes and the Firmicutes, affect the metabolic potential of the mouse gut microbiota. And they also demonstrated that the microbiota from obese mice have a greater capability to harvest energy from the diet.

The laboratories found more details in 12 obese people.¹⁷ They randomly assigned these people to either a fat-restricted (FAT-R) or to a carbohydrate-restricted (CARB-R) low calorie diet. The composition of their gut microbiota was monitored over the course of one year by sequencing 16sR ribosomal RNA genes from stool samples. The resulting data set of 18,348 bacterial sequences revealed that 70 percent of the 4074 identified phylotypes were unique to each person (see also **16S rDNA screens shed light on the diversity in our gut**). Despite these marked interpersonal differences in species-level diversity, members of the Bacteroidetes and Firmicutes divisions dominated the microbiota in all people – obese and not obese (92.6 percent of all 16S rRNA sequences). But the relative contribution of Bacteroidetes was only one or two percent in the obese people at the starting point of this study, while in lean controls the percentage was about 20 percent. However, the abundance of Bacteroidetes increased during body weight reduction, and the abundance of Firmicutes decreased, irrespective of diet type. This change was division-wide and not due to blooms or extinctions of specific bacterial species: bacterial diversity remained constant.

It would appear that the gut of obese people probably has as-yet uncharacterized properties that tip the balance towards the Firmicutes and more efficient digestion. However, this is not the only mechanism involved. Bäckhed showed that microbiota can also influence molecular signals involved in body weight regulation. His laboratory analyzed the effect of the Fasting induced adipose factor (FIAF).¹⁸ This is a protein that is increasingly expressed during fasting. Remarkably, germ-free, transgenic mice that cannot make FIAF develop diet-induced obesity, in contrast to wildtype germ-free mice. The study further revealed that the gut flora suppresses intestinal expression of FIAF, which results in higher LPL activity and more triglyceride storage in adipocytes and thus more body weight. Without gut microbiota (i.e. without suppression of FIAF) mice are protected against this stimulation of triglyceride storage.

These new insights indicate that manipulation of gut microbial communities could be another approach in the treatment of obesity.

5. Stress affects mucosal barrier

¹⁴ Ley et al, PNAS 2005

¹⁵ Bäckhed et al, PNAS 2004

¹⁶ An obesity-associated tug microbiome with increased capacity for energy harvest, Turnbaugh et al, Nature 2006

¹⁷ Ley et al, Nature December 2006

¹⁸ Bäckhed et al. PNAS 2007

Intestine-related diseases such as Crohn's disease and Irritable Bowel Syndrome are not only characterized by an imbalance in the gut microbiota, but also by impaired mucosal barrier function. But what are the external forces that effect this barrier? Chronic stress and early life traumas, explained Mary Perdue from McMaster University in Hamilton (Canada) and Johan Söderholm from the Linköping University (Sweden). And, said Perdue: "Probiotics taken during stressful periods can ameliorate the intestinal problems."

At least they do so in rats. During the past ten years, several laboratories have performed experiments with rats to uncover the mechanisms through which stress alters gut function. In one of the first experiments, it was shown how deeply stress can influence the gut physiology. Wild type rats and transgenic, mast cell deficient rats were exposed to Water Avoidance Stress for ten days (one hour a day on a platform surrounded by water). In less than two weeks, this chronic stress resulted in dramatic changes in the gut as well as in other systemic effects. All stressed animals had lost weight, while all controls had gained weight. In addition, the stressed rats defecated five times as much as the control rats. A pronounced effect was seen upon electron-microscope analysis of the mucosal barrier: wild type rats displayed prolonged barrier dysfunction and ion secretion, apoptosis of epithelial cells, bacterial adherence/penetration into the epithelium and low grade mucosal inflammation.¹⁹

So if human are comparable to rats, patients with intestine related diseases could end up in a vicious circle: chronic stress results in mucosal barrier defects, which lead to bacterial-epithelial attachment and penetration, in turn resulting in inflammatory cell influx and mast cell activation, ending in chronic stress. Can probiotics break this circle? The research groups from Canada and Sweden determined the effect of *Lactobacillus rhamnosus* and *Lactobacillus helveticus* (in a 20:1 ratio) on bacterial interactions with epithelium in stressed rats.²⁰ Rats were given probiotics in drinking water for 7 days before and during the ten days of water avoidance stress. And indeed, bacterial adherence and penetration was prevented by probiotics.

Mast cells apparently play an important role in this stress- induced process. This has already been seen in the rats exposed to water avoidance stress: the mucosal alterations were not found in the mast cell deficient rats. To elucidate the role of the mast cells further, researchers in Perdue's laboratory implanted mini pumps delivering the stress mediator Corticotropin-Releasing Hormone (CRH) in rats. And indeed, chronic exposure to this stress mediator mimicked the effects of chronic stress on weight and corticosterone in the blood, on mucosal barrier properties, and on bacterial adherence to mucosal surface. So it appears that this stress hormone CRH does play a role in the process, and therefore the mast cells do as well, because rat mast cells express two CRH receptors that are involved in the permeability and regulation of ions and macromolecules.

Scientists at McMaster University have also studied the effect of an early psychological trauma, maternal separation (individual separation of the pups from the mother, for three hours a day, two weeks before weaning). Maternal separation, Perdue concluded, induces long-term behavioural changes, such as more hesitant adults. It alters the susceptibility to mild stress in adult life.²¹ In addition, maternal separation causes altered gut barrier function and increased bacterial-epithelial interactions in the distal colon. The rats separated from their mothers had higher levels of serum corticosterone, and enhanced release of Acetylcholine

¹⁹ Santos et al, 2001; Soderholm et al, 2002

²⁰ Zareie et al, 2006

²¹ Soderholm et al, 2002

from enteric nerves. This resulted in a greater ion secretion and permeability in response to electric field stimulation of enteric nerves.²²

In these rats with an 'early life trauma', the long term effects of maternal separation could be prevented by using probiotics (the same *L. rhamnosus* and *L. helveticus* mixture). Neonatal rats were given probiotics orally and rectally during the period of daily separation. These treatments normalized macromolecular permeability, and reduced adherent bacteria. Remarkably, the rats only received probiotics in their first two week, but the beneficial effects were maintained into adulthood.

Söderholm concluded that probiotics can strengthen the mucosal barrier, but it is still unclear how they do this. Probably through a variety of different modes of action, such as inhibiting adhesion and invasion of pathogenic bacteria, preventing apoptosis of epithelial cells, and influencing specific biochemical pathways involved in the permeability of the barrier. Obviously more studies are needed, because the effects of a given probiotic cannot be generalized to all probiotics, and in vitro and in vivo effects also still differ. Söderholm: "It works, but we still have a long way to go before we know what bug or mixture is suitable for each occasion."

6. Probiotics may fulfil very specific tasks in our body

In order to select a probiotic strain for a given, specific application, in-vitro cellular models or experimental animals are often used. It is well recognized that the ultimate in-vivo effect in the human host may be different. Nevertheless, a reductionist approach can reveal unexpected properties of different probiotic strains, on which to build future applications.

Dennis Kasper from Harvard Medical School in Boston (the "king of reductionism") showed how a single polysaccharide molecule (PSA) found in only one phylotype, *Bacteroides fragilis*, can be responsible for regulation of one critical step in the development of the immune system: CD4+ T cell maturation. This is an important step in the immunomodulatory process, because these mature T helper cells play a crucial role in activating and directing many other cells types of the immune system.

The Kasper lab compared the numbers of CD4+ cells in the spleen of germ-free mice (mice lacking all gut bacteria) and in wild type mice. The germ-free mice had fewer CD4+ cells in the spleen (11 % of the total cell number) than wild type mice (17 %). When these mice were colonized with *B. fragilis*, the number of CD4+ cells was almost the same as in normal mice. So only *B. fragilis* appears to have this effect. However, when these *B. fragilis* lacked PSA, the therapy didn't work: the mice still suffered from a reduced number of CD4+ cells in the spleen. Therefore it was only the polysaccharide that was responsible. What's more, this special polysaccharide from *Bacteroides fragilis* seems to be quite selective. Splenic CD8+ T cells and CD19+ B cells - two other types of lymphocytes - were unaffected by colonization with these Bacteroidetes.

Kasper showed that the *B. fragilis* PSA has more immunomodulatory effects in germ-free mice; it corrected splenic ultrastructural defects, it induced Th1 cytokine production and it stimulated T regulatory cells to produce the potent immunoregulatory cytokine IL 10.

According to Kasper, IL-10 down-regulates inflammatory response and can down-regulate both Th1 and Th2 mediated disease. So *B. fragilis* can be regarded as a potent probiotic for

²² Gereau et al, 2006

patients suffering from allergic disorders. Alternatively - in keeping with the reductionist approach - PSA itself could be used as a potent immunomodulator.

During the workshop, more phylotypes were shown to be capable of inducing the secretion of IL-10. Two of these are *Lactobacillus reuteri* and *L. casei*. The research group of Yvette van Kooyk from the VU University Medical Centre in Amsterdam is investigating the mechanisms underlying their capacity to induce IL-10. They showed that both *Lactobacilli* bind dendritic cells (DC) *in vitro*. And Van Kooyk could also tell through which receptor they bind - the pathogen recognition receptor DC-SIGN. So these probiotics can induce regulatory T cells (*in vitro*), and thus the secretion of IL-10 by interaction with a single receptor. Another set of receptors which are a target for probiotics are the Toll-like receptors (TLR). TLR's are a class of single membrane-spanning non-catalytic receptors that recognize structurally conserved molecules derived from microbes. Elke Cario from the University Clinic Essen (Germany) showed that triggering of TLR-3 with poly I:C (a synthetic double-stranded RNA that is used experimentally to model viral infections *in vivo*), protected against induced colitis in rats. Also triggering of TLR9 with CpG DNA had a protective effect. The role of TLR4 in colitis is less clear, although polymorphisms in TLR4 are associated with disease susceptibility. In mice, the absence of SIGIRR, which is a signalling molecule for TLR4, renders the animals extremely susceptible to DSS colitis.

Eduard Stange from the Robert-Bosch-Krankenhaus in Stuttgart showed the capacity of the probiotic *Escherichia coli* Nissle to induce a specific defensin, named human beta-defensin-2, in intestinal epithelial cells. Defensins are endogenous antimicrobial peptides that kill pathogens by pore formation in the bacterial membranes. They are crucial when the mucosal barrier is damaged. By using flagellin mutants, the German researchers were able to prove that the induction of human-beta-defensin 2 is mediated through the flagellin of this phylotype. Other probiotics, including two *Lactobacilli*, can also activate the promoter of this human defensin, but not as powerfully as *E. coli* Nissle.

French and Belgian research groups have recently discovered an unexpected property of *L. acidophilus* and *L. salivarius*. These phylotypes can induce the expression of opioid and cannabinoid receptors in human epithelial cell cultures and in rats and mice. Administration of these strains to the animals also modulated perception of intestinal pain – the effect was comparable to that of morphine. The researchers evaluated the ability of five well-known and representative probiotic bacteria and two *E. coli* strains. Only *L. acidophilus* and *L. salivarius* seemed to have the ability to induce the expression of these promoters. According to several speakers, this research indicates a promising effect of these probiotics in patients with Irritable Bowel Syndrome.

Riitta Korpela, Vice President Research from the Finnish company Valio showed the capacity of *Lactobacillus helveticus* to degrade beta-casein of milk in bioactive tripeptides. Several clinical studies have already demonstrated that two of these tripeptides - Ile-Pro-Pro and Val-Pro-Pro - lower blood pressure and improve peripheral circulation. Valio first launched Evolus fermented milk with these two tripeptides in Finland in 2000.

An ability to interfere with pathogen Quorum Sensing (QS) mechanisms would also be desirable, explained Michael Blaut from the German Institute of Human Nutrition in Potsdam. Quorum Sensing means the regulation of gene expression in response to bacterial cell-population density. Many pathogens use QS mechanisms to attack their host. Together, organized in a biofilm or expressing the same virulence factors at the same time, they are more effective. It has not yet been proven that probiotics can prevent quorum sensing by pathogens. But it is a worthwhile avenue to explore, because it is already known that

probiotics also possess genes that are involved in quorum sensing. “If we develop the capability to interfere with quorum sensing in enteropathogens, probiotics could become a powerful tool for preventing the outbreak of infections.”

7. Clinical trials promising for probiotics in IBS and Necrotizing Enterocolitis (NE) (Studies still disappointing for Crohn’s disease)

Selecting a probiotic strain or mixture of strains requires laboratory studies, as outlined above. However, only controlled, randomized clinical studies can prove that probiotics are really effective. A number of promising clinical studies in this field were presented during the workshop. All speakers were positive about the prospects of probiotic strains for patients with Irritable Bowel Syndrome, Crohn’s disease, allergy, pancreatitis, urthritis and other inflammatory conditions and infections. However, most of them think it is still too early to draw definite conclusions about the effect of particular strains and doses for specific clinical situations. For general recommendations, hospitals will have to wait for larger comparative clinical studies.

Eamonn Quigley from University College Cork in Ireland presented clinical studies that have been done on patients with Irritable Bowel Syndrome. Ten to fifteen percent of the Western population suffers to a varying degree from IBS syndrome, characterized by ‘recurrent abdominal pain or discomfort at least 3 days per month.’ Of the nine probiotic studies Quigley found in the literature, five reported positive results. However, all of these studies were small, and could not be compared with each other because different definitions of IBS and different probiotic strains were used and the trial design and endpoints were also different.

So researchers from Cork, the University of Manchester and Procter & Gamble company set up a large, double-blind, placebo controlled study involving 362 women with IBS, who were recruited in primary care.²³ The women received milk containing *Bifidobacterium infantis* in three different bacteria concentrations or a placebo milk for four weeks. Throughout the study, patients daily recorded how they felt in terms of abdominal pain/discomfort, bloating/distension, sense of incomplete evacuation, straining at stool, urgency of bowel movement, passage of gas and mucus and bowel habit satisfaction. The patients also recorded their general assessment of relief.

At the end of the four-week study, the milk with the *B. infantis* concentration of one hundred thousand million cfu/dose (colony forming units per dose) was observed to be superior to the placebo milk for most forms of discomfort. However, the placebo milk also had a surprisingly positive effect, as was also found in the Danone study presented by Irene Lenoir. This placebo-effect is probably due to the high expectations of this new, promising therapy. The placebo effect was even significantly superior to the effect of the highest concentration *B. infantis* milk (one hundred times the middle concentration), and almost the same as that of the one hundred times lower concentration milk. The highest concentrations of bacteria turned into gel and therefore could not be delivered. Quigley’s conclusion was that some specific probiotics show promise for treating IBS. “Evidence from human studies continues to accumulate.”

However, Philip Marteau from the Hopital Lariboisiere & Paris University warned against excessive enthusiasm and extrapolating too quickly from positive studies. Until now the

²³ Whorwell et al, Am J Gastro July 2006

placebo controlled trials with Crohn's Disease patients have been quite disappointing, he indicated. Of the seven done in hospitals, four were negative. Also disappointing was the trial with 98 subjects operated on Crohn's Disease in which the Hopital Lariboisere collaborated.²⁴ The patients had taken a high concentration of *L. johnsonii* LA1 milk or a placebo for 6 months, but the differences were not significant. "Why have Probiotics failed so far in the treatment of Crohn's disease?" Marteau asked. "The researchers may have used the wrong strains, the wrong doses, clinical end points, patient populations or the wrong statistical methods. Probiotics do work for intestinal disorders in some situations," he concluded. "But in general, the effect of probiotics has not yet been sufficiently proven, with the exception of three clinical situations: lactose intolerance, gastroenteritis and antibiotic-associated diarrhoea."

But according to Cathy Hammerman from the Shaare Zedec Medical Centre in Jerusalem, there is already a fourth clinical situation in which probiotics are proving to be effective: probiotic supplementation to Low Birth Weight infants can reduce both the incidence and severity of Necrotizing Enterocolitis (NE). NE is one of the most feared diseases in neonates because it can progress rapidly from mild abdominal distension and feeding intolerance to fulminant septic shock, necrosis of the entire intestine and death (overall mortality is 20-50%). Necrotizing Enterocolitis is affecting an increasing number of neonates (4%-20% of infants weighing less than 1,5 kg), as doctors are now able to treat problems of immature lungs, which used to be the most important cause of death. Hammerman compared four clinical studies in which a total of 1784 neonates had received probiotics during the first weeks of their life, and 1832 babies that had received a placebo. In all studies the probiotics showed a positive effect, including her own study in which probiotics were administered to 72 neonates (73 were administered a placebo).²⁵ Seventeen babies in the control group died - three of Necrotizing Enterocolitis while only 6 babies died in the probiotic group, and none died of NEC. These results indicate prospects for the future, but more has to be elucidated. For example, the selection of strains for NEC patients has been relatively random up to now. "Our task for the future," she concluded, "is to select the right strains, to determine the best timing – when to begin treatment and for how long –, the right doses and the right treatment groups."

8. Ongoing studies of patients at risk of acute pancreatitis and allergy: a glimpse into the future

Hospitals can improve their clinical studies by collaborating on research. In the last three years, several multi centre studies have been organized in Europe. The PANDA study (Probiotics AND Allergy), was set up by UMC Utrecht and Wilhemina Children's Hospital in Utrecht. The researchers are investigating the effects of a probiotic produced by the Dutch company Winclove Bio Industries, on children at risk from allergy. In 2001, Finnish researchers showed a positive effect of a *Lactobacillus rhamnosus* strain in 53 at-risk children.²⁶ This preliminary study has often been cited, but Ger Rijkers from the UMC Utrecht warned against extrapolating too quickly from one positive study. He compared eight studies on the effect of probiotics in children at risk of developing allergic disease: two studies showed a positive effect, two did not, and three are still ongoing.

²⁴ Droault-Holowacz et al, DDW May 2007

²⁵ Bin-Nun et al, J Pediatr 2005: 147:192-196

²⁶ Kalliomaki et al. Lancet 357 (2001) 1076

In the PANDA study, 157 families have been divided into 79 placebo and 78 probiotic families. The researchers chose for a mix of *Bifidium bifidum*, *B. infantis* and *L. lactis* because of their good IL-10 inducing capacity as well as their efficient inhibition of IL-5 and IL-13 in vitro.²⁷

The mothers take the probiotic daily during the last two months of their pregnancy. After birth, they give the probiotic to the baby daily until he or she is one year old.

The researchers already have sampled some biomarkers in the babies of 122 families, and indeed, they were able to show an effect on the amount of the antibody IgE for egg, cow's milk, house dust mite, peanut and cat in the blood. In the probiotics group (59 children) none of the children had these IgE antibodies, whereas in the placebo group (63 children) seven children had one of these IgE antibodies. And, more importantly, the probiotic group had a reduced TH2 cytokine production and, at the age of three months, a lower incidence of atopic eczema. We now have to wait for more results, Rijkers explained. The scientists are determining the composition in the intestinal microbiota, the effects on the immune system and the effects on the clinical development of asthma and allergy.

A nationwide Dutch double-blind multi-centre study is PROPATRIA, a trial in which 293 adult patients with a first episode of predicted severe acute pancreatitis received a multispecies probiotic preparation from Winclove Bio Industries (Ecologic 641) or a placebo.²⁸ Pancreatitis is a severe, potentially lethal disease that is characterized by (small intestine) bacterial overgrowth, mucosal barrier failure and impaired host defence. The bacterial dysbiosis may be caused by hypomotility of the ileum, (for example due to morphine administration), starvation, malnutrition or antibiotic administration. This dysbiosis is the driving force behind bacterial translocation from the intestine to the pancreas, which turns the disease into a life-threatening situation. The trial is taking place in 15 Dutch hospitals. The researchers are investigating the effect of the probiotic on a number of factors including total number of infectious complications, mortality, use of antibiotics, total costs and bacterial resistance.

Harro Timmerman from Winclove Bio Industries presented the results of a comparable animal study.²⁹ Thirty-eight rats with a Boston model of acute pancreatitis were given Ecologic 641 or a placebo starting 5 days before induction of pancreatitis and continuing until 6 days after. Fewer animals died in the probiotics treated group (24% vs. 48%). After 7 days of pancreatitis, the Dutch researchers examined the rats that still were alive (62 %). And indeed, the probiotic strains had a positive effect. The number of pathogens had been reduced in the duodenum, spleen, pancreas and the blood of the probiotic rats. Further research showed that probiotics prevented mucosal barrier failure, resulting in less translocation of bacteria through the mucosal barrier.

Remarkably, the probiotic treated rats had significantly more of a specific commensal bacterium in the ileum. Presence of this bacterium was associated with improved pancreas pathology, reduced bacterial overgrowth in the small intestine and protection against bacterial translocation from the intestine. It appears, concluded Timmerman, that probiotics not only reduce pathogens, but also protect or stimulate commensal bacteria. "The question is: would probiotics have an effect without commensal bacteria anyway?" A negative answer has implications for the use of antibiotics, because antibiotics not only destroy pathogenic bacteria, but also commensal bacteria.

²⁷ Niers et al. *Clinical Experimental Allergy* 2005;35:1481-1489

²⁸ Besselink et al. *BMC Surg.* 2004 4:12 see also: www.pancreatitis.nl

²⁹ Van Minnen et al, *Surgery* 2007 (4): 470-80

9. Multi-centre studies comparing antibiotic treatments with probiotic treatments in patients with (urogenital tract) infections

Infections are often treated with antibiotics. However, antibiotics not only reduce the number of pathogens but also the numbers of commensal bacteria. And what's more, many pathogens are resistant to an increasing number of antibiotics. Because of these negative side-effects, it would be interesting to compare probiotic with antibiotic treatments.

This is the goal of a second nationwide Dutch multi-centre study, named Napruti (Non-Antibiotic versus Antibiotic Prophylaxis for Recurrent Urinary Tract Infections). Women with recurrent urinary tract infections, caused by a translocation of *Escherichia coli* into the bladder, have often been prescribed different antibiotics without a sustainable effect. In addition, they often have a reduced amount of commensals in the urogenital tract. Might probiotics be an alternative for antibiotics? Eight Dutch hospitals are participating in this trial. Two hundred women with recurrent urinary tract infections are taking an antibiotic or a probiotic for one year. The results are expected in 2009.

The Dutch decided to use *Lactobacillus rhamnosus* GR-1 from the Canadian R&D Centre for Probiotics in Ontario. This centre has demonstrated that *L. rhamnosus* can be delivered to the vaginal environment even if the probiotic is taken orally. Several studies have already confirmed this. Gregor Reid from this Canadian centre, presented a trial in which he had investigated whether this oral lactobacilli therapy is able to reduce the rise in urogenital pathogens.³⁰ He also compared this probiotic treatment with an antibiotic treatment in women with Bacterial Vaginosis. Twenty of them inserted two vaginal gelatine capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 at bedtime for five days; twenty women applied 0.75 % metronidazole vaginal gel twice a day for five days. The effect was remarkable: the signs and symptoms declined significantly in the probiotic group compared with the antibiotic group, even after 30 days. "The *Lactobacillus* strains *L. GR-1* and *RC-14* help to restore and maintain a healthy urogenital tract in women," Reid concluded. Reid also expects to see an effect of these probiotic strains on HIV. Unfortunately, probiotics have not yet been introduced in Africa, Reid remarked. "So their impact on reducing the risk of HIV in women still needs testing."

Ed Kuijper from Leiden University Medical Centre argued in favour of probiotic trials in patients at risk from *Clostridium difficile* associated diseases. The emergence of this hospital-acquired, cholera-like diarrhoea, is threatening the elderly in many hospitals in North America and Europe³¹, and the current antibiotic treatments are not successful. Probiotics may do better, but this needs to be confirmed in multi-centre trials.

According to Jürgen Schrezenmeir from the Federal Research Centre of Nutrition and Food in Kiel (Germany), probiotics may help also in reducing the risk of winter colds and other common infections. He presented the results of an open, randomized controlled study with 360 elderly people. The elderly had received either 2 bottles of Actimel (*Lactobacillus casei*) or a 'normal' fermented milk products per week during three weeks.³² The mean duration of the winter infections in that season was 7 days in the probiotic treated group, versus 8.7 days in the control group.

10. EU rules for probiotics: claims and safety

³⁰ Reid et al. 2003

³¹ Kuijper et al. *Clinical Microbiology and Infection* Oct 2006

³² Turchet et al, *J. Nutr. Aging*, 7 (2003), 75

Having read the above sections, the reader might think that companies will market their new probiotics as medicines. But this will not be the case. The probiotics which are already on the market are sold as food supplements and the same is likely to happen with the new probiotics. However, whereas the general probiotics use a “general health claim” (e.g. probiotic x supports the body's natural resistance), these clinically tested supplements will probably use a “reduction of disease risk claim” (e.g. probiotic y reduces the risk of allergy in at-risk children).

The EU distinguishes these different types of claims in its new Regulation on Nutrition and Health Claims, which came into force on July 1st 2007. Bart Degeest of Yakult explained the conditions for using these claims. Companies are allowed to use a general health claim when this claim is part of the “article 13.1 list” for health claims with “generally accepted scientific evidence”. All national authorities are currently submitting their own list, and the officials expect to have compiled an authorized EU list in 2010. This document will probably contain dozens of health claims including their scientific references and conditions of use. Clinical studies are not necessary for a product to be placed on the list. Animal studies, authorities and in-vitro data are enough to establish that the scientific evidence is “generally accepted”. In addition, food companies don’t need to compile their own scientific dossier. “All probiotics should definitely be part of the article 13.1 list,” concluded Degeest. “Then we can be sure that a substantial amount of generally accepted evidence exists.”

Probiotics also have the potential however to meet the “reduction of disease claim”, described in article 14.5, according to Degeest. But before using this claim, food companies have to submit their own scientific dossier, first to a national authority, then to the European Food Safety Authority (EFSA) in Parma.

Degeest expects ten guidelines for the evidence-based review system

1. Persuasiveness of each relevant study
2. Consistency of results across different studies
3. Consistency between various populations and within them
4. Magnitude of the effect
5. Strength of the association
6. Dose-response relationships
7. Temporal relationships
8. Biological plausibility
9. Specificity of the effect
10. Statistical validity

Experts from the EU programme PASSCLAIM have already elucidated the principles for the assessment. They have distinguished four levels of evidence. In-vitro studies, animal studies and small uncontrolled human studies are “insufficient”. Small controlled human studies supported by laboratory data make the claim “possible”. For the category “probable”, companies also need large-scale human studies, supporting epidemiological data, and positive reviews from independent experts. Only when independent expert bodies have accepted the evidence, will they be placed in the highest category “convincing”. “We need to continue research,” Degeest concluded.

Research is also necessary to guarantee the safety of strains that were not in use before 1997, because these new probiotics have to be judged according to the Novel Foods regulation (258/97/EC). Recently, the EFSA has proposed a simple decision tree to evaluate safety, a

Qualified Presumption of Safety (QPS). Although this decision tree consists of rather vague questions (e.g. what is the identity, does the available knowledge indicate safety concerns, and is the body of knowledge sufficient?), Degeest expects that most probiotics can be evaluated according to this simple tree. “In general, probiotics are safe.”