Lactobacillus plantarum 299v

CONTENT

Consumption of live lactic acid bacteria

Consumption of live lactic acid bacteria (LAB), included in fermented foods, has been a regular part of the human food intake for a long time. In fact, there are archaeological signs that humankind has used this technique from the beginning of time; it was presumably invented 1.5 million years ago by the early humanoids (Leakey 1993; Leakey 1995). Thus, humans have in this way consumed large numbers of live LAB throughout their entire history.

Fermentation is the simplest and often the safest way to preserve food, and before the Industrial Revolution, fermentation was applied just as much in Europe as it still is in many rural areas of the World. Thus, it could very well be that the human digestive tract evolved to adapt to a more or less daily supply of live LAB. This supply of live LAB ceased in many industrialized countries during the twentieth century, which eventually may have led to increased frequency of gastro-intestinal (GI) and immunological dysfunctions in urbanised humans.

When beneficial effects of certain types of live bacteria have been discussed, these types of bacteria have been gradually called "probiotics". The original concept of probiotics implies that the balance between beneficial and harmful bacteria in the microbiota of the GI-tract can be positively affected by eating the right type of live microorganisms (Parker 1974; Fuller 1989). However, the concept of probiotics is today used more generally for describing live bacteria that after ingestion, exercise health beneficial effects beyond conventional nutrition. It is presupposed that these health beneficial effects have been scientifically proved.

Functional groups and taxonomically based taxa

Lactic acid bacteria

The bacteria performing the conversion of carbohydrates to carboxylic acids, mainly lactic acid in traditional fermented foods, are called lactic acid bacteria (LAB). Food microbiologists used the term early, and 1919 the Danish bacteriologist Orla Jensen tried to define key features of LAB, unaware of the fact that LAB is not forming a systematically defined group based on evolutionary relationships; instead it can be regarded as a functional group used by food microbiologists, aiming at those bacteria that occur and multiply spontaneously in traditional lactic acid fermented foods. Furthermore, it is understood that LAB are harmless to human health. Already 2002, it was shown in meta-analyses of published clinical trials that different kind of LAB can be used to prevent antibiotic associated diarrhoea (D'Souza *et al.* 2002) and shorten the duration of acute diarrhoeal illness in children (Huang *et al.* 2002).

From the taxonomic point of view, LAB means a relatively wide variety of different taxonomically based groups (taxa). The only absolute condition for organisms involved in lactic acid fermentation of food must be that the bacteria mainly produce lactic acid and that they are harmless to consume in high numbers, even for consumers with underlying sicknesses that may have weaken their immunological defence. The different kind of lactic acid producing bacteria frequently occurring in high numbers in traditional, spontaneously fermented foods belong to genera as *Lactobacillus*, *Pediococcus*, *Weissella*, *Leuconostoc*, *Oenococcus*, *Lactococcus*, and the species *Streptococcus thermophilus* (and similar species).

The genera *Lactobacillus* and *Pediococcus* belong to the family *Lactobacillaceae* which also includes the relatively new genera *Paralactobacillus* and *Sharpea*. They can all be included in the trivial expression "lactobacilli".

Leuconostoc, *Weissella* and *Oenococcus* belong to the family *Leuconostocaceae* together with the genus *Fructobacillus*.

Lactococcus and *S. thermophilus* have from the phylogenetic point of view relatively little in common with *Lactobacillaceae* and *Leuconostocaceae* even if

they all are included in the order of *Lactobacillales*.

The species *Lactobacillus plantarum*

L. plantarum is one bacterial species in the huge and relatively diverse genus of *Lactobacillus*, which comprises about 90 validly named species and subspecies. By tradition, the *Lactobacillus* spp. have been divided into three functional groups depending on their fermentation abilities; the obligate homofermentatives (Group I), the facultative heterofermentatives (Group II) and the obligate heterofermentatives (Group III) (Kandler and Weiss 1986). Group I ferment hexoses exclusively to lactic acid, and can't ferment gluconate or pentoses, while Group II also ferments hexoses to lactic acid but is additionally able to ferment pentoses and/or gluconate. Group III ferments hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide. *L. plantarum* is facultatively heterofermentative. The type strain of *L. plantarum* is ATCC 14917^T (Kandler and Weiss 1986).

L. plantarum differs from many other *Lactobacillus* spp. in the following points:

1) *L. plantarum* has a relatively large genome in comparison with many other *Lactobacillus* spp. This indicates an adaptive ability for many different conditions (Kleerebezem *et al.* 2003).

2) *L. plantarum* can ferment many different carbohydrates.

3) *L. plantarum* has a high growth requirement for manganese and can accumulate high intercellular levels of manganese (Archibald and Fridovich 1981b). Manganese provides a defence for *L. plantarum* against oxygen toxicity by the reduction of oxygen free radicals to hydrogen peroxide (H_2O_2; Archibald and Fridovich 1981a). The produced H_2O_2 can then be converted to oxygen (O_2) and water by manganese cofactored pseudocatalase (Kono and Fridovich 1983a, 1983b).

4) *L. plantarum* have a high tolerance to low pH (Daeschel and Nes 1995). The fact that *L. plantarum* frequently predominate in spontaneously, lactic acid fermented foods where the final pH usually is below 4.0, and also can survive the passage through the acid conditions of the human stomach (Johansson *et al.* 1993), points to the high resistance to acid conditions.

5) *L. plantarum* can possess tannase activity (Osawa *et al.* 2000; Vaquero *et al.* 2004) and are also able to metabolise phenolic acids (Barthelmebs *et al.* 2000; Barthelmebs *et al.* 2001). Furthermore, a strain of *L. plantarum* (IFPL935) was able to metabolize a flavan-3-ol enriched grape seed extract by means of galloyl-esterase, decarboxylase and benzyl alcohol dehydrogenase activities (Tabasco *et al.* 2011).

L. plantarum frequently occurs and multiply spontaneously to high numbers

in most lactic acid fermented foods, especially when the foods are based on plant material, for example, in brined olives (Fernández Gonzalez *et al.*1993), capers (caper berries; Pulido *et al.* 2005), sauerkraut (Dedicatoria *et al.*1981; Plengvidhya *et al.* 2007), salted gherkins (McDonald *et al.* 1993), sour-dough (Lönner and Ahrné 1995), Nigerian ogi (made from maize or sorghum) (Johansson 1995a), Ethiopian kocho (made from starch from *Ensete ventricosum*) (Gashe 1985; Nigatu 1998), Ethiopian sour-dough made out of tef (*Eragrostis tef*) (Gashe 1987; Nigatu 1998) and cassava (Oyewole and Odunfa 1990; Moorthy and Mathew 1998). *L. plantarum* also occurs in grape juice and wine (Vaquero *et al.* 2004). Thus, it is obvious that individuals consuming traditionally fermented products of plant origin that haven't been heat-treated also consume large amounts of live *L. plantarum*. Not surprisingly, *L. plantarum* frequently occurs in the human GI-tract, from the mouth to the rectum (Molin *et al.* 1993; Ahrné *et al.* 1998).

In order to get an idea how humans acquire immune tolerance against harmless, food-associated bacteria, van Baarlen *et al.* (2009) studied the stimulating effect of *Lactobacillus plantarum* (strain WCFS1) on the immune system of adult, healthy volunteers in a randomized double-blind placebo-controlled cross-over study. The subjects ingested either live or heat-killed *L. plantarum*. The expression profiles in biopsies taken from the intestinal duodenal mucosa were analyzed using whole-genome microarrays and by biological pathway reconstructions. The expression profiles displayed differences in modulation of NF-*kappa*B-dependent pathways, notably after consumption of live *L. plantarum*. In other words, it was seen that the mucosal gene expression patterns and cellular pathways correlated with the establishment of immune tolerance after consumption of live *L. plantarum* (van Baarlen *et al.* 2009). This demonstrates a close relationship between *L. plantarum* and the immune-affected physiology of humans.

Furthermore, genotyping of twenty different strains of *L. plantarum* from various sources have been assessed by microarrays containing a subset of small genomic fragments from the strain, *L. plantarum* WCFS1 (Molenaar *et al.* 2005). It was shown that genes involved in sugar transport and catabolism were highly variable between strains while those involved in biosynthesis or degradation of structural compounds like proteins, lipids and DNA were conserved (Molenaar *et al.* 2005).

The bacterial strain, *Lactobacillus plantarum* 299v

Taxonomic considerations

The strain *L. plantarum* 299v (=DSM 9843) has been isolated from healthy human intestinal mucosa (Molin *et al.* 1993; one of the two isolates labelled 299 in Table 1 of the paper). *L. plantarum* 299v was granted patent in Europe

and USA amongst others. Taxonomically, *L. plantarum* 299v is included in a genetic subgroup within the species *L. plantarum* (Johansson *et al.* 1995b) where the members mostly originate from human intestinal mucosa, but also can be found in traditional lactic acid fermented foods. *L. plantarum* strains of this particular genomic subtype can be found dominating the total *Lactobacillus* flora of healthy individuals, both on oral and rectal mucosa (Molin *et al.* 1993; Ahrné *et al.* 1998).

Closely related strains of *L. plantarum* can be defined and identified by restriction endonculease analysis (REA) of total chromosomal DNA by the use of relatively frequently cutting restriction enzymes such as *Eco*RI and *Cla*I, and the fragment pattern can be visualised by traditional agarose gel electrophoresis (Johansson *et al.* 1995b). This method was successfully used for strain-definition and re-isolation of *L. plantarum* 299v from mucosal biopsies obtained in an administration study in humans (Johansson *et al* 1993). *L. plantarum* 299v could be re-isolated after oral administration from mucosal biopsies taken from jejunum and rectum (Johansson *et al* 1993). In some individuals *L. plantarum* 299v could be found as the dominating part of the mucosal lactobacilli-flora, even 11 days after the administration (Johansson *et al* 1993).

L. plantarum 299v contains four plasmids of the size 4, 9, 15 and 21 Mda (Johansson *et al.* 1995d). The strain has the same genomic ribopattern (Restriction fragment length polymorphism of the 16S rRNA gene) as the type strain of *L. plantarum* (ATCC 14917[T]) with four bands (operons) showed after cleavage with the endonuclease *Eco*R1 and five bands after cleavage with *Hind*III (Johansson *et al.* 1995d).

When the genome of *L. plantarum* 299v was compared (by microarrays containing a subset of small genomic fragments of the strain *L. plantarum* WCFS1) with the genomes of 19 other *L. plantarum* strains (Molenaar *et al.* 2005), *L. plantarum* 299v was shown to be genomically different from all the other test-strains, but was closest related to the strain, *L. plantarum* 299 (=DSM6595) (Molenaar *et al.* 2005).

Tolerance to environmental stress

L. plantarum 299v can grow in 6% NaCl under otherwise optimal conditions, and it shows sub-lethal growth up to 16 % NaCl (Melgar-Lalanne *et al.* 2014). Thus, *L. plantarum* 299v can be considered as halotolerant.

L. plantarum 299v possess a wide pH-tolerance, and grow at pH between 4.0 and 8.0; with a sub-lethal growth down to pH 2.0 and up to 9.0 (Melgar-Lalanne *et al.* 2014)

L. plantarum 299v is able to grow in the presence of bile salts up to 2%

(Melgar-Lalanne *et al.* 2014).

Adhesion and cell surface

Strains of the "*L. plantarum* 299v subgroup" (see above) have a pronounced ability to attach to human mucosa cells *in vitro* and the adhesion is dependent on a mechanism for mannose-binding adherence (Adlerberth *et al.* 1996; Ahrné *et al.* 1998). These results were confirmed by Tallon *et al.* (2007) that also showed that *L. plantarum* 299v adhere to mucin. A mannose adhesion-encoding gene in *L. plantarum* has been identified Pretzer *et al.* (2005). The mannose-binding adherence mechanism has been shown to be crucial for the immune modulating ability of *L. plantarum* 299v in human HT-29 colonic epithelial cell line (McCracken *et al.* 2002). The adherence mechanism was also important for the ability of *L. plantarum* 299v to decrease translocation in a septic rat model (Mangell *et al.* 2006).

The glycolytic enzymes glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and enolase (ENO) are normally regarded as intracellular but nevertheless they have been isolated from outer cell surface of *L. plantarum* 299v (Glenting *et al.* 2013). When the adhesive properties of these two enzymes were characterized, it was demonstrated that both have a highly specific binding to plasmogen and fibronectin while GAPDH but not ENO also showed weak binding to mucin. Furthermore, a pH-dependant and specific binding to Caco-2 cells was found for both enzymes (Glenting *et al.* 2013). It seems as these two enzymes could play a role in the adhesion of *L. plantarum* 299v to GI-mucosa.

GAPDH can also be regarded as a housekeeping enzyme (Saad *et al.* 2009). The cell wall GAPDH (cw-GAPDH) formed by *L. plantarum* 299v were characterized and shown to be identical homotetrameric active enzymes. It was demonstrated that the increase in cw-GAPDH concentration from the early exponential growth-phase to the late stationary phase is closely related to an increase in plasma membrane permeability. Also, it was established that cells with impaired membrane manifested five times more cw-GAPDH than unaltered cells. Thus, plasma membrane permeability of *L. plantarum* 299v appears to be closely related to the efflux of GAPDH on the bacterial cell surface (Saad *et al.* 2009).

Ability to digest polyphenols

It has been demonstrated that *L. plantarum* 299v are able to metabolize the tea derived polyphenol, theaflavin 3,3′-digallate (TFDG) into pyrogallol, gallic acid, theaflavin, theaflavin 3-gallate and theaflavin 3′-gallate (Chen *et al.* 2012).

ProViva: Fermented oatmeal in a fruit drink

Content and production

L. plantarum 299v is included in a Swedish functional food product with the brand name ProViva (Molin 1995; Molin and Ahrné 1999; Molin 2001; Molin 2003; Molin 2007; Molin 2008). ProViva is a fruit-based beverage that has been produced and marketed in Sweden since 1992.

The lactic acid fermented component in the ProViva is an oatmeal beverage (formula) that has been fermented with *L. plantarum* 299v (Figure 1).
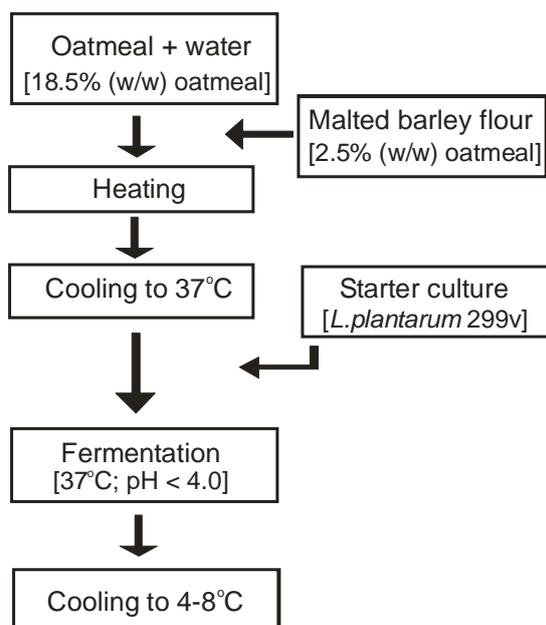
Figure 1. Flow scheme of the production of lactic acid fermented oatmeal formula to be used in ProViva.

The fermentation results in about 10^{11} colony forming units (CFU) of *L. plantarum* 299v per ml of oatmeal formula. This fermented oatmeal formula was originally developed as a new concept for enteral feeding (nasogastric feeding) (Molin *et al.* 1991a). The lactic acid fermented oatmeal formula is an integral part of ProViva, where 5% fermented oatmeal formula has been mixed with different types of fruit drinks, including for example, rose hip, blueberry, blackcurrant, raspberry and tropical fruits. In the final product (ProViva) there is about 5×10^7 CFU of *L. plantarum* 299v per ml of fruit drink. The

process to produce the lactic acid fermented oatmeal beverage was patented (Molin *et al.* 1991a).

The viscosity of the products is during the process lowered by a supplement of malt flour (malted barley) in combination with the heat treatment, followed by the decreased pH in the lactic acid fermentation. When the fermented oatmeal formula was intended to provide a base for a nutritional formula for enteral feeding, low viscosity and high energy density were prerequisites (Molin *et al.* 1991a). Without added malt flour, the oat meal formula with the stated concentration of flour (18.5%; w/w) will form porridge impossible to administer through a thin tube (Molin *et al.* 1991a; Marklinder and Lönner 1994; Marklinder 1996). The decrease in viscosity is presumably due to degradation of starch, and malted barley is rich in amylases. Also the solubility of *beta*-glucans is increased by the process, and if higher amounts of malt are used, or extra malt flour is added after the heat treatment, there will be a substantial reduction in total amount of *beta*-glucans (Marklinder and Lönner 1994; Marklinder 1996). However, the *beta*-glucans are considered valuable as they are believed to delay intestinal absorption and beneficially affect cholesterol and glucose metabolism. The process does cause a small reduction of the total content of *beta*-glucans.

At the time, the lactic acid fermented oatmeal formula provided about 76% of the energy and 70% the protein and 99% of the carbohydrate content compared to the average nutrient content in commercial nutritive solutions intended for enteral feeding (Marklinder and Lönner 1994). The formula is relatively rich in *beta*-glucans, thiamine, phosphorus, iron, copper and manganese (Marklinder and Lönner 1994).

Insulin demand

The impact of the fermented oat meal formula supplemented with bilberries on glycaemic and insulinaemic responses in humans have been evaluated (Granfeldt and Björck 2011). Glycaemic and insulinaemic responses in young healthy adults were measured in different combinations of fermented oatmeal (fermented with *L. plantarum* 299v) and added bilberries. It was found that the insulin index was remarkably lower than expected in view of the carbohydrate content of the treatment product. The authors concluded that the "*fermented oat meal drink added with bilberries induced a lower insulin response than expected from the glycaemic response. The mechanism for the lowered acute insulin demand is still unclear, but may be related to some bio-active component present in the bilberries, or to the fermented oat meal base*" (Granfeldt and Björck 2011).

Beneficial health effects

Intestinal microbiota

Survival of L. plantarum 299v

It is a well established fact that high numbers of lactobacilli counteract many pathogenic and potentially pathogenic bacteria, regardless of whether the ecological niche is lactic acid fermented food or human intestines (De Vuyst and Vandamme 1994a, 1994b). The original concept of probiotics implies that the balance between beneficial and harmful bacteria in the microbiota of the GI-tract can be positively affected by eating the right type of living microorganisms (Parker 1974; Fuller 1989).

L. plantarum 299v was after oral administration to humans found in high numbers on the rectal mucosa (Nobaek et al. 2000) and in faeces (Johansson et al. 1998; Nobaek et al. 2000; Önning et al. 2003; Goossens et al. 2003; Berggren et al. 2003; Goossens et al. 2005; Goossens et al. 2006a; Goossens et al. 2006c). Furthermore, L. plantarum 299v already adhere to the tonsillar mucosa directly after oral intake (Stjernquist-Desatnik et al. 2000).

The in vivo gene expression of L. plantarum 299v in the human gut has been demonstrated (de Vries et al. 2006; Marco et al. 2010). Prior to surgery, three patients diagnosed with colon cancer ingested L. plantarum 299v (10^{11} CFU per d) for one week. Total RNA was isolated from the mucosa of surgically removed intestinal segments of tissue, and hybridized to a DNA microarray comprising clones covering the L. plantarum genome. The presence of living L. plantarum 299v on the mucosa was confirmed, and the ingested L. plantarum 299v cells were metabolically active in all subjects as demonstrated by the detection of about 10% expressed genes by the DNA microarrays (de Vries et al. 2006; Marco et al. 2010).

Antagonistic effects against adverse microorganisms

The current definition of probiotics has changed somewhat since Fuller (1989). Today probiotics are "live microorganisms with beneficial health effects when administrated to animals and humans", but even so, the original concept of counteracting deleterious bacteria in the GI-tract still remains. A crucial question is what components of the intestinal flora that should be suppressed?

That it would be beneficial if probiotics can inhibit pathogens is self-evident, but true pathogens are not supposed to be part of the resident microbiota. Examples of frequently occurring components of the human intestinal microbiota that can have negative health implications and therefore should be counteracted are *Bacteroides fragilis* and the different genera and species of the family *Enterobacteriaceae* (for example, *Escherichia coli* and *Klebsiella pneumoniae*). Members of the *Enterobacteriaceae* family can be found in high numbers in the normal microbiota are also frequently involved in abdominal infections and sepsis. They synthesise lipopolysaccharides (LPS; also called endotoxins) associated to the cell walls. LPS have strong proinflammatory effects if it enters the circulation.

Rats pre-treated with the Gram-negative, *Bacteroides fragilis*, before the onset of an acute liver injury, developed a significantly poorer liver status than control rats with the liver injury but without bacterial pre-treatment (Adawi *et al.* 1999a). Some strains of *B. fragilis* can also secrete toxin that activates T-cell factor dependant β-catenin nuclear signalling in intestinal epithelial cells. It has been suggested that this event may contribute to oncogenic transformation in the colon (Wu *et al.* 2003).

L. plantarum 299v possesses anti-microbial activity *in vitro* against potentially pathogenic species such as *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Yersinia enterocolitica*, *Citrobacter freundii*, *Enterobacter cloacae* and *Enterococcus faecalis* (Jacobsen *et al.* 1999), and relatively strong antagonistic properties against *Salmonella enterica* subsp. *enterica* (Hütt *et al.* 2006), and more intermediate antagonistic activity against *Helicobacter pylori* (Hütt *et al.* 2006). *L. plantarum* 299v has also strong inhibitory effect *in vitro* against *Streptococcus mutans* and *Candida albicans* which both are suggested to be associated to caries (Hasslöf *et al.* 2010). The ability of *L. plantarum* 299v to mitigate *S. mutans* (biofilm formation *in vitro*) was confirmed by Söderling *et al.* (2011).

When healthy volunteers consumed a mixture of lactobacilli strains, including *L. plantarum* 299v, there was a decrease in the level of Gram-negative anaerobes, *Enterobacteriaceae* and sulphite-reducing clostridia (Johansson *et al.* 1993).

Enterobacteriaceae is a family including many pathogenic and opportunistically pathogenic taxa, and even normally non-pathogenic taxa of *Enterobacteriaceae* can have a pathogenic potential in situations where the immunological defence of the host is failing. The inhibitory effect of *L. plantarum* 299v against *Enterobacteriaceae* (Mao *et al.* 1996a; Adawi *et al.* 1997; Wang *et al.* 2001; Osman *et al.* 2005) and Gram-negative anaerobes (Mao *et al.* 1996a) has been demonstrated *in vivo*, in rat-models simulating severe clinical conditions.

L. plantarum 299v inhibits adhesion of enteropathogenic and enterohemorrhagic *Escherichia coli* to intestinal epithelial cell-cultures by inducing mucin expression in the epithelial cells, i.e. intestinal epithelial cells produced more mucin which limited the access for *E. coli* to bind to their surface (Mack *et al.*1999; Mack *et al.* 2003). The ability of *L. plantarum* 299v to reduce secretory response of intestinal epithelial cells to enteropathogenic *E. coli* (EPEC) has been shown *in vitro* (Michail and Abernathy, 2002). The observed effect was due to reduced attachment of EPEC to epithelial cells (Michail and Abernathy, 2002). Furthermore, *L. plantarum* 299v increased Muc3 protein and mRNA expression *in vivo* (rats) in jejunum and ileum (Dykstra *et al.* 2011).

Colonization of *L. plantarum* 299v competes with that of *E. coli* in gnotobiotic rats (Herías *et al.* 1999). It has been shown that increased levels of *E. coli* in pregnant rats resulted in pups with increased systemic inflammation (Fåk *et al.* 2008a). No such negative effects were seen when the rat mothers were administrated *L. plantarum* 299v, instead, the gut maturation improved (Fåk *et al* 2008b).

Gram-negative anaerobes are noxious from the viewpoint that they often are involved in secondary infections after abdominal surgery (Nichols 1980; Offenbartl and Bengmark 1990; Wittman 1991). Furthermore, Gram-negative bacteria contain lipopolysaccharides (LPS) and they can initiate, even when present in small numbers, violent inflammatory reactions. Gram-negative anaerobes are also suggested to be producers of carcinogenic substances in the intestine (Rowland 1992; Roberfroid and Gibbson 1994). The inhibitory effect of *L. plantarum* 299v against *Bacteroides* was shown in a placebo controlled study in patients with inactive ulcerative colitis (Goossens *et al.* 2006b).

Sulphite-reducing clostridia includes species with toxin producing capacity, and sulphite-reducing clostridia generally produce hydrogen sulphide (H_2S) that is a gentoxic agent in concentrations as low as 250 µmol/litre which is in agreement to that found in the human colon (Attene-Ramos *et al.* 2006). The well known pathogens, *Clostridium perfringens* and *Clostridium difficile* belongs to *Clostridium* cluster I and *Clostridium* cluster XI of Collins, respectively. *L. plantarum* 299v has been found to reduce recurrence of *Clostridium difficile*-associated disease, i.e. enteral administration of *L. plantarum* 299v to critically ill patients treated with antibiotics reduced colonisation with *C. difficile* (Klarin *et al.* 2008).

In a study in Tanzania, *L. plantarum* 299v was used as a starter culture for producing the cereal based lactic acid fermented beverage Togwa. *L. plantarum* 299v was used for producing 50% of the test-togwa while the other 50% was made by traditional back sloping (Kingamkono *et al.* 1999). Spontaneously fermented togwa is frequently dominated by *L. plantarum* (Mugula 2001). The product was given to children (<5 years) once a day for 13 consecutive days and the presence of faecal enteropathogens such as

Campylobacter, enteropathogenic *Escherichia coli*, *Salmonella* and *Shigella* was evaluated. The proportion of children with isolated faecal enteropathogens decreased significantly (P<0.001) among children given Togwa during the study period (Kingamkono *et al.* 1999).

Effects on the gastro-intestinal environment

The ingestion of LAB can positively alter the bacterial flora of the GI-tract as has been seen by the decreased plate counts of *Enterobacteriaceae* and sulphite reducing clostridia after ingestion (Johansson *et al.* 1993). In a randomized, placebo controlled, double blinded study in healthy volunteers that consumed *L. plantarum* 299v in a fruit drink ($2x10^{10}$ CFU/day for 3 weeks), the total level of carboxylic acids in faeces increased (Johansson *et al.* 1998); it was the concentration of acetic acid and propionic acid that increased (Johansson *et al.* 1998). The carboxyl acids are produced by the GI-microbiota, and this change in acid composition points at significant changes in the composition of the microbiota. The increased concentration of especially propionic acid must be regarded as beneficial from a health-perspective. Short-chain fatty acids are the major energy source of the colonic mucosa cells. Therefore, an increased level of short-chain fatty acids in the lumen should be beneficial for the condition of the mucosa. Moreover, absorbed propionic acid comes via the portal blood to the liver where it can have positive effects on both lipid metabolism and inflammatory response. Healthy subjects receiving *L. plantarum* 299v experienced a decrease in flatulence during the treatment period (Johansson *et al.* 1998), which might indicate that the concentration of gas-producing bacteria in the GI-tract was decreased.

In a double blind, placebo controlled study in patients with cardiovascular disease it was shown that a daily intake of *L. plantarum* 299v increased the diversity of the colonic microbiota, i.e. administration of this single-strain increased the bacterial diversity on the rectal mucosa (Karlsson *et al.* 2010). The results suggest that administration of *L. plantarum* 299v might be a strategy to favour a diverse intestinal microbiota and prohibit overgrowth by, for example *E. coli*. A healthy mucosa decrease the risk of translocating of "pathogen-associated molecular patterns" that negatively affects atherosclerosis.

Intestinal mucosal condition and reduced translocation

Liver injury

Bacterial translocation increases with a decreasing mucosal condition (translocation = the passage of viable bacteria through the epithelial mucosa into the *lamina propria* and then to the mesenteric lymph nodes and possibly

other tissues [Berg and Garlington 1979]). Translocation can be studied in rats with an acute liver injury, induced by an injection with D-galactose-amine which selectively causes inflammation in the liver (Kasravi *et al.* 1996a; Kasravi *et al.* 1996b). Twenty-four hours after the onset of the liver inflammation, translocating bacteria from the gut can be found in organs such as the liver and spleen, and in the blood. The liver injury does not directly affect the intestinal mucosa but the immunological defence of the animal is severely weakened, which allows the translocating bacteria to travel beyond the mesenteric lymph-nodes and the liver. However, by pre-treating the animals with *L. plantarum* 299v before the onset of the liver inflammation, the translocation was significantly decreased (Adawi *et al.* 1997; Adawi *et al.* 1999a; Kasravi *et al.* 1997; Wang *et al.* 2001; Osman *et al.* 2005).

It is interesting to identify what sort of bacteria that are translocating in rats with liver failure (Wang *et al.* 2001). In rats that not had received any probiotic treatment, the majority of the bacteria found in the liver originated from the dominating population of the intestinal mucosal microbiota, i.e. *Lactobacillus animalis*, *Lactobacillus reuteri* and *Lactobacillus acidophilus* which often is part of the dominant microbiota in rodents, but also more aggressive species were found, i.e. *Proteus vulgaris*, *Bacteroides distasonis*, *Enterococcus faecalis* and *Staphylococcus aureus* (Wang *et al.* 2001). Furthermore, *P. vulgaris* and *S. aureus* were also found in the arterial blood (Wang *et al.* 2001). However, pre-treatment for 8 days with *L. plantarum* 299v before the liver injury did not only decrease the rate of translocation to the liver, but no bacteria whatsoever translocated to the blood and only *L. animalis*, *L. reuteri* and *L. acidophilus* were found in the liver (Wang *et al.* 2001). Thus, the *L. plantarum* treatment did not only decrease the rate of translocation, it obviously had a controlling impact on the intestinal microbiota and enhanced the domination of *Lactobacillus*. It can be noted that *L. plantarum* 299v never was found in extra-intestinal sites in spite of the large treatment doses (Wang *et al.* 2001).

Many of the intestinal bacteria that translocate in rats with liver failure will end-up in the liver which will enhance the inflammation of the liver and the condition of the liver will deteriorate. This deterioration can be measured by the concentration of liver enzymes in the blood. In the liver injury model, it was shown that pre-treatment with *L. plantarum* 299v decreased the concentration of the liver enzymes asparate-transaminase (AST) and alanine-transaminase (ALT) in the blood, indicating that the liver status was improved by the treatment (Adawi *et al.* 1997; Kasravi *et al.* 1997; Adawi et al. 1999b).

Mucosal status

The effect of *L. plantarum* 299v on the mucosal status (condition) and barrier function has been extensively studied in rat models. When the status of the intestinal mucosa was evaluated using the content of protein, or content of

rRNA and DNA as markers, an improvement in the mucosal status was found in rats with acute liver injury that had been pre-treated with *L. plantarum* 299v (Kasravi *et al.* 1997; Adawi *et al.* 1999b). An improved mucosal status was also seen in rats with enterocolitis that had been treated with *L. plantarum* 299v (Mao *et al.* 1996a). In the study of Mao *et al.* (1996a), the permeability of EDTA through the mucosa was measured and found to decrease in animals receiving *L. plantarum* 299v (Mao *et al.* 1996a).

In a maternal model with healthy rats, the consumption of *L. plantarum* 299v of dams affected gastrointestinal growth and function in the suckling pups (Fåk *et al.*, 2008b). The weight of the small intestine, pancreas and liver increased in 14 days old pups from dams treated with *L. plantarum* 299v in the drinking water compared with control pups from dams given only water without lactobacilli. Furthermore, the *L. plantarum*-treated pups had an improved gut barrier function (decreased permeability; Fåk *et al.*, 2008b). Also direct exposure of pups to *L. plantarum* 299v resulted in lower permeability of the gut (Fåk *et al*, 2008c).

Translocation in different in vivo *models*

The preventive effect of *L. plantarum* 299v on bacterial translocation from the GI-tract has been seen in several different experimental *in vivo* models:

1) Pre-treatment of rats with *L. plantarum* 299v in the drinking water for one week inhibited *E. coli*-induced permeability of the intestine (Mangell *et al.* 2002). This was studied in intestinal segments mounted in an Ussing chamber where the permeability of mannitol was measured. Exposure to *E. coli* in the Ussing chamber normally increases the permeability, but the pre-treatment of the living rats with *L. plantarum* 299v abolished this increase in permeability (Mangell *et al.* 2002).

2) *L. plantarum* 299v significantly reduced the translocation in rats with enterocolitis, induced by the chemo-therapeutic drug, methotrexate (Mao *et al.* 1996a). In this model, the mucosa became inflamed and damaged. The lactobacilli administration to the enterocolitis rats mitigated the mucosal injuries induced by the chemotherapy (Mao *et al.* 1996a).

3) In an experimental rat model with pancreatitis, a decreased translocation was observed by treatment with *L. plantarum* 299v (Mangiante *et al.* 2001).

4) In a DSS (dextran sulphate sodium) induced colitis model in rat, a decreased translocation was observed by treatment with *L. plantarum* 299v (Osman *et al.* 2004).

5) In a septic rat model, a decreased translocation was observed by treatment with *L. plantarum* 299v (Mangell *et al.* 2006).

Protective mechanisms

There can be several explanations as to how *L. plantarum* 299v improve the mucosal status and decrease the translocation rate from the intestines, i.e. (*i*) by a beneficial immunomodulation, (*ii*) a stimulation of the mucin-production of the intestinal epithelial cells, (*iii*) improved function of tight junctions of the epithelial cell layer of the mucosa, or the more traditional explanation that (*iv*) the administered probiotic strain counteracts adverse bacteria. Aggressive, adverse bacteria in the resident microbiota are more prone to induce and maintain an inflammation as they more easily translocate and have pro-inflammatory compounds in their cell-walls. It is possible that the probiotic strain not only counteracts adverse components of the microbiota, probiotics may also stimulate beneficial taxa in the resident flora. Such a stimulating effect was indicated in humans when the amount of propionic acid in faeces increased after consumption of *L. plantarum* 299v, since propioinic acid is not produced by 299v, instead some other component of the gut microbiota able to produce propionic acid must have been stimulated (Johansson *et al.* 1998).

It has been shown in septic rats that the mannose sensitive adhesion-ability of *L. plantarum* 299v was important for the translocation-blocking capability (Mangell *et al.* 2006). Furthermore, the domination of resident intestinal lactobacilli of rats increased after treatment with *L. plantarum* 299v (Wang *et al.* 2001).

In humans, the overall bacterial diversity of the gut flora was increased after administration of *L. plantarum* 299v (Karlsson *et al.* 2010).

Translocation in humans

In a prospective randomised controlled study in patients undergoing elective abdominal surgery, it was pointed out that the concentration of IgM antibodies at the mucosal surface in specimens of normal small bowel was increased in the control group while it was constant in the patients given *L. plantarum* 299v prior to surgery (Woodcock *et al.* 2004). An increase in IgM can be seen as a marker for bacterial translocation (Woodcock *et al.* 2001; Woodcock *et al.* 2004).

In patients in intensive care, *L. plantarum* 299v improved the gut barrier function (Klarin *et al.* 2008).

In patients with obstructive jaundice undergoing biliary drainage, the gut barrier function was improved by administration of *L. plantarum* 299v (Jones *et al.* 2013). The authors concluded "*Pretreatment with probiotic LP299v improves intestinal permeability after biliary drainage and attenuates the*

inflammatory response. However, a larger multicentre trial is required to determine the effect on clinical outcome" (Jones *et al.* 2013).

Immune modulation

Expression of cytokines in cells, in ex vivo *systems*

The cytokine response of human peripheral blood mononuclear cells differs between different *Lactobacillus* spp. It has been shown that different strains of *L. plantarum* of intestinal origin are able to induce the production of the cytokines IL-12 and IL-10 from blood mononuclear cells (Hessle *et al.* 1999). Compared to *E. coli*, less IL-10 was produced but considerably more IL-12 was produced. In the same study, *L. paracasei* induced the production of a higher proportion of IL-12, and *L. rhamnosus* induced a higher proportion of IL-10. The response of the mononuclear cells was more balanced in respect to production of IL-10 and IL-12 when they were exposed to *L. plantarum*, compared to the other two tested *Lactobacillus* spp. (Hessle *et al.* 1999).

The cytokine response of bone marrow-derived, murine, dendritic cells when exposed to different probiotic strains of *Lactobacillus* have also been shown to vary (Christensen *et al.* 2002). Substantial differences could be seen between strains in their capacity to induce IL-12 and TNF-α production in dendritic cells. The ranking among the tested strains was as follows: *L. casei* subsp. *alactus* CHCC3137 >> *L. plantarum* Lb1 > *L. fermentum* Lb20 > *L. johnsonii* La1 > *L. plantarum* 299v >> *L. reuteri* DSM 12246 (Christensen *et al.* 2002). Similar but less pronounced differences were observed among the test strains in the induction of IL-6 and IL-10.

The ability of the proinflammatory cytokine tumour necrosis factor, TNF-α to influence epithelial IL-8 responses to *L. plantarum* 299v has been analysed in HT-29 colonic epithelial cell line (McCracken *et al.* 2002). The results showed that TNF-α sensitises HT-29 cells to *L. plantarum* 299v and the IL-8 mRNA expression was increased above levels induced by TNF-α alone. However, even if the expression had been increased, the IL-8 secretion was most unexpectedly decreased in the HT-29 cells that had been exposed to *L. plantarum* 299v. This means that even if *L. plantarum* 299v sensitises the HT-29 cells, the bacteria exert a protective effect by down regulating IL-8 secretion (IL-8 is a proinflammatory cytokine) (McCracken *et al.* 2002). In a way, this gives an explanation to the paradox that *L. plantarum* 299v is able to both up-regulate the immunological response and exercise an anti-inflammatory effect.

Macroscopically normal colonic tissue from human colon that had been immunologically provoked by phobol 12-myristate 13-acetone and ionomycin was used to test if different probiotic strains were able hinder inflammation associated signals (Bäuerl *et al.* 2013). Exposure of the colonic tissue to *L.*

plantarum 299v down regulated different genes related to inflammation and triggered a global change of the transcriptional profile that indicated a homeostasis restoring effect and a decrease in signals produced by activated T cells (Bäuerl *et al.* 2013).

Experimental in vivo *models*

After the administration of *L. plantarum* 299v to rats with Methotrexate-induced enterocolitis, the subnormal levels of secretory IgA-antibodies in the intestines increased and approached a more normal level, (Mao *et al.* 1996b). Also the level of CD4 and CD8 lymphocytes in the intestinal *lamina propria* increased to more normal levels after treatment with *L. plantarum* 299v (Mao *et al.* 1996b).

The levels of total serum IgA antibodies increased, and the IgA and IgM antibody levels against *Escherichia coli*, were marginally higher in gnotobiotic rats colonized with *E. coli* together with *L. plantarum* 299v, compared with rats that were colonized with *E. coli* alone (Herías *et al.* 1999). The group treated with *L. plantarum* 299v also had a significantly higher density of CD25-positive cells in *lamina propria*, and displayed a decreased proliferative spleen cell response after stimulation with ConA one week after colonization. The results indicated that *L. plantarum* 299v can modulate a response to antigens presented via the gut (Herías *et al.* 1999).

Rats were fed *L. plantarum* 299v before they underwent laparotomy and bile duct ligation which increase bacterial translocation from the gut (Badger *et al.* 2013). It was found that the treatment with *L. plantarum* 299v slightly altered the inflammatory response to portal endotoxemia, and it was concluded that the inflammatory modulation may benefit patients undergoing interventional procedures (Badger *et al.* 2013).

Pigs were used to study the interaction between the host and *L. plantarum* 299v (Hulst *et al.* 2015). Six weeks old pigs were repeatedly administered high doses of *L. plantarum* 299v or just bacterial suspension medium without bacteria. Gene expression was assessed with pooled samples of RNA from different sections of the intestines and it was shown that *L. plantarum* 299v affected metabolic and immunological processes in the especially the ileum. *L. plantarum* 299v seems to (*i*) regulate the activity of adipocytes and/or different subsets of B cells, and (*ii*) induce repression of NFKB-mediated transcription and peroxisome proliferator-activated receptor gamma (PPARG) signalling. It was concluded that the observed effects may temper inflammation (Hulst *et al.* 2015).

Immune response in HIV positive children

Children congenitally exposed to human immune-deficiency virus (HIV) have received *L. plantarum* 299v in a fermented oatmeal formula (freeze dried), in a pilot-study. The results suggested that *L. plantarum* 299v elicits specific systemic immune responses after oral supplementation (Cunningham-Rundles *et al.* 2000; Cunningham-Rundles *et al.* 2002).

Systemic inflammatory response in critically ill patients

One-hundred and three critically ill patients were randomised to receive an oral preparation containing *L. plantarum* 299v (ProViva, strawberry) in addition to conventional therapy (treatment group, n=52) or conventional therapy alone (control group, n=51) (McNaught *et al.* 2005). On day 15, serum IL-6 levels were significantly lower in the treatment group compared to controls (McNaught *et al.* 2005). IL-6 is a cytokine produced by many cell types, including lymphocytes, fibroblasts and monocytes. It has a variety of systemic effects including activation of B and T lymphocytes and induction of acute phase protein production in the liver. IL-6 appears to be a good indicator of activation of the cytokine cascade and predicts subsequent organ dysfunction and mortality (Blackwell and Christman, 1996). Thus, the enteral administration of *L. plantarum* 299v to critically ill patients was associated with a late attenuation of the systemic inflammatory response (McNaught *et al.* 2005). This was associated with a change in EndoCAB levels in the patients administered *L. plantarum* 299v, indicating a decreased endotoxin exposure (McNaught *et al.* 2005).

Cell-mediated immunity in healthy humans

Lymphocyte subsets and cytokine responses to *L. plantarum* 299v among term and pre-term infants and adults were compared (Peoples *et al.* 2009): Cytokine responses to *L. plantarum* 299v in neonates were generally weaker than in adults, but with one exception, the IL-12 response was stronger in term infants.

In a blind, placebo-controlled study, the effect of a daily intake for 5 weeks of *L. plantarum* 299v on the innate and acquired immune system was investigated *in vivo* (Rask *et al.* 2013). Blood lymphocyte subsets were quantified by flow cytometry (FACS) and the expression of activation and memory markers was determined. *L. plantarum* 299v was also examined for its capacity to be phagocytosed by human peripheral blood mononuclear cells (PBMCs). Intake of *L. plantarum* 299v significantly increased the expression of the activation marker CD25 on CD8+ 11 T cells and the memory cell marker CD45RO on CD4+ 12 T cells. The phagocytic activity of granulocytes towards *Escherichia coli* was also increased. Thus, as conclude by the authors, *L. plantarum* 299v

seemed to be an activator of acquired T cell immunity (Rask *et al.* 2013).

Iron absorption

The effect of *L. plantarum* 299v in a fermented oat meal formula on non-haem iron (Fe) absorption from a low-Fe bioavailability meal was compared with different versions of the fermented oat meal formula without live *L. plantarum* 299v (Bering *et al.* 2006). In a crossover trial, 24 healthy young women were served (*i*) fermented oatmeal formula with live *L. plantarum* 299v, (*ii*) pasteurised fermented oatmeal formula, (*iii*) pH-adjusted non-fermented oatmeal formula, and (*iv*) non-fermented oatmeal formula with added organic acids. The meals were extrinsically labelled with 55Fe or 59Fe and consumed on four consecutive days. Fe absorption was determined from isotope activities in blood samples. The fermented oatmeal formula with live *L. plantarum* 299v increased Fe absorption significantly compared with the pasteurised and non-fermented formulas. Thus, the fermented oatmeal formula increased non-haem Fe absorption from a phytate-rich meal in young women, indicating a specific effect of live *L. plantarum* 299v (Bering *et al.* 2006).

Risk-factors for coronary artery disease

Animal model

The effect of a fruit drink (Goodbelly) with *L. plantarum* 299v and *Bifidobacterium lactis* Bio-07 on circulating cytokine levels and severity of ischemia/reperfusion injury in the heart was evaluated in rat (Lam *et al.* 2012). The treatment resulted in a decrease in circulating leptin-levels by 41%, smaller myocardial infarcts (29% reduction), and greater recovery of postischemic mechanical function (23%). However, pretreatment with leptin abolished the cardioprotectional effect. Lam *et al* (2012) concludes "*this proof-of-concept study is the first to identify a mechanistic link between changes in intestinal microbiota and myocardial infarction and demonstrates that a probiotic supplement can reduce myocardial infarct size*".

Human trials

L. plantarum 299v has been shown to decrease different risk factors for coronary artery diseases in individuals at risk. In a small randomized, placebo controlled and double blind study on men with slightly elevated cholesterol levels, it was shown that the concentrations of total cholesterol and of LDL-cholesterol were decreased after consumption of *L. plantarum* 299v in ProViva rosehip (Bukowska *et al.* 1998). The study included 30 individuals divided into

two groups, where the treatment group consumed 200 ml fruit drink, containing 5×10^7 CFU per ml, for 6 weeks and the placebo group consumed fruit drink without lactobacilli. The fall in cholesterol level was small but statistically significant (Bukowska *et al.* 1998). Furthermore, it was shown in the same study that the fibrinogen level in serum also decreased (P<0.001), representing a reduction of 13.5% (Bukowska *et al.* 1998). Fibrinogen is an acute phase protein that reflects the systemic inflammatory status of the individual. Fibrinogen is an independent risk factor for coronary artery disease (Kannel *et al.* 1987).

In a subsequent, placebo controlled randomized double blind study, with thirty-eight healthy smokers, consumption of 400 ml ProViva rosehip daily for six weeks did not only significantly decrease the level of fibrinogen, but also the levels of F_2-isoprostans and IL-6 (Naruszewicz *et al.* 2002). Moreover, *L. plantarum* 299v positively affected the systolic blood pressure, and the insulin and leptin response (Naruszewicz *et al.* 2002).

Sixteen males, with atherosclerotic plaque on the carotid wall, were randomly selected from a larger cohort and included in a double blind, placebo controlled study. Interestingly, the consumption of *L. plantarum* 299v increased the bacterial diversity of the rectal mucosa (Karlsson *et al.* 2010). The administration of *L. plantarum* 299v might be favourable for the condition of the mucosa, and a healthy mucosa decrease the risk of translocation while translocation affects atherosclerosis negatively.

Antioxidative capacity in serum

Effects in an in vivo *model*

Food is important for the recovery of the body after physiological stress, training and other stresses. Otherwise, oxidative stress could give rise to reactive oxygen species (ROS) that can cause damage to body tissue. Antioxidants may protect the body against damage from ROS, and food with high content of antioxidants are believed to have preventive effects on different diseases such as arteriosclerosis and cancer.

Ischemia/reperfusion (I/R) of the colon is an inflammatory condition leading to tissue injury where ROS play a central role. In an I/R-model in mouse the antioxidative activity of probiotics and other antioxidants can be evaluated *in vivo*. The combination of *L. plantarum* 299v and rosehip which is rich in biologically active polyphenols with antioxidative properties (which may be important in prevention of lipid peroxidation) was studied in the I/R-model (Håkansson *et al.* 2006). *L. plantarum* 299v possesses enzymatic activity towards polyphenols (tannins) and can split up the tannins to flavonoids and thus increase the availability and the antioxidative capacity *in vivo* of the

phenolics in rosehip. Administration of rosehip and *L. plantarum* 299v, together, significantly decreased lipid peroxidation (the content of malondialdehyde [MDA] was taken as an index of lipid peroxidation) in caecum tissue. Also the number (viable count) of *Enterobacteriaceae* in caecum content was decreased. A positive correlation between MDA levels and *Enterobacteriaceae* counts was found. The results support a synergistic/additive role of rosehip and *L. plantarum* in reducing lipid peroxidation (Håkansson *et al.* 2006).

Effects in humans

In a placebo-controlled trial with healthy volunteers that consumed a drink containing a mixture of antioxidants and *L. plantarum* 299v (ProViva Active®, Skånemejerier, Malmö), the total plasma antioxidant capacity of serum was increased together with the content of selenium and selenprotein P (Önning *et al.* 2003). However, the eventual role of *L. plantarum* 299v in these effects was not addressed separately. The total load of lactobacilli in faeces increased in the treatment group (Önning *et al.* 2003).

Irritable Bowel Syndrome (IBS)

Irritable bowel syndrome (IBS) is a common deficiency, but its cause is largely unknown. It is not a single condition, but rather a collection of disorders causing similar symptoms of abdominal pain, diarrhoea, constipation or variability of bowel habit. The absence of strict pathogenic features has made IBS a disease without a proper diagnosis. Early attempts were made to develop criteria for a positive diagnosis of IBS (Manning *et al.* 1978; Thompson *et al.* 1992). 20-50% of patients coming to gastroenterology clinics are suffering from IBS, even if most patients with IBS do not seek medical care (Maxvell *et al.* 1997). IBS is a chronic relapsing condition that perhaps occurs in most adults at some point in their lives. Symptoms begin before age 35 in 50% of patients, and 40% of patients are aged 35-50 (Maxvell *et al.* 1997).

The effects of *L. plantarum* 299v have been studied in a murine IBS-model where the intestinal dysfunction was created by rectal administration of 1% allyl isothiocyanate (oil of mustard) in 30% ethanol (Waugh *et al.* 2009). *L. plantarum* 299v was given daily through gavages for up to 28 days, beginning either 7 days before (pre-treatment) or 8 days after oil of mustard administration (post-treatment). *L. plantarum* 299v reduced inflammation and normalized intestinal transit rates in the mice (Waugh *et al.* 2009).

L. plantarum 299v in the fruit drink ProViva (rosehip) was administrated to patients with IBS in two, double blinded, placebo controlled studies, one in

Poland (Niedzielin *et al.* 2001) and one in Sweden (Nobaek *et al.* 2000). In both studies, the patients were divided into two groups, one was given *L. plantarum* 299v and the other a similar rosehip drink without *L. plantarum* 299v (placebo). In the Swedish study, patients with slight to moderate symptoms, mainly bloating and pain, were included (Nobaek *et al.* 2000) while the Polish study requited patients that besides bloating and pain also had problems with irregularity in defecation and stool consistency (Niedzielin *et al.* 2001).

The results of the Polish study were that the magnitude of several of the experienced IBS symptoms decreased in the *L. plantarum* group, and a higher proportion of the patients became free from symptoms in the treatment group than in the placebo group (Niedzielin *et al.* 2001). In the Swedish study, *L. plantarum* 299v significantly decreased the subjectively experienced bloating during the treatment period (Nobaek *et al.* 2000). Pain was also significantly reduced in both the treatment-group and in the placebo-group, but the decrease was more rapid and more pronounced in the *L. plantarum* group. Twelve months after the treatment, the patients given *L. plantarum* 299v in the study, still experienced a better overall gastrointestinal function than the placebo-patients (Nobaek *et al.* 2000).

The bloating and pain experienced by IBS-patient might be due to abnormal colonic fermentation giving rise to an excess of gas production, especially of hydrogen (King *et al.* 1998). In a small randomised placebo controlled study on *L. plantarum* 299v in ProViva, the gas production and composition was measured after 4 weeks consumption. However, no difference was seen between the placebo and the treatment group (Sen *et al.* 2002). On the other hand, if the patients were provoked by consuming 20 g lactulose, the hydrogen in the breath was significantly decreased in the group pre-treated with *L. plantarum* 299v. Thus, the intestinal microflora must have been changed in some way. It should be pointed out that the study of Sen *et al.* (2002) was performed with a cross-over design that in this case might disfavour differences between the groups.

Freeze-dried *L. plantarum* 299v in a capsule was given to subjects between 18-70 years with IBS in a double blind, placebo controlled, parallel-designed study (Ducrotté *et al.* 2012). In total 214 IBS patients were recruited to the study by general practitioners in four clinical centres in India. The test product contained 10^{10} CFU per capsule in potato starch while the placebo product just contained potato starch. Patients consumed one capsule per day for 4 weeks. The primary endpoint of the study was improvement of the frequency of abdominal pain episodes, and secondary endpoints were changes in severity of abdominal pain, changes in frequency and severity of abdominal bloating and in feeling of incomplete rectal emptying. *L. plantarum* 299v significantly decreased both pain severity and daily frequency of pain episodes. Similar results were obtained for bloating. The conclusion of the authors were that "*a four week treatment with* L. plantarum *299v provided effective relief of*

symptoms, particularly of abdominal pain and bloating, in IBS patients fulfilling the Rome III criteria" (Ducrotté *et al.* 2012).

Freeze-dried *L. plantarum* 299v in a capsule was given to IBS-patients in a double blind, placebo controlled, parallel-designed study (Stevenson *et al.* 2014). Eighty-one patients (79 women and 2 men), recruited at a private gastroenterology clinic in Port Elizabeth (South Africa), were randomized into groups. The overall study length was 12 weeks with an 8 weeks treatment phase. The study product was well tolerated, but no statistical significance was seen in IBS-symptom severity score (IBS-SSS), and in improved quality of life, between the placebo and study group. However, the placebo-effect was strikingly high for both groups which might have muddled eventual differences. Another factor that may have interfered with the result is the large difference in number of patients between the placebo-group and the study group (27 versus 54). Furthermore, the patients in the study group had a history of slightly higher percentage use of anti-IBS treatment than the placebo group. Also, the drop-outs from the study group were strikingly higher than that from the placebo group (14 versus 2) (Stevenson *et al.* 2014).

Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a chronic inflammation along the GI-tract. It can be limited to the large bowel (ulcerative colitis) or it can be situated anywhere along the GI-tract (Crohn's disease). Ulcerative colitis is a relatively superficial ulcerative inflammation, while Crohn's disease is a transmural, granulomatous inflammation. IBD is thought to be due to an abnormal and uncontrolled immune response to normally occurring constitutes in the intestine. The aetiology of IBD is unknown. Microbial agents appear to be involved in the pathogenesis of IBD, and intestinal bacteria seem to be an important factor in development and chronicity (Ardizzone *et al.* 1999; Campieri and Gionchetti 2001; Schutltz and Sartor 2000). Under these conditions, there are a complex interaction of bacteria, mucosa and immune system but this interaction is far from clear (Campieri and Gionchetti 2001).

The potential of *L. plantarum* 299v to counteract intestinal inflammation has been studied in different animal models:

1) In rats with enterocolitis induced by Methotrexate, administration with *L. plantarum* 299v mitigated the mucosal injuries induced by the chemotherapy (Mao *et al.* 1996a).

2) In rats, the inflammation in the intestinal mucosa after radiation was decreased by administration of *L. plantarum* 299v in a fermented oatmeal formula (Liu *et al.* 2001).

3) In interleukin-10 deficient mice, in germ-free and specific pathogen-free (SPF) environments, *L. plantarum* 299v was able to attenuate the established colitis when the bacterium had colonized the gastrointestinal tract of the mouse before the mouse was transferred to the SPF environment (Schultz *et al.* 1998; Schutltz and Sartor 2000; Schultz *et al.* 2002). It was also demonstrated that a mono-association with *L. plantarum* 299v (i.e. *L. plantarum* 299v was the only bacterium in the animal) did not induce colitis but only initiated a very mild immune response. Shultz *et al.* (2002) concluded "*these results demonstrated that L. plantarum can attenuate immune-mediated colitis and suggest a potential therapeutic role for this agent in clinical inflammatory bowel diseases*". It has also been shown that *L. plantarum* 299v have a more active role than the probiotic strain *Lactobacillus rhamnosus* GG in preventing the onset of colitis in gnotobiotic IL-10 mice on an inbreed 129SvEv background, colonized with SPF bacteria (Veltkamp *et al.* 1999).

4) In DSS (dextan sulphate sodium) induced colitis in rat, *L. plantarum* 299v decreased the Disease Activity Index (DAI), i.e. the severity of the colitis (Osman *et al.* 2004). DSS was given in the drinking water and after 5 days the animal develops colitis. The DSS-induced lesions and the location of the lesions (mainly the left colon) have resemblances to ulcerative colitis in humans.

The efficacy and safety of an oatmeal gruel fermented with *L. plantarum* 299v (see Figure 1 above) supplemented with lecithin (Profermin®) in inducting remission in patients with active ulcerative colitis have been evaluated (Krag *et al.* 2012). 39 patients with mild to moderate ulcerative colitis were treated twice daily for 24 weeks in an open label study design. The authors concluded "Profermin® is safe and may be effective in inducing remission of active ulcerative colitis (Krag *et al.* 2012).

Antibiotic and *Clostridium difficile* associated diarrhoea

Diarrhoea is a frequently occurring side-effect of antibiotic therapy. Antibiotic treated, hospitalised patients were receiving *L. plantarum* 299v in a fruit drink (treatment product) or a fruit drink without probiotics (placebo): The overall risk of developing loose or watery stools was significantly reduced among patients receiving *L. plantarum* 299v, and so was the development of nausea (Lönnermark *et al.* 2010). The results indicate that intake of *L. plantarum* 299v can have a preventive effect on gastrointestinal symptoms during antibiotic treatment.

Recurrent *Clostridium difficile* associated diarrhoea is a serious condition that often requires prolonged treatment with antibiotics, but these treatments often fail to prevent further recurrences. In a double-blind, placebo-controlled trial the ability of *L. plantarum* 299v to prevent recurrent episodes of *Clostridium*

difficile associated diarrhoea was tried (Wullt *et al.* 2003). Recurrence of clinical symptoms was seen in 4 out of 11 patients who received metronidazole in combination with *L. plantarum* 299v and in 6 out of 9 treated with metronidazole in combination with placebo. The investigation was limited to 21 patients, and the results were not statistically significant. Nevertheless, there was a tendency towards fewer recurrences in the lactobacillus group in comparison with the placebo group and this tendency was maintained for additional 3 months after the study period. To reach statistical significance with a power 80%, 40 patients must be included in each arm. The study encourages the performance of large multicentre studies (Wullt *et al.* 2003).

L. plantarum 299v was shown to affect the faecal concentrations of short-chain fatty acids during and after metronidazole treatment in 19 patients with recurrent *C. difficile*-associated diarrhoea (Wult *et al.* 2007). After intake of metronidazole a significant decrease in total short-chain fatty acids was seen in the placebo group but not in the group with *L. plantarum*. Thus, *L. plantarum* 299v reduced the negative effects of an antibiotic treatment on the colonic fermentation (Wult *et al.* 2007).

Critically ill patients are often treated with antibiotics and are at high risk of developing *Clostridium difficile*-associated disease. Patients in the intensive care unit (ICU) were investigated regarding the impact of *L. plantarum* 299v on *C. difficile* colonisation, and it was shown that enteral administration of *L. plantarum* 299v to critically ill patients treated with antibiotics reduced colonisation with *C. difficile* (Klarin *et al.* 2008).

Antiviral effects

The capacity of *L. plantarum* 299v in preventing rotavirus infection was evaluated by plaque assays and genomic analysis. Plaque assays revealed that priming with *L. plantarum* 299v decreased the concentration of live viruses at least by 100-fold. The gene expression suggested that homeostasis in the gut is maintained in probiotic-primed cells despite infection with rotavirus (Thompson *et al.* 2010).

Safety aspects

The safety of consuming high numbers of live bacteria has been addressed, and there are reports that *Lactobacillus* spp., including *L. plantarum*, have been isolated from diseased sites (Aguirre and Collins 1993). However, the potential of *Lactobacillus* spp. to cause serious infections is low. For example, it has been shown by studying the prevalence of bacteremia due to *Lactobacillus* spp. during a 4 year period in Finland, which indicated that the pathogenic potential of *Lactobacillus* spp. in general is low (Saxelin *et al.* 1996).

The fact that many traditional lactic acid fermented foods spontaneously contain high numbers of *L. plantarum* (Dedicatoria *et al.* 1981; Gashe 1985; Gashe 1987; Oyewole and Odunfa 1990; Fernández Gonzalez *et al.* 1993; McDonald *et al.* 1993; Lönner and Ahrné 1995; Johansson *et al.* 1995c; Moorthy and Mathew 1998) and that these products in the public mind, all over the world, have a reputation of being safe and wholesome, strongly indicates that live *L. plantarum* can be consumed without risk. This becomes especially obvious if the long historical tradition of the lactic acid fermented foods is taken into account. However, in the case of the strain *L plantarum* 299v, the safety has been more directly confirmed:

L. plantarum 299v has been given in a daily dose of 10^{10} CFU to two patients with small bowel bacterial overgrowth in short bowel syndrome (with D-lactic acidosis; Vanderhoof *et al.* 1998). No negative effects of the *L. plantarum* 299v administration were noted. Instead, it was concluded for the whole case-study, including six patients, that "*Preliminary experience with probiotics to change the flora to nonpathogenic organisms is promising and may demonstrate greater effectiveness and results in fewer long-term complications*" (Vanderhoof *et al.* 1998).

L. plantarum 299v has been given in doses of $2x10^{10}$ CFU per day to 64 patients undergoing elective major abdominal surgery for at least a week preoperatively and in the postoperative period, without any negative signs, e.g. increased translocation due to the increased bacterial load (McNaught *et al.* 2002).

L. plantarum 299v has been given in high doses to immune-compromised children with HIV, for extended time periods, without any adverse effects (Cunningham-Rundles *et al.* 2000; Cunningham-Rundles *et al.* 2002).

L. plantarum 299v has been given, to critical ill patient in the intensive care without any adverse effects (Klarin *et al.* 2005; McNaught *et al.* 2005). Eventual bacteraemia (bacteria in the blood) was followed by Klarin *et al.* (2005). *L. plantarum* 299v was never found in the blood.

Intensive care unit (ICU) patients with expected mechanical ventilation ≥48 h and/or expected ICU stay ≥72 h received *L. plantarum* 299v two times daily (Oudhuis *et al.* 2010). The 130 critically ill patients receiving *L. plantarum* 299v did not show increased ICU mortality or mortality at day 28 compared with 124 patients receiving selective decontamination of the digestive tract with antibiotics (Oudhuis *et al.* 2010).

The risk of endocarditis has been tested in an experimental rat model (Adawi *et al.* 2002). A catheter was passed down the right common carotid artery into the lumen of the left ventricle. The catheter was tied in place and the neck incision was closed. After 48 hours, 10^8 CFU of *L. plantarum* 299v was injected (0.5 ml of bacterial suspension) through the tail vein. Four days after the injection of the *L. plantarum* strain, the rats were sacrificed and the blood, heart tissue and catheter were sampled for bacteria. No *L. plantarum* 299v could be found in any of the sample sites (Adawi *et al.* 2002). Thus, even with this animal model, using a very unusual and challenging situation where a high dose of the bacteria is injected directly into the blood stream of an animal with an implant of artificial material in the artery and heart, the *L. plantarum* strain was removed from the system without causing any damage. It has been stated in Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project that "for *in vivo* assessment of safety by investigating strain pathogenicity in animal models, the rat endocarditis model appeared to be the most reliable model tested in the PROSAFE project" (Vankerckhoven *et al.* 2008).

L. plantarum 299v has been evaluated in the EU funded PROSAFE project: The identity of the strain was confirmed and no acquired antibiotic resistance could be detected (PRO SAFE report on strain *Lactobacillus plantarum* 299v).

The safety of *L. plantarum* 299v has also been evaluated at "Depto. de Graduados e Investigación en Alimentos" at "Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional" ("Carpio y Plan de Ayala, Col. Sto. Tomás", Mexico), and the strain was declared safe (Melgar-Lalanne *et al.* 2014).

Miscellaneous

Hypertension is associated with abnormal activity of rennin-angiotensin-aldosterone, where angiotensin converting enzyme (ACE) is critical for the regulation of blood pressure. Pea seeds fermented by *L. plantarum* 299v were digested *in vitro* under GI-like conditions (Jakubczyk *et al.* 2013). No ACE inhibitory activity was found after the fermentation but when the fermented product had been digested, potentially antihypertensive peptides were released (Jakubczyk *et al.* 2013).

Galacto-oligosaccharides (GOS) that commonly is produced from lactose in whey permeate by an enzymatic process have been shown to have prebiotic effects, i.e. promoting growth of beneficial components of the GI-microbiota. It has been shown that GOS containing whey permeate is a suitable substrate for growth of *L. plantarum* 299v (Golowczyc *et al.* 2013).

The survival and effects of *L. plantarum* 299v in Spanish fermented sausage has been evaluated (Rubio *et al.* 2013). *L. plantarum* 299v maintained high counts and prevented growth of *Enterobacteriaceae* throughout the entire ripening process, and "producing functional sausages with satisfactory overall sensory quality" (Rubio *et al.* 2013).

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