CHARACTERIZING *PROPIONIBACTERIUM FREUDENREICHII* SSP. *SHERMANII* JS AND *LACTOBACILLUS RHAMNOSUS* LC705 AS A NEW PROBIOTIC COMBINATION: BASIC PROPERTIES OF JS AND PILOT *IN VIVO* ASSESSMENT OF THE COMBINATION

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ABSTRACT

Each candidate probiotic strain has to have the documentation for the proper identification with current molecular tools, for the biological properties, for the safety aspects and for the health benefits in human trials if the intention is to apply the strain as health promoting culture in the commercial applications. No generalization based on species properties of an existing probiotic are valid for any novel strain, as strain specific differences appear e.g. in the resistance to GI tract conditions and in health promoting benefits (Madsen, 2006). The strain evaluation based on individual strain specific probiotic characteristics is therefore the first key action for the selection of the new probiotic candidate.

The ultimate goal in the selection of the probiotic strain is to provide adequate amounts of active, living cells for the application and to guarantee that the cells are physiologically strong enough to survive and be biologically active in the adverse environmental conditions in the product and in GI tract of the host. The *in vivo* intervention studies are expensive and time consuming; therefore it is not rational to test all the possible candidates *in vivo*. Thus, the proper *in vitro* studies are helping to eliminate strains which are unlikely to perform well *in vivo*.

The aims of this study were to characterize the strains of *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705, both used for decades as cheese starter cultures, for their technological and possible probiotic functionality applied in a combined culture. The *in vitro* studies of *Propionibacterium freudenreichii* ssp. *shermanii* JS focused on the monitoring of the viability rates during the acid and bile treatments and on the safety aspects such as antibiotic susceptibility and adhesion. The studies with the combination of the strains JS and LC705 administered in fruit juices monitored the survival of the strains JS and LC705 during the GI transit and their effect on gut wellbeing properties measured as relief of constipation. In addition, safety parameters such as side effects and some peripheral immune parameters were assessed. Separately, the combination of *P. freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 was evaluated from the technological point of view as a bioprotective culture in fermented foods and wheat bread applications.

In this study, the role of *P. freudenreichii* ssp. *shermanii* JS as a candidate probiotic culture alone and in a combination with *L. rhamnosus* LC705 was demonstrated. Both strains were transiently recovered in high numbers in fecal samples of healthy adults during the consumption period. The good survival through the GI transit was proven for both strains with a recovery rate from 70 to 80% for the JS strain and from 40 to 60% for the LC705 strain from the daily dose of 10 $\log_{10}$ CFU. The good survival was shown from the consumption of fruit juices which do not provide similar matrix protection for the cells as milk based products. The strain JS did not pose any obvious safety concerns *in vitro* and no harmful side effects were reported *in vivo* by healthy adults or by institutionalized elderly during the consumption of the combination of the JS and LC705 strains. A decreasing frequency in level of IgA secreting cells (p=0.02) was reported in the healthy adults; however this change may not be biologically relevant. A relief of constipation (from 2.1 to 2.6 times/week; p=0.04) was reported in the institutionalized elderly and a slight, but insignificant tendency of increased defecation frequency was observed in healthy adults. The bioprotective antimicrobial role against yeasts and molds in fermented foods and against ropiness causing *Bacillus* sp. in wheat bread was proven for the strains.

LIST OF ABBREVIATIONS

ATR	acid tolerance responce
BIF	bifidobacteria
BCFA	branced chain fatty acid
CFU	colony forming unit
DCs	dentritic cells
DM	dry matter
DNA	deoxyribonucleic acid
EFSA	European food safety authority
FDA	Food and drug administration
GALT	gut-associated lymphoid tissue
GC content	guanine-cytosine content
GIT	gastrointestinal tract
GMO	genetically modified micro-organism
GRAS	generally recognized as safe
IBD	irritable bowel disease
IBS	irritable bowel syndrome
Ig (A/G/M)	immunoglobulin A/G/M
ISC	immunoglobulin secreting cells
LAB	lactic acid bacteria
MIC	minimal inhibitory concentration
QPS	Qualified presumption of safety
PAB	propionic acid bacteria
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PPA	YEL medium buffered with β-glyserolphosphate
RAPD	randomly amplified polymorphic DNA
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
SCFA	short chain fatty acid
SD	standard deviation
Ssp.	subspecies
TTA	total titratable acidity
YEL	yeast extract lactate medium

ORIGINAL PUBLICATIONS

The thesis is based on the following original publications which are referred to in the text by Roman numerals I-V.

I. Suomalainen, T. and Mäyrä-Mäkinen, A. 1999. Propionic acid bacteria as protective cultures in fermented milks and breads. *Lait* **79**, 165-174.

II Ouwehand, A.C., Suomalainen, T., Tölkkö, S. and Salminen, S. 2002. In vitro adhesion of propionic acid bacteria to human intestinal mucus. *Lait* **82**, 123–130.

III Ouwehand, A. C., Lagström, H., Suomalainen, T., and Salminen, S. 2002. Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly. *Ann Nutr Metab* **46**, 159–162

IV Suomalainen, T., Lagström, H., Mättö, J., Saarela, M., Arvilommi, H., Laitinen, I., Ouwehand, A.C., and Salminen, S. 2006. Influence of probiotic drinks containing *Lactobacillus rhamnosus* on intestinal well-being and immune response in healthy adults. LWT **39**, 788-795

IV Suomalainen, T., Sigvart-Mattila, P., Mättö, J., and Tynkkynen, S. 2008. *In vitro* and *in vivo* gastrointestinal survival, antibiotic susceptibility and genetic identification of *Propionibacterium freudenreichii* ssp. s*hermanii* JS. *Int Dairy J* **18**, 271-278

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1. REVIEW OF THE LITERATURE

1.1. Introduction: from dairy starters to candidate probiotic cultures

Propionic acid bacteria (PAB) have been used for decades for technological applications in the food industry.

The original aims in applying lactic and propionic acid bacteria in foods was as technological starters for improving the shelf life, texture and flavour of the products. The new era for these bacteria was raised at time when Metchnikoff published his early findings of beneficial effects of *Lactobacillus* sp. as probiotics (from Greek words "for life"), and currently probiotics are becoming increasingly important in the context of human nutrition and wellbeing. Scientific evidence accumulates in rebalancing the disrupted gastrointestinal (GI) –tract and in alleviating infectious disease especially in children (reviewed by Walker et al., 2006; Pham et al., 2008). In particular, certain strains belonging to *Bifidobacteria* and *Lactobacillus* species are the most widely studied and exploited probiotics. Of the dairy propionibacteria, species *P. acidipropionici* and *P. freudenreichii* are introduced as possible candidate probiotics (Mantere-Alhonen, 1995; Bougle et al., 1999; Jan et al., 2002, Ouwehand, 2004).

Interest in various types of food products and dietary supplements with probiotic bacteria is continuously expanding. Fermented foods and dairy products are by far the most popular human applications for probiotics due to traditionally known positive health image of fermented foods and, on the other hand, due to the fact that consumers are familiar with these products containing living microorganisms (Heller, 2001). Additionally, dairy products are excellent matrixes for the delivery of probiotics because of the buffering capacity of the milk (Salminen & Playne, 2001) and milk fats (Stanton et al., 1998) against acid secretions in the upper gastrointestinal tract. In parallel with growing consumer awareness on the benefits of probiotics, interest in supplementing other food products than fermented dairy products with the probiotics is one of the current issues in research and product development. The latest innovation in probiotic products with a market volume of more than 1000 million kg, is the daily-dose drinks in small bottle (Buss, 2004). Other novel food applications are e.g. infant foods, non-dairy foods, soft drinks and dietary supplements (for review, see Champagne et al., 2005).

The probiotic strains are challenged in food or supplement applications by different stresses during the production and in the gastrointestinal tract as illustrated in Table 1 (modification from Lacroix & Yildrim, 2007) and therefore the basis for the strain selection is the strain's ability to adapt to different stresses during production and during the transit in the intestine. The ultimate goal in the selection of the strains is to provide adequate amounts of active, living probiotic cells for the application and to guarantee that the cells are physiologically strong enough for the environmental conditions in the product and especially in GI tract of the host. The strain selection is therefore a key action in the development of the new probiotics.

Table 1. Main factors affecting the viability of probiotics from production to the gastrointestinal tract (modified after Lacroix & Yildirim, 2007)

Steps from production to gastrointestinal tract			
Fermentation	**Downstream process**	**Storage**	**Gastrointestinal tract**
Composition of growth medium	Mechanical stress	Acidity of food	Acidic conditions in stomach
Dissolved oxygen	Oxygen stress	Oxygen stress	Enzymatic activities
Toxic by-products (e.g. organic acids)	Extreme temperature during spray or freeze drying	Competition with other microbes in the product	Composition of environment
Final cell mass	Cell dehydration	Temperature	Bile salts in the small intestine
		Moisture content	

1.2. Probiotics

1.2.1. Definition

Since the first definition of probiotics as "micro-organisms stimulating the growth of another" by Lilly and Stillwell (1965) the term has been redefined several times from the initial improvement in intestinal microbiota to the health effects outside the intestine (Salminen et al.1998; Schrezenmeier and de Vrese, 2001). The latest definition is given by FAO/WHO expert panel as "**live micro-organisms which, when consumed in adequate amounts, confer a health benefit on the host**" (Food and Agriculture Organization & World Health Organization, 2002). At present, scientific evidence is sufficient for indicating the potential of probiotic foods with well characterized strains to provide health benefits and that those specific strains are safe for human consumption (FAO/WHO, 2002). It is noteworthy that different strains may have different effects to humans and therefore the current concept of the probiotics states that the beneficial effects are based on the specificity of the clearly defined strain, applied either as a monoculture or as a part in a multistrain mixture.

1.2.2. Current probiotic strains and their clinical use

The microbes mostly studied and currently used as probiotics are specific strains belonging mainly to *Lactobacillus* or *Bifidobacterium* species. The interests in studying these species as probiotic are based on their association with healthy human tract (Boyle et.al., 2006) and on their presumed safety for healthy and immunosuppressed individuals (Borriello et al., 2003). The

number of probiotic strains studied and claimed to have probiotic activity is high, but only a handful of species dominate the market (Table 2).

Table 2. The main probiotic strains applied commercially in the EU member states

	Supplier	**Commercial brand name(s)**
Bifidobacterium animalis ssp. *lactis* BB-12	Chr.Hansen A/S	Several brands
B. lactis HNO19	Danisco	Howaru™ Bifido
B. animalis ssp. *animalis* DN 173 010	Danone	Bifidus Digestivum; Actifit
Lactobacillus casei DN-114-001	Danone	Lactobacillus Defensis; Actimel
L .rhamnosus HN001	Danisco	Howaru™ Rhamnosus
L. casei shirota		Yakult
L. plantarum 299v	Probi AB	Proviva
L. reuterii ATTC 55730	Arla-Ingman	RELA
L. rhamnosus GG ATTC 53013	Valio Ltd	Gefilus; LGG, Vifit; Cultura, Actifit, etc.

The positive effects in treating gastrointestinal disorders, especially in the incidence or duration of rotavirus diarrhea in children are well evidenced by placebo-controlled intervention trials (de Vrese & Marteau, 2001; Gill & Guarner, 2004). Based on meta-analysis of Cochrane reviews (Allen et al., 2004) and D'Souza et al. (2002), sufficient evidence exists to recommend the use of *L.rhamnosus* LGG in milk or in capsules as an adjunct to oral rehydration therapy for the treatment of rotavirus diarrhea. Also, well documented evidence is supporting the balancing role of probiotics during antibiotic treatments both in children (Johnston et al., 2006) and in adults (Cremonini et al., 2002; Engelbrektson et al., 2009). Putative, positive indications exist for the management of inflammatory bowel syndrome (IBS) (Kajander et al., 2005; 2008) which is a functional bowel disorder. In case of ulcerative colitis which is one of the different forms of inflammatory bowel disease (IBD), probiotics may provide modest benefits in mild to moderately severe ulcerative colitis, but the evidence is limited (Mallon et al., 2007).

Probiotics are usually consumed orally which enables the communication with the intestinal enterocytes and gut-associated lymphoid tissues (GALT) in the small intestine. This interaction of probiotic strain and hosts cells is important for the controlled production of cytokines and chemokines secreted by macrophages and dentritic cells (DCs) (see review Delcenserie et al., 2008). In the small intestine the low number of resident bacteria enables the communication between probiotics and intestinal cells more easily than in the colon and therefore probiotics may have better competition capability with luminal microbiota of the small intestine than in the colon (Walker et al., 2006). The positive effects of probiotics on gastrointestinal disorders may be explained most likely by the ability of probiotics to enhance the gut mucosal immune system, especially in the small intestine e.g. by increasing mucosal barrier function, preventing apoptosis of epithelial cells and decreasing antigen uptake (Walker et al., 2006). Certain probiotic strains have been shown in well conducted studies to stimulate and regulate several aspects of innate and adaptive immune responses (Hatakka & Saxelin, 2008b; Gill & Prasad, 2008). However, these

changes need to be connected to the physiological events before the effects on health and well being can be estimated.

Most of the probiotic products available are based on single strain monocultures. The combinations of different strains are rare; this may be due to the difficulty to prove the effects of the strains with different mechanisms functioning simultaneously or because the interactions between the strains may be difficult to predict or even due to the demanding authorization procedure. It is noteworthy that combinations may have a broader possibility in specific disorders and in certain conditions if the strains are acting synergistically. Only few groups of researchers have been active in studying the possible probiotic effects of multistrain culture combinations (Kajander et al, 2008; Myllyluoma et al., 2005; Gionchetti et al., 2007; Drouault-Holowacz et al., 2008). Those studies have demonstrated the beneficial effects of probiotic combinations *in vivo* for irritable bowel syndrome (IBS) (Kajander et al., 2008; Drouault-Holowacz et al., 2008) relieving abdominal pain and distension, for *Helicobacter pylori* eradication and alleviating of symptoms (Myllyluoma et al., 2005), and for preventing the onset of acute pouchitis and maintaining remission following antibacterial treatment of acute pouchitis (Gionchetti et al., 2007).

1.2.3. Legislative constraints

In Europe, regulation concerning the marketing of functional foods into which probiotic foods are included has so far been national and fragmented, and the situation has lead to probiotic product launches with diverse and even controversial messages to consumers. In some cases the launches were even applying the generic relation to the known probiotic strain of the same species without properly conducted intervention trials. These approaches confused consumers and health professionals and called for an EU-level consensus on evaluating and regulating the market.

The new legislation on nutrition and health claims entered into force in January 2007 (Regulation No. 1924/2006 of the European Parliament and of the Council). The regulation covers to all nutrition and health claims made in commercial communication of foods in EU member states. The new regulation establishes the claims that can be made, generates lists of generally accepted health and nutrition claims and establishes the procedure for the authorization of new claims. Claims for probiotic foods can be related to nutritional/functional (article 13) or reduction of a risk of a disease action (article 14). A list of accepted nutritional/functional claims e.g. generic claims should be published by EFSA in the beginning of the year 2010. In case of the article 14, only claims with clear evidence demonstrating the causality between consumption of nutrients and indented health benefits will be accepted after judging by EFSA. Besides, all foods having nutrition and health claims must meet nutritional requirements based on nutrient intake recommendations. At the time being, mainly generic claims are used in the current probiotic products in Europe, however, more specific strain-dependent health claims are of great interest but they do require more resources and investments from the marketing company because of the demanding authorization.

Generally, substantiated scientific documentation for the health benefits in conjunction to technical and administrative information is demanded and in practice the application should contain at least the information according to the Joint FAO/WHO Working group (2002) guidelines for the evaluation of probiotics in foods. From EFSA point of view safety and strain identification combined with performance and functionality are the main criteria to be fulfilled.

Currently, no positive opinion on applied probiotic dossiers has been given by the agency. Some of the main concerns EFSA has expressed on probiotic dossiers have been that strains have not been adequately characterized, and that some of the science referenced in dossiers referred to different strains to those present in the foods for which the claims were being made.

Table 3. General selection criteria of probiotic candidates

Strain selection

- Safety and identification
  - The strain must be safe for consumption, free of pathogenic and toxic effects.
  - The strain should be properly identified with the current molecular methods (e.g. PFGE DNA/DNA hybridization; 16S RNA sequencing).
  - The strain deposition in an official culture collection is recommended.
- Biological properties
  - The strain should be able to resist gastrointestinal fluids (acids and bile) and should be able to adhere to the human intestinal mucosa.
- Technological suitability
  - The strain should have good technological properties such as the ability to survive during the industrial processing and in the final product in order to maintain high viability and activity in the product and at its site of action.
  - The strain should be genetically stable and maintain phenotypic properties.

Performance and functionality

- Documented beneficial health effects
  - The strain must have clinically proven health benefits which are proven at least in one well defined human study.
  - In case article 14 claims are used, at least two independent DBPC tests should be performed.
- Functional characterization

In 2008, the FDA Center for Food Safety and Applied Nutrition published a guidance for industry on "Substantiation for Dietary Supplement Claims" (The Federal Food, Drug, and Cosmetic Act (21 U.S.C. 343(r)(6), section 403(r)(6)). The guidance covers probiotic foods and supplements. Claims allowed for probiotics can be of structure/function, qualified health or significant scientific agreement claims. Structure/function statements of food are related to normal functioning of the human body and these claims are used most typically on probiotic food products as no approval or notification is required for these claims. The pre-approval from FDA is needed for the claim for reduction of the risk of disease.

The investigations of the current single strain probiotics have focused primarily on applications of *Lactobacillus* or *Bifidobacterium* species rather than on dairy *Propionibacterium* species although dairy propionibacteria have a long history of safe use as a dairy starter culture and *P. freudenreichii* species are bearing QPS acceptance in EU member states and the GRAS status in

the U.S.A. Interestingly, PAB are easily adapting to different environments and they pose several interesting features with potent health promoting effects.

1.3. Propionic acid bacteria in food applications and in the gastrointestinal tract

1.3.1. Technological applications of propionic acid bacteria in foods

The genus *Propionibacterium* belongs to a large heterogeneous group of firmicutes in the class of *Actinobacteria* with high G+C content (>50%) (Stakebrandt et al., 1997). The genus *Propionibacterium* comprises two distinct groups from different habitats, strains found on human skin, referred to as the "Cutaneous propionibacteria", and strains isolated from milk and dairy products, referred to as "dairy" or "classical" propionibacteria. The dairy group of propionibacteria includes species *P. freudenreichii*, *P. acidipropionici*, *P. jensenii*, and *P. thoenii* (Cummins and Johnson, 1986). Recently, two new species have been included to the dairy PAB group, namely *P. cyclohexanicum* and *P. microaerophilium* (Dherbecourt et al., 2006). Especially *Propionibacterium freudenreichii* species have been exploited for decades in big eye (Swiss type) cheese production in which their key role is in hole(eye) and flavour formation. In addition, dairy type *Propionibacterium* species are used in vegetable fermentations, in preservation of feeds and for the biosynthesis of propionic acid and vitamin B_{12}. *P. freudenreichii* subspecies *freudenreichii* and *shermanii* have been granted Qualified Presumption of Safety (QPS) status in Europe (Barlow et al., 2007) and the same subspecies are classified as "Generally Recognized As Safe" (GRAS) for use in cheese manufacture (Mogensen et al., 2002) in the USA.

<u>Applications of LAB and PAB as bioprotective cultures</u>

The interests in minimal processing systems and in bioprotection of foods and feed have derived researchers to seek alternative methods for traditional use of chemicals and high temperature treatments for ensuring the high microbial quality of the products. The use of the bioprotective capacity of lactic acid bacteria (LAB) and propionibacteria is one of the approaches. The antimicrobial action of LAB and PAB is based on various active compounds such as organic acids, H_2O_2, diacetyl, bacteriocins and bacteriocins-like substances acting in most cases synergistically. The antimicrobial action of organic acids such as lactate, propionate and acetate is based on their ability in undissociated, lipid soluble form to passively diffuse through the cell membrane and after entry into the cytoplasm, dissociate into proton and charged derivatives to which the cell membrane is impermeable. This intracellular acidification affects the cell deleteriously by disrupting the transmembrane delta pH, deactivating the acid-sensitive enzymes, and damaging the proteins and DNA. Also charged, anionic moiety of dissociated organic acids has detrimental effect on cellular physiology due to a chelating interaction with essential elements (Adams, 2001).

Sorbic, benzoic and acetic acids and their salts are used widely in the food industry as preservatives against fungi whereas propionic acid and its salts have been used in bakery industry

to prevent moulds and *Bacillus* occurring in breads (Ponte and Tsen, 1987). Antimicrobial proteinaceous compounds derived from LAB and PAB have been investigated and proposed for use in food preservation (Holo et al., 2002; Klaenhammer, 1993), but so far only nisin, produced by certain *Lactococcus* species, is allowed with restricted use as a food additive (EU-guideline, No.95/2EG). Nisin is active only against gram-positive bacteria and its antimicrobial spectrum does not cover Gram-negative bacteria, yeasts or moulds (Daeschel, 1989). Antifungal compounds produced by LAB and PAB would be of great importance for food industry as fungi are a frequent cause of spoilage in foods manufactured without chemical additives. Most isolated and identified antifungal compounds produced by LAB are low molecular-mass organic compounds such as capric acids and short-chain fatty acids (Corsetti et al., 1998), phenyl-lactic acids and cyclic dipeptides (Lavermicocca et al.,2003; Ström et al., 2002). Acetate and propionate produced by PAB have inhibitory effect towards various fungi (Lind et al, 2005; Paster et al., 1999; Razavi-Rohani & Griffiths, 1999) but the production of short chain fatty acids (SCFA) and the antifungal activity varies depending on the growth conditions and on the species within propionibacteria (Lind et al., 2005). Additionally, antifungal activity of *Propionibacterium freudenreichii* species have been reported to produce phelyllactic acid (PLA) similar to LAB (Thierry and Maillard, 2002; Lind et al., 2007). Interestingly, the synergistic properties of cocultures of *P. jensenii* and *L. paracasei* subsp *paracasei* has proved to be more effective against food spoilage yeasts than the cultures separately (Miescher Schwenninger & Meile, 2004).

Although the activity in studies connected to antimicrobial agents has been intense, only few applications have been exploited commercially in food industry, for example MicroGard™ fermentates by Danisco A/S. The new era for “green” chemicals may within a few years awaken a renewed interest in existing PAB and LAB application for the wider commercial exploitations.

1.3.2. Gut and its microbiota as an ecosystem

The human GI tract can be subdivided into three distinct microbial communities; the upper GI tract, the ileum, and the colon. Within these different regions different physicochemical environments occur and are reflected in the microbiological populations which are primarily modified by different host factors such as age (Abbott, 2004) and host genotype (Bäckhed et al., 2005). In the upper GI tract rapid transit time, acidic conditions and gastric juices make the regions hostile and restrict the microbial populations. Only aciduric gram-positive lactobacilli and streptococci can be detected in low levels. Besides the harmless species, the potential pathogen, *Helicobacter pylori*, is able to survive the harsh conditions in about 50% of humans (Go, 2002) and under certain circumstances to cause chronic gastritis and eventually even peptic ulcer disease (Fox and Wang, 2001).

In the ileum (distal small intestine) the conditions turn less hostile for microbes and the bacterial populations comprised of versatile species composition reach cultivable levels between 6 to 8 $\log_{10}$ CFU g^{-1} of luminal content. The dominant species are gram-negative facultative anaerobic bacteria (family Enterobacteriaceae) and obligate anaerobes such as *Bacteroides*, *Veillonella*, *Fusobacterium* and *Clostridium* species with reasonable levels of lactobacilli and enterococci.

The colon is the main region harboring the majority of the fermentative microbes of the gastrointestinal tract, the microbial composition of the colon is complex reaching from levels of 10 to 12 log_{10} CFU g^{-1} of content consisting of the microbes belonging to main phyla of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrocomicrobia* (Andersson et al., 2008; Eckburg et al., 2005). This resident microbiota of the intestine has shown to be essential for the well-being of the host as the microbiota take part in nutrient processing, regulation of host's fat storage and protection against pathogens (Ley et al., 2006; Xu and Gordon, 2003). Besides, the intestinal microbes have a major influence on the physiology of the intestinal cells, evidenced by the expression of more than hundred genes of the intestinal cells by the resident microbes in the colon via bacteria-bacteria and bacteria-cell dialogue (Hooper & Gordon, 2001) and by the maturation of a developing intestinal immune system (Rautava & Walker, 2007). On the other hand, the malfunctioning of this GI tract microbiota has been linked to certain gastrointestinal diseases (Turroni, 2008). Within the near future the advances in modern molecular technology will considerably improve the understanding of the interaction between the intestinal microbiota and the intestinal cells (for review, see Hooper et al., 2002; Bäckhed et al., 2005).

1.3.3. The role of propionibacteria in the human intestine

The incidence of propionic acid bacteria (PAB) in the human GI tract has not been established as well as the incidence of LAB and BIF. Thus, only few studies have been published concerning the abundance and occurrence of PAB in the gut. The dairy type of propionic acid bacteria are not considered as typical of human microbiota (Rinta-Koski et al., 2001) and the basal counts of propionibacteria in human are reported to be below 5 log_{10} CFU g^{-1} (Bougle et al., 1999; Myllyluoma et al., 2007, Jan et al., 2002, Herve et al., 2007) giving an indication that PAB are probably minor, transient members of the intestinal microbiota in humans on the contrary to digestive tract of ruminants (Jarvis et al., 1998; Cheong and Brooker, 1999, Rinta-Koski et al., 2001).

In vitro studies have indicated that certain dairy PAB could be able to withstand the conditions of the gastrointestinal transit. Interestingly, this has been verified in human intervention studies which show that certain strains of dairy PAB (Table 4) have the potential to survive through upper GI and to reach the viable cell levels over 6 log_{10} CFUg^{-1}, which is generally considered sufficient for a probiotic to exert beneficial effects on colon ecology and host physiology (Collins and Gibson, 1999).

Table 4. Dairy propionic acid bacteria strains, doses and cell counts in after administration in different matrixes in human intervention studies

Subjects (n)	**Strain and Carrier**	**Dose $\log_{10}$ CFU d^{-1}**	**Concentration in , $\log_{10}$ CFU g^{-1}**	**Ref.**
Male healthy adults (n=17)	*P. freudenreichii* SI41 and SI26 freeze dried in powder	10,7	1) 6.37±0,89	Bougle et al., 1999
Healthy adults (n=7)	*P. freudenreichii* S141 in classical capsules	Low,9.7 High,10.7	Low, 5.5±1.11 High, 6.25±0.66	Jan et al., 2002
Adults in *H. pylori* eradication treatment (n=23)	*P.freudenreichii* ssp. *Shermanii* JS in milk-based fruit drink	Total 10.9	7	Myllyluoma et al., 2007
Healthy adults (n=38)	*P. freudenreichii* ssp. *shermanii* JS in capsules	10.6	4.3	Hatakka et al., 2008a
Healthy adults (n=38)	*P. freudenreichii* ssp.*shermanii* JS in raspberry-blueberry juice with prebiotic (GOS or PDX)	10.3	6-7	Tiihonen et al., 2008a
Healthy adults (n=5)	*P. freudenreichii* S141 in fermented milk	11	7-8.5	Herve et al., 2007
Healthy adults (n=17)	*P. freudenreichii* ssp.*shermanii* JS in milk based fruit juice	10.5	8.3 $\log_{10}$ genome copies g^{-1}	Kekkonen et al., 2008

1.3.4. Metabolic activities of the gut microbiota and the possible effects of PAB in the gut

Balanced microbiota

The microbiota has an essential impact on energy production, renewal and differentiation of GI epithelial cells, formation of a barrier against pathogens, synthesis of vitamins and development and function of the immune system (Guarner, 2006; Ley, et al., 2006; Turnbaught et al., 2006). The intestinal tract is colonized by an extensive and diverse microbial community, which at the time is estimated to cover over 1000 species (Egert et al., 2006), however, showing a vast spatial, temporal and functional variation (Zoetendal et al., 2004). In a balanced state the complex ecosystem of gut does not have adverse acute effects on the wellbeing of the host, but it may be disturbed temporarily by factors such as antibiotic treatments, enteric infections or dietary

stresses. The probiotic approach is developed for balancing of the disturbances. Probiotics may not necessarily affect greatly the intestinal microbiota composition or number of cells, but the microbiota may respond functionally to probiotics without actual changes in the composition or cell numbers (Walker et al., 2006). Still, the precise impact of the ingestion of probiotic bacteria on the functioning of the human gut microbiota is open for discussions (Turroni et al., 2008).

Producers of short chain fatty acids (SCFA) in the gut

The major metabolic function of the anaerobic microbes in the gut is the fermentation of non digestible carbohydrates. The metabolism of these complex carbohydrates provides the production of the main SCFA (acetate, propionate, and butyrate) and gases hydrogen (H_2) and carbon dioxide (CO_2). Additionally, the protein fermentation leads to formation of SCFA and branched chain fatty acids (BCFA). The intestinal epithelium derives 60–70% of its energy needs mainly from butyrate, although the other SCFA contribute to some extent (Cumming and Macfarlane, 1997). Butyrate is not only the energy source for the colonic epithelium but it also has important effects on the development and gene expression in intestinal cells (Scheppach and Weiler, 2004). In addition, it is generally thought to play a protective role against colorectal cancer (Archer et al., 1998) and colitis (Christl et al., 1996).

The concentrations of SCFA are dependent on equilibrium between microbial activity and colonic absorption. In adults the dominant intestinal microbiota is relatively stable within an individual leading to stable microbial activity profiles (Ley et al., 2006). However, the colonic absorption may be more variable within an individual, as it is affected by factors such as gut transit time, diet, and aging, diseases, drugs and epithelial turnover times (see review, Macfarlane & Macfarlane, 2003). Due to the absorption and the effect of diet, the accurate quantification of SCFA is challenging. However, the production rate of SCFA is estimated to be of the order of 300-400 mmol/d in human colon (Cummings, 1997).

The major members of colon microbiota able to produce SCFAs are *Clostridium*, *Eubacteria*, *Fusobacterium* and *Roseburia* (Hold et al., 2003; Aminov et al., 2006). *Lactobacillus* or *Bifidobacterium* species may have an impact on fermentation of complex carbohydrates by producing acetic and lactic acids. Interestingly, *Bifidobacterium adolescentis* has been shown to cross-feed the butyrate producing *Roseburia* sp., *Anaerostipes caccae* and *Eubacteria hallii* with the lactate and acetate formed in human gut (Belenguer et al., 2006). Theoretically, propionic acid bacteria have the potential to impact on intestinal energy balance by utilizing the metabolism of carbon substrates via Wood-Werkman pathway (Wood 1981), which leads to production of two moles of propionate and one mole of acetate in parallel with CO_2 (Figure 1). Prerequisite for this metabolic activity is the survival and adhesion of PAB in substantial numbers within the colon content, at least transiently. Evidence is already supporting the ability of selected propionic acid bacteria to survive and even to maintain the metabolic activity during the GI transit. Humans with high population of dairy propionibacteria have been shown to contain enhanced concentrations of SCFA (Jan et al., 2002). In addition, certain *P. freudenreichii* ssp. *shermanii* strains have shown *in vivo* to transcribe the genes of mRNA encoding the 5S subunit of the multimeric transcarboxylase in human microbiota associated rats (Lan et al. 2007) and in healthy humans (Herve et al., 2007) indicating the metabolic activity of the strain in the intestinal tract. This enzyme, transcarboxylase (methylmalonyl-CoA carboxyltransferace, EC 2.1.3.1.), is an essential enzyme in propionic acid fermentation. The subunits 12S, 1.3S and 5S are involved in the transfer of CO_2 from methylmalonylCoA to puruvate to yield propionylCoa and oxaloacetate

(Deborde, 2002; Hall et al., 2004). The subunit 5S is specific for *P. freudenreichii* ssp. *shermanii* species (Herve et al., 2007).

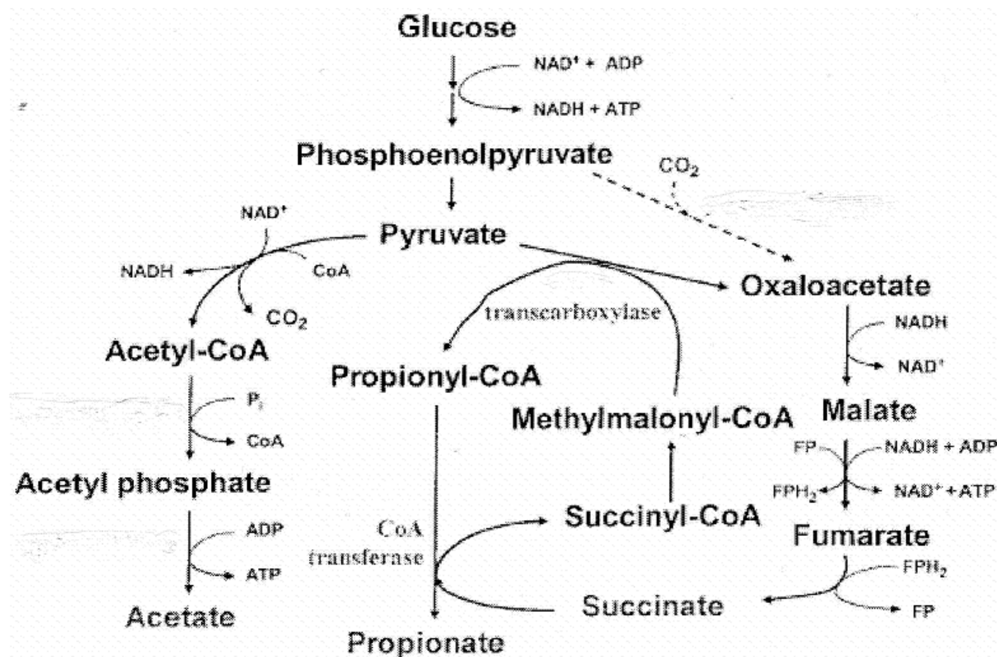


Figure 1. Overall fermentations of glucose to propionic, acetic and succinic acids by *Propionibacterium* (Suwannakham and Yang, 2005)

<u>Bifidogenic effect</u>

One of the mechanisms with which probiotic bacteria may confer the health of hosts is the modulation of intestinal microbiota for example by stimulating bifidobacteria whose presence is correlated with a healthy intestinal microbiota, especially in infants (Ouwehand et al., 2002a). In adults the population of bifidobacteria varies depending on age and physiological status (Reuter, 2001; Hopkins et al., 2002) and the actual role of bifidobacteria in adults is still under discussion. Nevertheless, certain strains of *P. freudenreichii* have shown to be able to enhance the growth and populations of bifidobacteria in the gut. First indication was reported by Kaneko *et al.* (1994) of an *in vitro* bifidogenic effect for cell-free extracts of *P. freudenreichii* and later the effect was reported to be due to the presence of 2- amino-3-carboxy-1,4-naphthoquinone (Mori *et al.*, 1997) and 1,4-dihydroxy- 2-naphthoic acid (Isawa *et al.*, 2002). Moreover, independent studies have shown that ingestion of *P. freudenreichii*, under the form of whey cultures, either heat-inactivated (Satomi *et al.*, 1999) or not (Hojo *et al.*, 2002), or of freeze dried live bacteria (Bougle *et al.*, 2002), resulted in a higher fecal bifidobacteria concentration in humans. This was linked to an increased number of defecations in constipated female volunteers (Hojo *et al.*, 2002). Thus, through the bifidogenic effect by the selected PAB strains the dysfunctioning of gut may be normalized.

Production of vitamins

Vitamin B_{12} is essential for the growth and health of humans as it is a crucial cofactor in fatty acid, amino acid, carbohydrate and nucleic acid metabolism (Quesada-Chanto et al. 1994). Natural synthesis of B_{12} occurs only in microbes e.g. in the colon. Propionibacteria utilizing the Wood-Werkman cycle use the multimeric transcarboxylase (EC 2.1.3.1) which needs cofactors B_{12} (cobalamin), B_9 (folic acid), and biotin for the rearrangement of C1 compounds during the fermentation. This phenomenon has been known for long and therefore dairy propionibacteria have been used for the food-grade industrial production of B_{12} by fermentation. From 1 kg of propionibacteria biomass, approximately 330 g of B_{12} can be isolated (Deborde, 2002). No studies have been carried out of the possible role of PAB as B_{12} producers in the human intervention trial with PAB.

Modulation of enzymatic activities

a) Enhancement of β-galactosidase activity

Lactose intolerance is a common discomfort and a disorder in humans caused by decrease in the lactase activity of human enterocytes which in most cases occurs already in childhood (Wang et al., 1998). Intolerance, if neglected may lead to malabsorption causing damage for the small intestinal mucosa or increase of GI transit time (Schaafsma, 2007). Lactose entering the colon may be hydrolyzed by colon microbes having beta-galactosidase activity. One of the benefits listed for probiotics is the reduction of lactose intolerance symptoms either by the beta-galactosidase they produce or by changing the composition of the colon microbiota. Certain propionibacteria do have beta-galactosidase activity as shown in study of Zarate et al. (2000) in which expression of genes for the beta-galactosidase in *P.freudenreichii* and *P.acidipropionici* species was reported.

b) Suppression of procarcinogenic enzyme activities

Binding and degradation of pro-carsinogens in the gut is one of the positive outcomes that have been connected to probiotics. Intestinal microbes produce several procarcinogenic enzymes, of which β-glucuronidase is assumed to contribute to the release of carcinogenic moieties from the glucurone compounds and β-glucosidase is connected to the formation aglycone compounds in the colon (Rowland, 1992; Goldin, 1990). Of the colonic microbes species such as clostridia and *Bacteroides* are known to have high procarcinogenic enzyme activities (Wollowski et al., 2001). Contradictionary results with different PAB strain of their effects on intestinal enzyme load have been reported. In mice fed with P. *freudenreichii* CRL 757, or SG1 or *P. acidipropionici* Q4 or CRL1198 strains, *P. freudenreichii* strains caused no change in β-glucosidase activity of the intestinal microbes whereas *P. acidipropionici* strains significantly reduced β-glucosidase activity of mice microbiota (Perez Chaia et al. 1999). In a human study of Hatakka et al. (2008a) *P. freudenreichii* ssp. *shermanii* JS in combination with *L. rhamnosus* LC705 was shown to decrease the activity of β-glucosidase producing microbes parallel to increasing propionibacteria counts. Thus, certain strains of PAB may be useful for preventing elevated levels of β-glucosidase activities linked loosely to increased risk of colon cancer (Reddy et al., 2000) although the evidence is based on limited data.

Modulation of the innate immune system

The cross talk between intestinal microbes and intestinal epithelial cells trigger a series of immunological mechanisms. Immunomodulating mechanisms that have been demonstrated with certain probiotics include the induction of mucus production, enhanced natural killer cell and phagocyte activity, enhanced production of pro- and anti-inflammatory cytokines, and induction of salivary secretory immunoglobulin A (sIgA) and neutrophils production (see review of Senok et al., 2005). In case of candidate probiotic PAB in animal studies, oral administration of *P.acidipropionici* was shown to stimulate phagocytotic activity of the innate immune system in mice (Perez Chaia et al., 1995) whereas orally administered *P. freudenreichii* ssp. s*hermanii* JS enhanced proliferation of T- and B-cells in mouse lymphocytes (Kirjavainen et al., 1999). Similarly, Adams et al. (2005) reported the enhancement of spleen lymphocytes proliferation by *P. jensenii* 702 strain in an animal study. These observations do not explain how these organisms might induce these effects nor do they explain which bacterial molecules or cellular receptors are responsible for them (Delcenserie et. al., 2008).

1.3.5. Safety assessments

Only foods containing novel compounds (Regulation (EC) 258/97) or. Genetically Modified Microorganisms (Regulation (EY) 1829/2003) have obtained an official authorization procedure requiring the assessment of the safety whereas no authorization procedure concerning the safety assessments of probiotic strains is available. Only the WHO/FAO guidelines (2002) list the recommendations for the safety assessments including parameters such as determinations of antibiotic resistance patterns, harmful metabolic activities and assessment of side effects during human studies. Qualified Presumption of Safety (QPS) approach of EFSA (Barlow et al., 2007) sets priorities within the risk assessment of those microorganisms used in food/feed production referred to EFSA. The use of any novel strain, unless listed on the QPS (Annex 3), should be properly tested for its safety parameters. The methods are left open for the applicant. In the U.S., Title 21 of the Code of Federal Regulations (21 CFR) and the FDA Office of Premarket Approval lists microorganisms which have a "generally recognized as safe" (GRAS) status.

Propionic acid bacteria belong to a heterogeneous group of *Actinobacteria* with a high G+C content and are included in the *Actinomycetale* order. Most probiotics currently are of low GC content (lactobacilli) or high GC content (bifidobacteria) Gram-positive bacteria (Saxelin et al., 2005). The first pure culture of *Propionibacterium* was isolated from Emmental cheese in 1906 by Orla-Jensen in the year 1909 and still a century later *Propionibacterium freudenreichii* is the main species used at the ripening of Swiss-type cheeses, so these species have a long documented history of use in foods. According to EFSA scientific committee (Barlow et al., 2007), based on their extensive application in the foods and feeds, *P. freudenreichii* species do not warrant specific safety evaluation and therefore *P. freudenreichii* is granted QPS status. In addition, in the USA the species *P.freudenreichii* is classified as GRAS for use in cheese (Mogensen et al., 2002).

The use of probiotics in humans has some theoretical adverse risks such as translocation of the cells and the potential for antibiotic resistance transfers from probiotic bacteria to potential pathogens within the GI tract (Senok et al., 2005). It is accepted that antibiotic non-susceptibility/resistance is not a hazard unless it renders the probiotic untreatable in rare cases of

infection or unless it can be transferred to potential pathogens (Borriello et al., 2003). The vancomycin resistance genes carried by many *Lactobacillus* species seem to be chromosomally located and therefore the genes are not easily transferable to other genera (Tynkkynen et al, 1999). Dairy PAB are typically resistant to aminoglycosides, the 1st and 2nd generation quinolones, colistin, metronidazole and phosphomycin (Madec et al.,1994; Chamba et al., 2007). Antibiotic resistance in dairy propionibacteria does not appear to be plasmid-encoded (Rehberger and Glatz, 1990) although the issue is not studied widely.

1.4. Approaches to overcome the technological and physiological challenges

In order to provide the desired health benefits, a probiotic strain must have good technological properties (industrial scale cultivation with high survival rates during processing and storage) and should be incorporated into food products without loosing viability and functionality and without creating unpleasant flavors or texture (Mattila-Sandholm et al., 2002; Ross et al., 2005). Ability to adapt to different stresses during the culture production and during the transit in the intestine is considered as an essential characteristic for a probiotic strain facing several challenges during the steps from manufacturing until the final destination at the host intestine (Lacroix & Yildrim, 2007). Tolerance to harsh conditions can be due to an intrinsic resistance or to an adaptive response (Segal and Ron, 1998) and many of the current probiotic strains have been chosen by their tolerance to the digestive stresses which have been studied by screening for an intrinsic resistance of acid and bile salts *in vitro*. If the strain does not have either intrinsic or adaptive response against the stresses, the cells can be protected with external layers as in microencapsulation (see review Champagne and Fusier, 2007).

Intrinsic systems

Microbes have evolved stress-sensing systems reacting on signals from the environment in order to keep the homeostasis within the cell in balance. The homeostasis in unfavorable environment is maintained with physiological changes controlled by gene expression (Segal and Ron, 1998). Genes implicated in stress response are numerous and for example in LAB the levels of characterization of their actual role and regulation differ widely between species. General stress responses rely on the coordinated expression of genes and the synthesis of stress-response proteins which alter different cellular processes such as cell division, DNA metabolism, membrane composition and transport (van de Guchte et al., 2002). In LAB, stress proteins synthesis has been studied in response to acidification, osmotic stress and high temperatures mainly with starter cultures. The current information is thoroughly reviewed by van de Guchte et al. (2002) and Lacroix & Yildirim (2007).

Propionibacteria applied technologically faces primarily the stresses caused by low pH and high temperature during the food processing and when applied as probiotic the stresses caused by gastrointestinal fluids during the passage through GI tract. PAB seem to have a good intrinsic ability to adapt and survive in harsh environments (Leverrier et al., 2004) and some *P.freudenreichii* strains withstand well the high temperatures and high saline concentrations e.g. during the Emmental cheese manufacture (Langsrud and Reinbold, 1973). However, a great diversity among strains exists in adaptation and survival in stressful conditions. Acid challenge

leads to drastic morphological changes (Jan et al., 2001) and at pH 2 to cell death caused by intracellular material leakage from non-adapted cells whereas the bile challenge is less deleterious (Leverrier et al., 2003). The acid tolerance response (ATR) can be triggered by a transient exposure to pH 5 which affords protection toward acid challenge at pH 2 both the physiological and morphological levels (Jan et al, 2001). The physiological state of the cells during lethal treatment plays an important role in the response to bile salts, as stationary-phase bacteria appear less sensitive than exponentially growing cells (Jan et al., 2000; Leverrier et al, 2003). Cross-protection between bile salts and heat adaptation increased the stress tolerance, whereas heat and acid responses did not present significant cross-protection in *P. freudenreichii* (Leverrier et al., 2004). In conclusion, the stress susceptibility of propionic acid bacteria seems to be species and even strain dependent (Jan et al., 2002; Anastasiou et al., 2006) and by proper strain selection or by stress adaptation, stress tolerant strain can be chosen.

External techniques for protecting probiotic cells

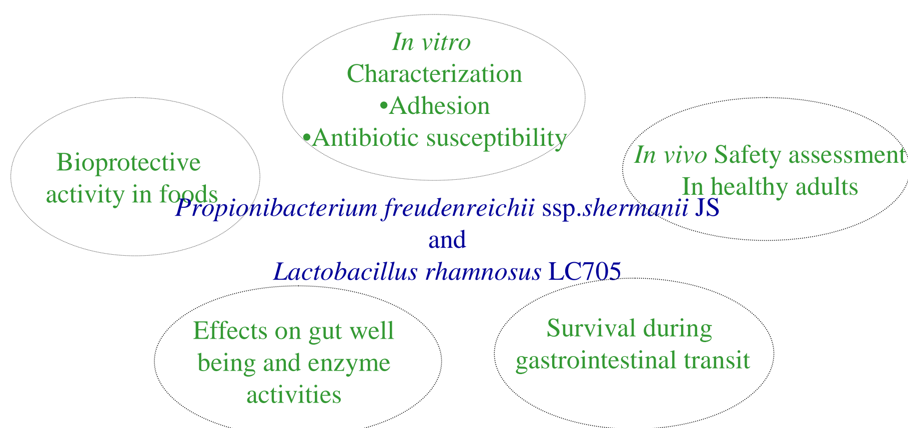
The viability of probiotic strain can be enhanced by cell protection with new technologies such as microencapsulation. In microencapsulation the inner matrix i.e. probiotic cells are sealed with a continuous coating inside of a microcapsule core which protects the cells from extremes of heat, moisture (Doleyres and Lacroix, 2005) and gastrointestinal fluids (Kailasapathy, 2002) and which provides controlled release of the probiotics at the target site (Chen et al., 2005). The semipermeable, thin and strong wall of the microcapsule allows nutrients and metabolites to diffuse through the material easily and at the same time keep the cells within the capsule (Chen et al., 2005). With respect to probiotics, the most often reported benefit of microencapsulation is enhanced viability in the different food matrixes. Microencapsulation techniques have not been explored widely for efficient delivery of probiotics in the GI tract (Champagne & Fustier, 2007).

2. Aims of the study

The purpose of the study was to assess the probiotic and the technological potential of *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 applied in a culture combination in different food matrixes. Additionally, the *in vitro* studies of *Propionibacterium freudenreichii* ssp. *shermanii* JS assessed the strain's probiotic parameters such as the viability during the acid and bile treatments and the safety aspects such as antibiotic susceptibility.

The specific aims of the studies were,

- To characterize the combined antimicrobial activity of the strains LC705 and JS by assessing the bioprotective effectiveness against common food spoilage yeasts/moulds and spore forming bacteria in food applications (Study I)
- To assess *in vitro* adhesion (Study II), upper GI tract tolerance (Study V) and antibiotic susceptibility (Study V) of the strain JS
- To assess the *in vivo* gastrointestinal survival of the strains JS and LC705 administered in whey-based orange juice by healthy adult volunteers (Study IV and V) and to assess the potential of the strains LC705 and JS to relief constipation in the elderly (III) and in the healthy adults (IV)
- To confirm the conventional biochemical identification of the JS strain in faecal samples by molecular biological methods (Study V)



3. MATERIALS AND METHODS

The detailed descriptions of the methods used in the studies are presented in the original articles and therefore the methods are not repeated in detail in this section.

3.1. Microbes

The probiotic candidates

The studies focused on two probiotic candidates (Table 5) which were assessed for their combined effects as a protective culture in fermented dairy foods and in wheat bread (Study 1), for their gastric survival in healthy adults (Study IV and V) and for their possible beneficial role on the treatment of constipation at the institutionalized elderly (Study III) and healthy adults (Study IV). Safety related parameters of strain JS were assessed in the Study II, III, and V.

The basic properties of *Lactobacillus rhamnosus* LC705 have been published by Tuomola et al. (2000) and Mäyrä-Mäkinen et al. (2002).

Table 5. The candidate probiotic strains

Strain	Culture collection	Growth condition	Study
Lactobacillus rhamnosus LC705	DSM 7061, Valio Ltd., Finland	MRS, LAMVAB 3 d / 37 C	I, II, III, IV
Propionibacterium freudenreichii ssp. *Shermanii* JS	DSM 7076, Valio Ltd., Finland	PPA, YEL and MRS/ 48 h and 7 d /30C	I, II, III, V

Other microbes

L. rhamnosus GG (ATCC 53103, Valio Ltd., Finland), *Bifidobacterium lactis* Bb12 (Hansen A/S, Denmark) and *B. longum* (nee *infantis*) BB99 (Valio Ltd., Finland) were used in the study II. *L.reuterii* ING 1 (Ingman, Finland) was used in the study III and *L. rhamnosus* E-97800 (VTT, Finland) was used in the study IV.

P. freudenreichii ssp. *freudenreichii* P131 (Valio Ltd., Finland), *P. freudenreichii* ssp. *freudenreichii* ITG P20 (Institut Technique Francais des Fromages; ITFF, France) and *P. freudenreichii* ssp. *freudenreichii* ITG P23 (ITFF, France), *P. freudenreichii* ssp. *freudenreichii* NCIMB 5959^T (National Collection of Industrial, Food and Marine Bacteria; NCIMB Ltd., UK) and *P. freudenreichii* ssp.*shermanii* NCIMB 10585^T (NCIMB Ltd., UK) were used in the study IV.

3.2. Antimicrobial activity of the P. *freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 culture (Study I)

The antimicrobial activities of the JS and LC705 as single strains and the culture combination were detected on conventional tests *in vitro,* previously (FI92498). The aim in antimicrobial study (study I) was to evaluate the bioprotective activity of the combination in real food systems. Commercial production processes of the products (yogurt, quark and wheat bread) were simulated and the products were artificially inoculated with contaminants isolated from the similar products (Table 6). The microbiological and chemical quality of the products was followed weekly during the storage time.

Table 6. Contaminants, growth parameters and their origin (Study I)

Product	**Contaminant**	**Origin**	**Growth parameters**
Yogurt and Quark	*Rhodotorula rubra* RHO	Fermented milk	YM 25C / 2 d
Yogurt and quark	*Pichia quilermondii* PQ	Fermented milk	YM 25C / 2 d
Wheat bread	*Bacillus subtilis* P.2.94	Ropy wheat bread	BHI 37C / 24 h
Wheat bread	*Bacillus licheniformis* P.1.94	Ropy wheat bread	BHI 37C / 24 h

Table 7. The growth media for the enumeration of microbes with plate counts (Study I)

Assay	**Medium**	**Conditions**
Total LAB	MRS-agara	3 d 37C
L. rhamnosus LC705	MRSV-agar with 0.005% (w/v) vancomycin	3 d 37C
Propionic acid bacteria	Mod. Sodium-lactate agar (YEL) with 1% (w/v) β-glyserolphosphate (PPA)	7 d 30C
Yeasts and molds	YCG-agar a	3 d 25C
Bacillus spp.	Phenol Red Egg Yolk Polymyxin agar a	20-24 h 37C

[a] LAB M, Bury, UK;

Preparation of contaminants (Study I)

Bacillus spore suspension in a wheat bread trial

Bacillus subtilis and *B. licheniformis* were isolated and identified from the ropy bread in the beginning of the study. The spore suspension of *Bacillus subtilis* and *B. licheniformis* strains was produced by harvesting the spores and vegetative cells grown on Nutrient agar enriched with 0.003% (w/v) $MnSO_4x4H_2O$ for 48 h at 37º C aerobically by centrifugation (4500 g for 10 min at 4ºC) and washing with sterile water (after which a stock solution was prepared in a sterile water.) In order to destroy the vegetative cells, the stock solution was heated for 10 min at 80ºC. The spore concentration in stock solution was 9.9 $\log_{10}$ CFU ml^{-1}. The inoculum level was 2.5 $\log_{10}$ CFU g^{-1} dough.

Yeasts suspensions in fermented milk products trial

Rhodotorula rubra and *Pichia quilermondii* were isolated from relevant contaminated fermented milk products in the beginning of the study. Yeasts were grown in YM-broth at optimal conditions for 2 d after which vegetative cells were inoculated in to products at a level of 2.5 CFU $\log_{10}$ g^{-1} quarks and 2.5 CFU $\log_{10}$ g^{-1} strawberry jam.

Table 8. Preparation of products (Study I, III, IV and V)

Product	**Protective strains**	**Active Dose**	**Additional technological starter**	**Study**
Quark	*L. rhamnosus* LC705 and *P. freudenreichii* ssp.*shermanii* JS	1) 7 $\log_{10}$ CFU g^{-1} quark 2) 6 $\log_{10}$ CFU g^{-1} quark	Probat 505*	I (technological use)
Yogurt	*L. rhamnosus* LC705 and *P. freudenreichii* ssp.*shermanii* JS	7 $\log_{10}$ CFU g^{-1} yoghurt	V2 *	I (technological use)
Wheat bread	*L. rhamnosus* LC705 and *P. freudenreichii* ssp.*shermanii* JS	8 $\log_{10}$ CFU g^{-1} sourdough	None	I (technological use)
3 Fruit juice®	*L. rhamnosus* LC705 and *P. freudenreichii* ssp.*shermanii* PS	8 $\log_{10}$ CFU ml^{-1} juice	None	III (probiotic use)
Whey-based fruit juice Hedelmätarha®	*L. rhamnosus* LC705 and *P. freudenreichii* ssp.*shermanii* PS	8 $\log_{10}$ CFU ml^{-1} juice	None	IV, V (probiotic use)

* supplied by Wiesby GmbH, Germany (Danisco A/S)

3.3. *In vitro* characterization of *P. freudenreichii* ssp. *shermanii* JS (Studies II and V)

3.3.1. *In vitro* gastric tolerance of *P. freudenreichii* ssp. *shermanii* JS

The *in vitro* gastric tolerance of *P. freudenreichii* ssp. *shermanii* JS was evaluated at low pH with HCl acid and at high bile concentration as follows, the propionibacteria strains were cultivated anaerobically in propioni medium (PA-broth, pH 7.3) for 3 d at 30°C (stationary growth phase). The survival of the propionibacteria strains during bile exposure was studied in PA-broth supplemented with 0.3 % or 0.5 % (w/v) oxgall powder (Bile bovine, Sigma B-3883, Sigma-Aldrich, St. Lois, MO, USA) by inoculating the broth with 1% of stationary growth phase culture and by enumerating the viable cell counts after 0 and 3 h of incubation at 30°C. The acid tolerance test was performed in whey permeate medium (WPM-broth) adjusted with 37% HCl to pH of 6.5, 4.0, 3.0 and 2.0. WPM-broth was inoculated with 1% of stationary growth phase culture and incubated for 1, 2 and 3 hours at 30°C, after which viable cell counts were enumerated. Viable cell counts from both experiments were determined on PPA-agar by incubating the plates anaerobically for 7 d at 30°C

3.3.2. Safety related *in vitro* studies

Safety related parameters such as adhesion and antibiotic susceptibilities of *P. freudenreichii* ssp. *shermanii* JS were evaluated with *in vitro* tests.

<u>Adhesion studies (II)</u>

In vitro adhesion of *P. freudenreichii* ssp. s*hermanii* JS was performed in mucus isolated from healthy part of resected human colonic tissue (Ouwehand et al., 2002b) and the non-specific adhesion was performed by using immobilized Bovine Serum albumin (BSA). Both the mucus and BSA were passively immobilized on polystyrene microtitre plates. The use of resected human intestinal material was approved by the joint ethical committee of the University of Turku and Turku University Central Hospital. The adhesion assay was performed according to the procedure described in details by Ouwehand et al. (2001). The bacteria were radiolabelled with methyl-1,2,-^{3}H thymidine and after the growth for late exponential phase at optimum growth conditions the cell concentrations were adjusted for the optimal densities of 7-8 $\log_{10}$ CFU ml^{-1} and added at a volume of 100 µl into mucus or BSA coated microtitre plate wells. Radioactivity was determined by liquid scintillation and the adhesion was expressed as the percentage of radioactivity recovered after adhesion, relative to the radioactivity in the bacterial suspensions added to the immobilized mucus.

<u>Minimal inhibitory concentration (MIC) of antibiotics (Study V)</u>

Determination of the minimal inhibitory concentration (MIC) of antibiotics was performed on VetMIC™ E-cocci microdilution panel (National Veterinary Institute, Uppsala, Sweden) by using the Lactic acid bacteria Susceptibility test Medium (LSM; Klare et al. 2005) consisting of Isosensitest broth (IST, Oxoid Ltd, Cambridge, UK) supplemented with 10% of MRS

medium (LabM, Bury, UK). The assay included the following antibiotics, ampicillin, erythromycin, virginiamycin, gentamicin, streptomycin, kanamycin, tetracycline, chloramphenicol, vancomycin, narasin, bacitracin and linezolid in a concentration range of 0.125 – 1024 µg mL^{-1}. Briefly, *P. freudenreichii* ssp. s*hermanii* JS, P131, NCIMB 5959^T and NCIMB 10583 were cultivated in LSM broth for 48 h at 30°C anaerobically. For the final inoculum, 1 mL of propionibacteria culture grown for 3 d at 30°C was suspended aseptically to sterile saline until a density corresponding to a spectrophotometric OD_{625} of 0.16-0.2 was reached. This saline suspension was then diluted 1:1000 in LSM broth to obtain a final concentration of 5.5-5.8 log_{10} cfu mL^{-1}. For each well in VetMIC™ E-cocci microdilution plate, a 100 µL of this inoculum was added within 30 min after the preparation of the final inoculum. The wells were sealed with a transparent covering tape and the panel was incubated for 3 days at 30°C, anaerobically. The growth of the culture in the wells was assessed visually. The MIC was defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth out of three repeated experiments.

Characterization of Enzyme Activity Profiles (Study V)

In vitro enzyme activity profiles were detected by the chromogenic, semiquantitative API ZYM system (bioMérieux sa, Marcy-l'Etoile, France) according to the manufacturer's instructions. Briefly, strains were harvested, washed and suspended into API ZYM medium and adjusted to cell count of 8 log_{10} CFU mL^{-1}. A 65 µl of this suspension was added to each well and then incubated for 4 h at 30°C. The reaction was stopped by adding the reagent Zym A and the colour development was obtained by adding the reagent Zym B. Enzyme activity was detected through colour reaction by comparing the colour intensity to the colour reading chart provided by the manufacturer and expressed as nM of substrate hydrolysed / 65 µL of culture on a scale of 0 (no activity) to ≥40nM (high activity). The cell numbers in the wells were enumerated by plate counting on PPA-agar for 7 days at 30°C and they were 8.0±0.1 log_{10} cfu mL^{-1} for the JS strain and 7.9±0 log_{10} cfu mL^{-1} for the P131 and NCIMB 5959^T strains. The test was performed twice for all strains.

3.4. Pilot assessment of the combination of *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 in human intervention studies (Studies III – IV)

The aim in the intervention studies was to assess *in vivo* gastrointestinal survival of the two candidate probiotics of *L. rhamnosus* LC705 (study IV) and *P. freudenreichii* ssp. *shermanii* JS (study V). The enumerations were performed by conventional plate counting (Table 10) from frozen fecal samples and isolates resembling the ingested probiotic strains were identified with current molecular methods (Table 11). The effects of the probiotic strains on intestinal well being of elderly (study III), and of healthy adults (Study IV) were followed by recording the gastrointestinal symptoms. The effects of the strains on humoral immune responses on the healthy adults were performed by enumerating the indicators of the innate immune system (immunoglobulin secreting cells (ISCs), expression of Fcα-receptors and complement receptors 1 and 3) from peripheral blood samples.

Elderly volunteers (n=28) were enrolled in the Study III, and healthy adult volunteers (n=22) were recruited for the studies IV and V. The studies were approved by the Ethical Committee of the Hospital District of Varsinais-Suomi. A signed informed consent was obtained from every volunteer.

Table 9. Study designs in human intervention trials (Studies III, IV and V)

Run-in	**Intervention**	**Washout**	**End point**	**Study**
3 wks Fruit juice	3 wks Fruit juice with LC705+JS	3 wks Fruit juice	• Defecation frequency • Fecal azoreductase and mucin content	III (Single blind parallel placebo controlled)
2 wks Fruit juice	2 wks Fruit juice with LC705+JS	2 wks Fruit juice	• *L. rhamnosus* Lc705 cell numbers • Humoral immune response (ISCs)	IV (Single blind parallel)
2 wks Fruit juice	2 wks Fruit juice with JS+LC705	2 wks Fruit juice	• *P. freudenreichii* ssp. *shermanii* JS cell number	V (Single blind parallel)

Table 10. Cell count enumerations of *L. rhamnosus* and *P. freudenreichii* ssp. *shermanii* (Studies IV and V)

Analysis	**Method**	**Conditions**	**Reference**
L. rhamnosus	LAMVAB	3 d; 37C; microaerophilically	Hartemink et al., 1997
P. freudenreichii ssp. *shermanii*	PPA	7 d; 30C; anaerobically	mod. Malik et al.

Colonies resembling *L. rhamnosus* LC705 on LAMVAB agar plates (study IV) were selected for genotyping with randomly amplified polymorphic DNA (RAPD) using primers listed in Table (11) and as described by Alander et al. (2001).

Presumptive propionibacteria colonies with light yellow colors were isolated and purified on PPA-agar (study V). Preliminary identification of the pure cultures of *P. freudenreichii* ssp. *shermanii* isolates was based on gram-staining, catalase activity, fermentation of lactose and sucrose, nitrate reduction (Nitrate test, Merck) and carbohydrate fermentation by API 50 CH (bioMérieux sa). The typing of the strains was performed with RAPD according to Fessler et al. (1998) with primers in combination listed in Table (11) and with pulsed field electrophoresis (PFGE) analysis with restriction enzymes *Xba*I and *Ssp*I (New England Biolabs) as described by Tynkkynen et al. (1999).

Table 11. Oligonucleotides used for the randomly amplified polymorphic DNA identification in the Study IV and V.

Typing	Name	Sequence (5’-3’)	Reference
L. rhamnosus	OPA-3	AGTCAGCCAC	Alander et al., 2001
	OPA-2	TGCCGAGCTG	
	OPA-5	AGGGGTCTTG	
	OPA-13	CAGCACCCAC	
	Primer-6	CCCGTCAGCA	
Propionibacterium	RAPD-primer	CGAGCCGTC	Fessler et al.,1998
	RAPD-primer	ACGCGCCCT	

Table 12. Parameters of gut wellbeing

Analysis	Method	Reference	Study
Constipation / Defecation frequency	Questionnaire		III and IV
Fecal mucin	Colorimetric assay with Alcian blue	Hall et al.,1980	III
ISCs	Enumeration of immunoglobulin secreting cells (ISCs)	Kaila et al.,1992	IV
CD89, CD35 and CD116	Expression of Fcα -, and complement 1 and 3 receptors	Grönlund et al., 1999	IV

3.5. Statistical analysis

Bacterial cell numbers were transformed into log10 values before any treatment. In the adhesion study (Study II) the results from the adhesion experiments were expressed as the average (± standard deviation) of at least three independent experiments. Each adhesion experiment was performed with four parallels. The statistical significance in adhesion between the strains, to the substrata and with the incubation time was tested by using the t-test (paired, two tailed). In the studies III and IV, The Mann-Whitney U-test was used for the comparison between the groups and Wilcoxon’s signed-rank test was used for the comparison within the group. Bacterial cell numbers in the human studies were presented separately for each subject. The statistical significance of differences in the cell counts of the strain LC705 (Study IV) and JS (study (V) at baseline versus after consumption and after follow-up within each individual was analyzed by using the t-test (paired, two tailed) (Windows XP, Microsoft Excel 2002).

4. SUMMARY OF RESULTS

4.1. Technological applicability as a bioprotective culture

In this study, the antimicrobial potential of the combination *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 was studied in laboratory scale in quark against yeast *R. rubra* RHO, in yogurt against yeast *P. quilermondii* PQ, and in wheat bread against *Bacillus* spp. Fresh quark mass was obtained from the commercial production process whereas the yogurt and wheat bread were prepared in the laboratory according to commercial manufacturing procedures. The products were contaminated with microbes isolated from similar products by adding contaminants at relevant levels to the products before packing.

The initial level of 0.1 % (v/v) of the protective culture equivalent to 7.3 $\log_{10}$ CFU g^{-1} product of both strain inhibited the growth of *R. rubra* RHO in quark whereas a one log lower concentration of the strains had no inhibitory effect on the growth of the contaminant RHO. The yeast RHO reached the cell count of over 6 $\log_{10}$ CFU g^{-1} product in three weeks of storage at 6°C rendering both the control and the test product with 0.01% of protective culture unacceptable. The production of acetic acid and diacetyl was higher in quark with protective culture of 0.1 % (v/v) than in control (Table 13). In yoghurt, both of the contaminants (RHO and PQ) were inhibited by the protective culture with a level of 7.3 $\log_{10}$ CFU g^{-1}. In control yoghurt, *R. rubra* RHO and *P. quilermondii* PQ reached cell counts of 3 $\log_{10}$ CFU g^{-1} and over 7 $\log_{10}$ CFU g^{-1} , respectively, after four weeks of storage at 6°C.

Table 13. Levels of organic acids, diacetyl, ethanol and yeasts in quark and yogurt manufactured with basic starter and with the addition of 0.1% protective culture of *L.rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS after storage for 3 weeks at 6° C

	Quark		**Yoghurt**	
	Control	Protective culture (log 7 CFU g^{-1})	Control	Protective culture (log 7 CFU g^{-1})
Lactic acid, %	0.72	0.73	0.94	0.94
Propionic acid, $mg \cdot 100\ g^{-1}$	<0.5	<0.5	<0.5	2.0
Acetic acid, $mg \cdot 100\ g^{-1}$	38.0	64.0	5.0	10.0
Diacetyl, $mg \cdot kg^{-1}$	0.6	49.0	<0.5	24.0
Ethanol, $mg \cdot kg^{-1}$	10.0	15.0	220.0	7.0
Bentzoic acid, $mg \cdot kg^{-1}$	Nt	Nt	<10	<10
Yeast count, log CFU g^{-1} product	> 6	2	3.2	1 (RHO)
			>7	3.9 (PQ)

Nt, Not tested

Wheat bread

The optimal process applying the combination of *L. rhamnosus* LC705 and *P. freudenreichii* ssp.*shermanii* JS for the use of sour dough in wheat bread was developed. The initial level of 8 log_{10} CFU g^{-1} and 8.5 log_{10} CFU g^{-1} sourdough fermented for 10 h with LC705 and JS, respectively, inhibited the growth of *Bacillus* species in wheat bread totally at the addition level of 10% of sourdough (v/v) to the final dough at RH<20% and at the level of 15% of sourdough (v/v) at RH 70% conditions. In the control breads *Bacillus* sp. reached the cell counts of over 4 log_{10} $CFUg^{-1}$ already in two days at RH 70%. No antimicrobial effect against *Bacillus* sp. was noticed in bread manufactured with 10% of sourdough fermented for 4 h. Changes in the lactic acid concentration and pH was observed in breads (Table 14).

Table 14. The effect of duration of sour dough fermentation on pH, total titratable acidity (TTA) and concentration of organic acids in wheat bread. Breads were made with sour dough fermented with an initial level of both *L. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS 1-3x10^8 cells g^{-1} dough for 4, 10 or 20 h by adding 20% of the sour dough for the final dough. Control breads were fermented without the bioprotective culture for 4, 10 or 20 h by adding 10% sour dough to the dough.

	Fermentation time					
	4 h		10 h		20 h	
	Control	PC	Control	PC	Control	PC
Lactic acid, %	<0.04	0.04	<0.04	0.17	<0.04	0.23
Propionic acid, mg·100 g^{-1}	1.0	4.0	1.0	5.0	1.0	5.0
Acetic acid, mg·100 g^{-1}	19.0	19.0	18.0	19.0	19.0	19.0
pH	5.5	5.5	5.5	4.8	5.5	4.6
TTA, mL	3.0	3.2	3.0	4.5	2.8	4.7

4.2. The *in vitro* characterization of *P. freudenreichii* ssp. *shermanii* JS as a candidate probiotic strain

According to the current probiotic concept, each candidate probiotic strain has to be characterized individually, therefore *P. freudenreichii* ssp. *shermanii* JS was studied for its acid and bile tolerance (study V) and for its adhesion to intestinal mucosa (Study II). In order to eliminate the possibility of harmful traits of *P. freudenreichii* ssp. s*hermanii* JS, *in vitro* assessment of safety related aspects were monitored by analyzing antibiotic susceptibility as minimal inhibitory concentration of antibiotics (MIC) on VetMIC™ E-cocci microdilution panel, and by monitoring enzyme activity profile on semiquantitative API ZYM system (Study V).

The strain *P. freudenreichii* ssp. *shermanii* JS survived well in the *in vitro* tests evaluating its ability to survive during the transit of the upper part of the gastrointestinal tract. The treatment for 3 hours at pH value over pH 2 did not have any lethal effects on cells, but at pH 2 the cells were suffering of the acidity and the count dropped drastically under the detection level (3 log_{10} CFU ml^{-1})(Table 15). Bile at a concentration of 0.5% was well tolerated by the strain JS.

Table 15. The effect of a low pH (pH 2, pH 3 and pH 4) and a high bile salt concentration (0.5%) on viability of *P. freudenreichii* ssp. *shermanii* JS during 3 hours treatment.

Strain	**pH and bile**	**Cell numbers log_{10} cfu ml^{-1} (std) during 3 hours acid and bile salt treatments**			
		0 hour	1 hour	2 hours	3 hours
JS	pH 4	7.8 (0.06)	7.8 (0.02)	7.9 (0.02)	7.8 (0.07)
	pH 3	7.8 (0.06)	7.8 (0.05)	7.8 (0.03)	7.7 (0.12)
	pH 2	7.8 (0.06)	6.7 (0.30)	3.6 (0.55)	<3.0 (0.0)
	Bile 0.5%	7.8 (0.09)	n.t.	n.t.	7.8 (0.05)

Data given as mean values and standard deviations expressed as log_{10}cfu/ml (std.), n=3
Detection limit 3 log_{10} g^{-1}, n.t. not determined.

Adhesion (Study II)

Adhesion was studied with mucus adhesion model with colonic mucosa as described by Ouwehand et al. (2001). *P. freudenreichii* ssp. s*hermanii* JS was observed to adhere only moderately to all tested substrata without significant difference in adhesion to intestinal mucus (0.4±0.2%) or BSA (0.2 ±0.1%). The influence of other bacteria adhering prior to the mucus did not have any effect on the adhesion of JS nor did the incubation time (Data in the Study II).

Enzyme activity profiles

P. freudenreichii ssp. *shermanii* JS showed a high β-galactosidase activity determined *in vitro* with the semiquantitative API ZYM system. No β-glucosidase activity was noticed (data in Study II).

Antibiotic MIC (Study V)

The antibiotic susceptibility of the *P. freudenreichii* strains JS did not differ from other propionibacteria strains measured on the VetMIC™ broth microdilution assay (Table 16). The strain JS showed low minimal inhibitory concentration (MIC <4 µg/ml) for ampicillin, erythromycin, virginiamycin, tetracycline, chloramphenicol, vancomycin, narasin, bacitracin, and linezolid, and high MICs (>4 µg/ml) for aminoglycosides (streptomycin, gentamicin, and kanamycin).

Table 16. Antibiotic susceptibilities of *P. freudenreichii* ssp.*shermanii* JS, *P. freudenreichii* ssp. *freudenreichii* 131, *P. freudenreichii* ssp. *freudenreichii* NCIMB5959^T and *P. freudenreichii* ssp. *shermanii* NCIMB10585 on VetMIC^{TM} broth microdilution assay.

	JS	131	NCIMB 5959^T	NCIMB 10585
Ampicillin, µg/ml	0.25	2.0	<0.125	0.25
Erythromycin, µg/ml	<0.25	<0.25	<0.25	<0.25
Virginiamycin, µg/ml	<0.25	1.0	0.50	0.50
Gentamicin, µg/ml	8	4	8	16
Streptomycin, µg/ml	8	16	16	32
Kanamycin, µg/ml	32	64	64	128
Tetracycline, µg/ml	1.0	1.0	<0.25	0.50
Chloramphenicol, µg/ml	2.0	1.0	0.50	1.0
Vancomycin, µg/ml	1.0	1.0	1.0	1.0
Narasin, µg/ml	0.125	0.50	1.0	0.50
Bacitracin, U/ml	<1	< 1	< 1	< 1
Linezolid, µg/ml	0.50	2.0	1.0	0.50

4.3. Pilot assessment of the combination of *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 in human intervention studies (Studies III – IV)

4.3.1. Safety related aspects of *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 administered by elderly subjects (Study III)

Parameters such as fecal azoreductase activity, pH and mucin content indicating changes in the intestinal microbiota were assessed in elderly subjects who were administering the combination of the strains *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 in fruit juice (Hedelmätarha®) with a daily dose of 10 $\log_{10}$ CFU for two weeks. A trend of a slight reduction in the fecal azoreductase activity in the elderly (Table 17) was noticed *in vivo* with the combination of JS and LC705 strains. The reduction was statistically significant ($p<0.05$) from the baseline to consumption. No statistically significant changes were observed in pH or in faecal mucin excretion (data in Study III).

Table 17. The effect of the consumption of *L. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS on fecal azoreductase activity in the elderly subjects (nmol/h/g ; average ±SD)

Group	Baseline	Treatment	Follow-up
Control	29.5 ±0.9	29.2 ±2.3	29.4 ±1.8
JS+LC705	30.3 ±2.4	28.1 ±3.0	28.2 ±2.5

4.3.2. Immunomodulatory parameters measured from healthy adults (Study IV)

Parameters such as the number of immunoglobulin secreting cells (ISCs) and the expression of Fcα-receptor for IgA and complement receptors 1 and 3 for neutrophils and monocytes were assessed from peripheral blood samples in healthy adults administering whey-based orange juice (Hedelmätarha®, Valio Ltd., Finland) daily for two weeks. During the administration of fruit juice supplemented with *L .rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS a decreasing frequency of IgA-secreting cells was observed from baseline to consumption period, mean number decreased from 1427 to 943 and the median change-% was 21.4% (p=0.02). A slight but not significant decrease was seen in the expression of Fcα-receptor on neutrophils during the same period, the mean number decreased from 8.63 to 7.79 and the median change was 7.45% (Table 18). No significant changes in IgG or IgM-secreting cells or in Fcα-receptor on monocytes were detected (data in publication IV).

Table 18. Number and median change-% of ISC/10^6 mononuclear IgA cells and of FcαR in neutrophils at baseline, consumption and follow up periods in subjects receiving juice supplemented with *L. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS.

Visit	Number of ISC/10^6, geometric mean (IQR)	Number of FcαR in neutrophils; fluorescence intensity (IQR)	Median changes (%) in ISC/10^6 (IgASC)	Median changes (%) in FcαR
Baseline	1427 (913-2955)	8.63 (7.74-9.39)		
Consumption	943 (560-1828)	7.79 (7.21-8.53)	-21.4* (-40 to -14)	-7.45 (+24.4 to -3.87)
Follow-up	1322 (1188-1758)	5.77 (5.05-10.10)	+27.5 (-11 to +99)	+10.49 (-34.7 to +23.1)

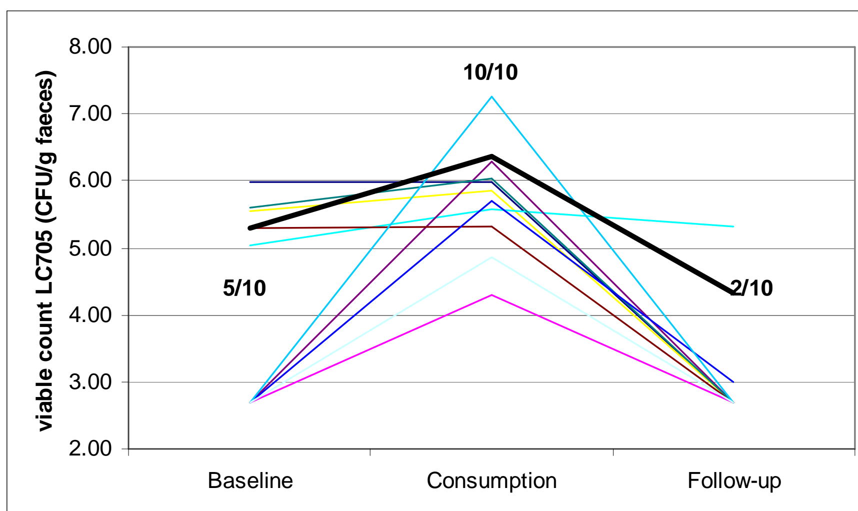
* Asteric denote for statistical significance (* p<0.05) tested by Wilcoxon test.

4.3.3. Survival of JS and LC705 *in vivo*

In vivo gastric survival of *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 was measured in single-blind randomized parallel intervention studies with 2-week periods (baseline, consumption, follow-up). The healthy adults were consuming the strains in whey-based orange juice (pH 3.8±0.2) Hedelmätarha® (Valio Ltd) with a daily dose of 10 log_{10} CFU during the study period. The fecal samples were collected after each period and analyzed by conventional cultivation methods. Isolates resembling the ingested strains were selected for further biochemical identifications and finally for genotyping in order to confirm the strain identification.

L. rhamnosus LC705 and *P. freudenreichii* ssp. *shermanii* JS survived at high numbers and were recovered at a concentration exceeding over 6 $\log_{10}$ CFU g^{-1} during the consumption period (Fig. 1a and 1b), JS reaching even the higher mean viable cell numbers (7.6 $\log_{10}$ CFU g^{-1}) than LC705 (6.4 $\log_{10}$ CFU g^{-1}). No isolates representing JS-type were found from the baseline samples whereas in five subjects isolates representing the identical RAPD type with LC705 strain were found. Total LAB numbers were not affected by the supplementation of LC705.

2 a)



2 b)

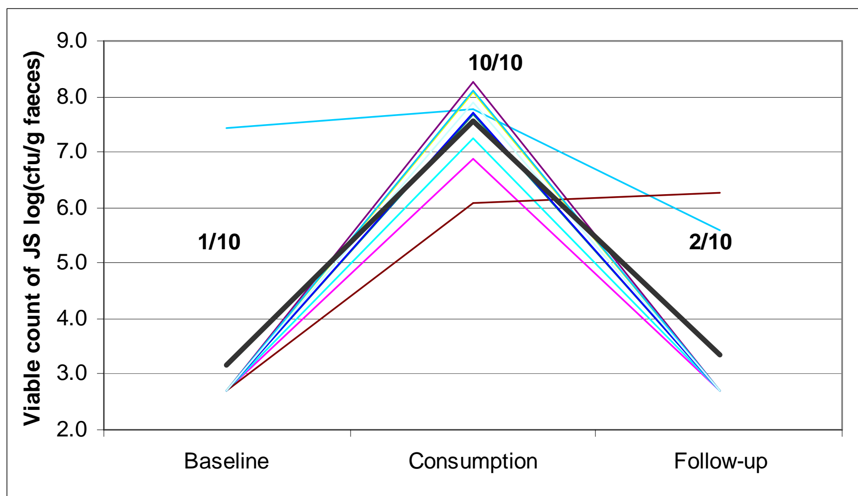


Figure 2a and 2b. Cell numbers and the detection frequency of *L. rhamnosus* LC705 (fig.2a) and *P. freudenreichii* ssp. *shermanii* JS (fig.2b) in faecal samples obtained at the end of baseline, consumption of fruit juice containing the strains LC705 and JS and follow-up periods (2 weeks each). The bold line shows the mean count of the strains in the faecal samples of 10 subjects and the thinner lines show the cell numbers of individual samples of each person.

4.3.4. Identification of the JS strain (Study V) and LC705 (Study IV)

The biochemical identification of *P. freudenreichii* ssp. *shermanii* JS isolates from the fecal samples was confirmed by 23S rDNA restriction analysis and by RAPD. The amplified 23S rDNA of strain JS gave a corresponding restriction pattern to the *P. freudenreichii*-type strain NCIMB 5959T, while the type strains *P. jensenii* ATCC 4868, *P. thoenii* ATCC 4874 and *P. acidipropionici* ATCC 4875 gave distinct restriction profiles. Isolates of strain JS did not reduce nitrate, which classified the strain to subspecies *shermanii*. Strain JS was differentiated from other strains representing the same subspecies by RAPD, which gave a unique RAPD-profile consisting of approx. 900-, 340-, and 250-bp fragments (Figure 3).

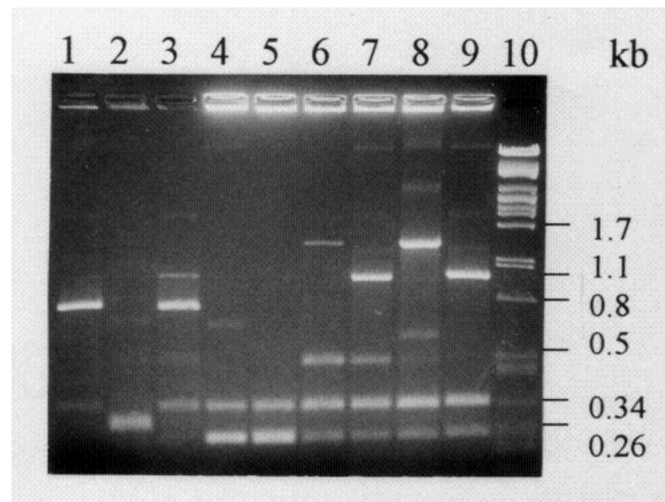


Figure.3. RAPD profile of *Propionibacterium* strains. Lanes 1-10, *P. jensenii* ATCC 4868^T (1), *P. thoenii* ATCC 4874^T, *P. acidipropionici* DSM 20272 (3), *P. freudenreichii* ssp.*freudenreichii* strain NCIMB 5959^T (4), 131 (5), ITG P20 (6), ITG P23 (7), *P. freudenreichii* ssp. *shermanii* strains NCIMB 10585 (8) and JS (9). Molecular weight marker λ *Pst*I (lane 10).

The identification of *L. rhamnosus* LC705 isolated from the fecal samples was confirmed by RAPD with OPA-3 primers. Additional primers were used in the RAPD analysis in order to increase the discriminatory power of the method. In total 135 isolates were compared by RAPD to the pure culture of LC705.

4.3.5. Gut wellbeing measured as defecation frequency (Study III and IV)

A slight increase in defecation frequency during the consumption of the juice containing *P. freudenreichii* ssp.*shermanii* JS and *L. rhamnosus* LC705 was observed in elderly institutionalized persons (Study III) (Table 19). In healthy adults (study IV) the defecation frequency showed increased tendency from the baseline to consumption period, but was unchanged from consumption to follow-up period (data in Study IV).

Table 19. The effect of the consumption of *L. rhamnosus* LC705 and *P. freudenreichii* ssp.*shermanii* JS on the defecation frequency (average/week ±SD) of the elderly (Study III).

	Baseline	**Treatment**	**Follow-up**
Control	2.2 ±1.4	1.7 ±0.5	2.2 ±0.4
LC05+JS	2.1 ±0.9	2.6 ±1.0*	2.3 ±0.8

* significantly different from the baseline levels ($p<0.05$)

5. Discussion

5.1. Technological and bioprotective applicability as a combined culture in foods

Alternative, natural techniques for ensuring the high microbiological quality of foods without chemical preservatives are of great interest. In this study the technological applicability of the strains *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 as a bioprotective culture in fermented milk products (quark and yoghurt) and wheat bread was studied. The effective concentration of the bioprotective culture against yeasts and molds was at a level of 7 $\log_{10}$ CFU g^{-1} fermented milk product. Similarly, the culture was active against *Bacillus* sp. in wheat bread at the level of 8 $\log_{10}$ CFU g^{-1} sour dough. The concentrations were economical and the procedures technologically feasible for the commercial applications. However, the efficacy of the antimicrobial activity may be contaminant and product dependent and therefore the antimicrobial spectrum cannot be generalized against all yeasts or molds causing spoilage in fermented products or against all *Bacillus* species in cereal products.

The combination of *P. freudenreichii* ssp. *shermanii* JS with *L. rhamnosus* LC705 was chosen for the bioprotective application study because the preliminary tests indicated that the synergistic effects of the strains JS and LC705 were more efficient against wide range of yeasts and molds than the effect of these strains alone (Mäyrä-Mäkinen & Suomalainen, 1995), an observation similar to Miescher Schwenninger & Meile (2004).

The mechanisms and possible compounds behind the antimicrobial action of the combination JS+LC705 were not studied further. Lactic and propionic acid with other non proteinaceous compound such as phenyl-lactic acid (PLA) may play a crucial role in the inhibition. Synergistic effects of acetate, lactate and propionate are likely to be important; however, Lind et al. (2005) reported that the effect of propionibacteria against certain yeasts and molds was not totally explained by acids. Also, bacteriocins may be involved in the bioprotection of foods (Holo et al., 2002). *P. freudenreichii* species are reported to produce bacteriocins (Gwiazdowska & Trojanowska, 2006; Warminska-Radyko et al., 2001) and similarly, bacteriocin production has been reported for *L. rhamnosus* (Weese, 2002) but as no studies for bacteriocin production of *P. freudenreichii* ssp. *shermanii* JS or *L. rhamnosus* LC705 has been conducted, this issue remains under further studies.

5.2. *P. freudenreichii* ssp. *shermanii* JS a as candidate probiotic strain

5.2.1. Biological properties

The current probiotic concept includes that each probiotic strain should have its own documentation even though applied in a culture combination. *L. rhamnosus* LC705 has been described for its biological properties otherwise (Tuomola et al., 2000; Mäyrä-Mäkinen et al., 2002). In this study, *P. freudenreichii* ssp. *shermanii* JS was characterized for its applicability as a candidate probiotic strain *in vitro*. At the selection phase the assays of safety characteristics and the tolerance to upper GI-tract conditions by *in vitro* tests are prime concerns. The excellent tolerance of *P. freudenreichii* ssp. *shermanii* JS to conditions encountering in upper gastrointestinal tract was shown *in vitro* for the cells. The cells of the strain JS tolerated with high cell numbers acidity until pH 2 in which the conditions caused drastic cell death. Bile salts were not affecting the JS strain. The survival of the strain JS is comparable with other potential probiotic *P. acidipropionici* and *P. freudenreichii* strains (Zarate et al., 2000; Huang and Adams, 2004; Herve et al., 2007). The activity of certain bacterial enzymes such as azoreductase, nitroreductase, β-glucuronidase, and β-glycosidase produced by the intestinal microbiota have been connected to the release of potentially carcinogenic compounds in the colon (Rowland, 1992; Chadwick et al., 1992) and therefore the enzyme profiles of potential probiotic strains have been characterized. In this study, the enzyme activity profiles of the strain JS was studied *in vitro* with the semiquantitative API ZYM system which showed no production of β-glucosidase, similarly as in the study of Perez-Chaia et al. (1999) with *P. freudenreichii* strains CRL 757 and SG1 or *P. acidipropionici* strains Q4 and CRL1198 . The other harmful enzymes of interests are not included in API ZYM test assay. Instead, a positive enzyme activity of β-galactosidase was detected with high intensity, similarly as evidenced in the study of Zarate et al. (2000) with *P. freudenreichii* and *P. acidipropionici*. It is suggested that β-galactosidases of PAB may be active within the gut (Zarate et al., 2002) and thus helpful in treating lactose intolerance.

Adhesion of the probiotic strain to the intestinal mucosa is considered an important factor at least for the immunomodulatory effects in which the close contact between the probiotic cells and the immunologically active cells is thought to be essential (Brassart & Schiffrin, 1997). Thus, *in vitro* adhesion to human mucus preparation may be an indication of possible *in vivo* adherence ability and it may be associated with the transient colonisation (Johansson et al., 1993). However, to study the *in vivo* adherence is demanding as adherence is associated to the mucosa-associated cells (Walker et al., 2006) and only invasive techniques such as colonyscopy could be used for the sampling of mucosa-associated cells (Alander et al., 1999). The colonoscopy demands ethical approval and it is laborious to perform, and therefore the use is generally restricted to severe cases. In this study the adherence of the strain JS was studied to human resected mucus *in vitro* and the assay showed a moderate adherence compared to the other PAB strains. Interestingly, the adhesion of the other PAB but not of the strain JS was enhanced in combination with selected probiotic bacteria, e.g. with well known LGG. Despite the *in vitro* adhesion results, it can be assumed that the strain JS does interact with the intestinal mucosa as in an intervention trial with three different bacteria genera, one of them being the strain JS, on immunomodulatory capacity (Kekkonen et al., 2008), all the strains evoked the immune responses in a strain specific manner, e.g. the strains JS and LGG

reducing the serum hsCRP level of the healthy adults, thus indicating a potential for anti-inflammatory effects. Thus, the relevance of *in vitro* adherence for indicating the biological activity is arguable.

5.2.2. Safety related aspects of the strain JS

In European member states, according to EFSA scientific committee (Barlow et al., 2007), any strain of microorganism of which the identity is established and assigned to a QPS group does not need any for further safety assessment than that conducted by EFSA. Based on that, *P. freudenreichii* species are freed by EFSA from safety assessment. Similarly, *L .rhamnosus* species are freed by EFSA.

A major area of concern has been the potential for antibiotic-resistance transfer in the gastrointestinal tract that might take place between probiotics and pathogenic bacteria (Salyers et al., 2004; Mathur and Singh, 2005). It is accepted that antibiotic resistance is not a hazard unless it renders the probiotic untreatable in rare cases of infection or unless it can be transferred to potential pathogens. Certain probiotic LAB and BIF cultures seem to carry acquired *tet*(W) gene which mediates ribosomal protection to tetracycline. High *Tet*(W) frequency among the intestinal and oral microbes of humans and animals suggest its transferability (Kastner et al., 2006) and that may cause extra safety concerns with these certain potential probiotic LAB and BIF cultures. Dairy propionibacteria exhibit a natural antibiotic resistance to oxacillin, aminoglycosides, 1st and 2nd generation quinolones, colistin, metronidazole and phosphomycin (Madec et al.,1994; Chamba et al.,2007). According to MIC distribution data based on VETMIC panel in this study, the *P. freudenreichii* ssp. s*hermanii* strain JS could be regarded susceptible to all other antibiotics, except for aminoglycosides (streptomycin, gentamicin and kanamycin), similarly as the other dairy PAB. The resistance to aminoglycosides is a common characteristic of anaerobic bacteria as they lack a cytochrome-mediated drug transport system (Bryan and Kwan, 1981). No study with the JS strain or with other current potential *P. freudenreichii* probiotics has been conducted to clarify if the antibiotic resistance is plasmid or chromosomally coded. The assumption lies on the chromosomal resistance genes as no differences in the antibiotic resistance patterns were seen between parent and plasmid-cured strains of dairy PAB (Rehnberger and Glatz, 1990). Additionally, metionidatzole resistance gene *nimA* in cutaneous PAB was detected in strains without plasmids, suggesting chromosomal location (Lubbe et al., 1999). All in all, the genotyping of the antibiotic resistances is recommendable.

5.3. *In vivo* performance and functionality of the culture combination of *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705.

5.3.1. *In vivo* survival

The preliminary intervention trials indicating *in vivo* survival pattern and possible probiotic traits are needed for the final assessment of the probiotic traits. In this study the probiotic traits of the strain JS were explored in a culture combination with *L. rhamnosus* LC705 administered in a fruit juice. The human intervention trials confirmed the *in vitro* tolerance to the upper GI tract conditions of the strains JS and LC705. The strains in administration level of 10 log_{10} CFU d^{-1}, survived the passage through the GI tract in high numbers with the

recovery of 60 to 80% for JS and 40 to 70% for LC705 from feces by conventional cultivation assays. Similar levels of fecal recovery have been reported for the JS strain in other studies (Myllyluoma et al., 2005; Kekkonen et al., 2008) and for *P. freudenreichii* strain S141 (Jan et al., 2002; Herve et al., 2007). Also, *L. rhamnosus* LC705 survival and colonization pattern was comparable to other former *L. casei* –group strains (Saxelin et al., 1999). It is assumed that probiotics do not colonize the intestine permanently as the abundant resident microbiota compete the transient exogenous species (Ouwehand et al., 2006). This was seen with the strains JS and LC705 as the cell counts decreased below the detection level within two weeks from the end of the consumption. Competitiveness or the activities of the strains JS and LC705 within the gut microbiota were not studied, but the changes seen in the faecal azoreductase activity in this study, in β-glucosidase activity in the study of Hatakka et al. (2008a) and in urinary aflatoxin-B-N^7-guanine, biomarker for increased risk of liver cancer in young men (El-Nezami et al., 2006) indicate that the strains of the combination may be able to compete and cause changes within the resident microbiota.

The efficacy of probiotics is dependent on the strain's ability to reach elevated population levels and to remain alive and biologically active in the gut. Variable daily doses from 6 $\log_{10}$ CFU to 9 $\log_{10}$ CFU have been recommended (Coeuret et al., 2004), but the generalization is not recommended as the dose responses are dependent on strain and carrier matrix as the matrix into which cells are incorporated prior to their consumption plays a crucial role in the survival of the strains (Saxelin et al., 2003). Unfortunately, only limited numbers of dose-response studies of probiotics have been performed, mainly with the probiotic LAB strains (reviewed in Lahtinen, 2007). In this study the strains were administered in acidic fruit juices with average pH level of pH 3.8. Both strains survived during the shelf life of the product and through the GI-tract in adequate numbers reaching the level of 6 $\log_{10}$ CFU in feces. According to the current opinion, the live cells recovered from the feces do not explain the biological activity in the intestine but they verify the good survival pattern of these strains.

5.3.2. Safety related aspects of the combination

P. freudenreichii ssp. *shermanii* JS and *L. rhamnosus* LC705 can be considered as safe for human consumption. No harmful side-effects on the institutionalized elderly or healthy adults during the intervention studies were reported and in the elderly no mucin degradation was observed. Most important, *P. freudenreichii* and *L. rhamnosus* species are considered as safe by EFSA.

5.3.3. Beneficial health effects measured as gut wellbeing

Gut health can be measured by several end points such as bowel movements and transit time, abdominal pains, or changes in the gut microbiota composition; however for any of these parameters the interpretation is complicated by the large individual variability even within the normal range (Aggett et al., 2005). In this study, the gut well being was characterized in constipated elderly and in healthy adults by following the defecation frequency. The combination *P. freudenreichii* ssp. *shermanii* JS and *L .rhamnosus* LC705 was shown to relief the constipation in the elderly (p=0.04) during the consumption period of 2 weeks with daily dose of 10 $\log_{10}$ CFU. A statistically insignificant tendency to increase the defecation frequency in the healthy adults was reported, too. Elderly individuals are considered more

susceptible to functional gastrointestinal symptoms than young adults, and therefore constipation is relatively common in elderly (Pitkälä et al., 2007; Tiihonen et al., 2008b). Interestingly, both *B.longum* and *B.lactis* isolated from the elderly and administered in fermented oat drinks normalized the bowel movements of the elderly (p=0.004 and p=0.002, respectively) (Pitkälä et al., 2007) and a multistrain combination consisting of *Bifidobacteria* sp. and *Lactobacillus* sp. increased the frequency of bowel movements in children with functional defecation disorder (p=0.009) (Bekkali et al., 2007). Studies performed on symptoms of constipation with *L. casei s*hirota (Koebnick et al., 2003) and with *B. infantis* DN-173 010 (Marteau et al., 2002; Meance & Turchet, 2001) in adults showed decrease in colonic transit time. The positive effect of probiotics in gut well being is hypothesized to be due to the production of organic acids by the strains resulting in a lowering of pH in the colon which in turn enhances the motility and subsequently decreases the transit time in the colon (Bekkali et al., 2007). In addition, the composition of colon microbiota influences the peristalsis of the colon (Picard et al., 2005) Especially in the elderly the counts of bifidobacteria have been reported to decline (Hopkins et al., 2002). Interestingly, certain propionibacteria are known to enhance the growth of bifidobacteria and this could be an approach to affect the bifidobacteria counts in the elderly and thus increase the amount of short chain fatty acids. The softening of the stools by stimulating water and electrolyte secretion may improve the gut wellbeing (Bongers et al., 2007). Especially ageing seem to affect the consistency of the stools by increasing the fecal dry matter (DM) content (Tiihonen et al., 2008b), but no probiotic strain has been reported to affect on DM content.

5.3.4. Immunological activity

Healthy intestinal mucosa contains abundant immunoglobulin A (IgA)–secreting cells, which are generated from B cells in gut-associated lymphoid tissues (GALT)(Rodrigo Mora et al., 2006). IgA secreting cells are mainly detected from the saliva or faecal samples. In this study, IgA, IgG and IgM were analyzed from the peripheral blood of the healthy adults. It remains uncertain whether the observed change in IgA secreting cells is biologically relevant, partly because the evaluation of the changes in blood cell immune responses does not explain the actual physiological effects (Delcenserie, 2008) and because the subjects were healthy adults with no reported side effects. In addition, no changes were seen in the frequency of IgG- or IgM secreting cells. The strain JS was not studied for any health promoting activity alone in any of these studies, but the study of Kekkonen et al. (2008) gives the indications that the strain JS as a single culture may pose a promising anti-inflammatory activity.

5.4. Confirmation of the biochemical identifications

The conventional cultivations of faecal samples are commonly used for the measurement of viability of ingested strains. The enumeration of the strain JS from feces was conducted by conventional cultivation on PPA medium which is a buffered modification of the traditional YEL-medium (yeast extract lactate-medium) with the supplementation of 1 % (w/v) β-glyserolphospate which was added in order to diminish the acidity stress caused by lactic acid bacteria in the faecal samples. A more selective PAL Propiobac® medium can be used instead of YEL-type medium (Jan et al., 2002; Herve et al., 2007), but as the discriminatory

power of PPA was sufficient (data not shown), PAL Propiobac® medium was not used in this study. The identification of *P. freudenreichii* isolates to the species level was performed by 23S rDNA restriction analysis and to the strain level by RAPD. The RAPD profile consisted of 900-, 340-, and 250-bp DNA fragments, of which 340- and 250-bp fragments are typical for *P. freudenreichii* species and the fragment 900-bp typical for the strain JS, only. The specificity of RAPD assay was confirmed with PFGE analysis which is the golden standard method for the strain identification.

For dairy *Propionibacterium* species identification, PCR-based methods are available for genus and species level (Meile et al., 1999; Tilsala-Timisjärvi and Alatossava, 2001) and for the confirmation of the strain identity, methods such as PFGE and RAPD-PCR are available (Gautier et al, 1995; Rossi et al., 1998). In this work, strain-specific RADP-PCR was used to identify the strain JS in feces and the further identification was confirmed with PFGE. Interestingly, the biochemical characteristics of *P. freudenreichii* species can be used for differentiating *P. freudenreichii* species from other dairy species (Dherbecourt et al, 2006) and within the *P. freudenreichii* subgroups, *P. freudenreichii* ssp. *shermanii* types can be differentiated from *P. freudenreichii* ssp. *freudenreichii* by their nitrate reduction and lactose fermentation. In this study, the biochemical identification was true for 94.5% of the preliminary isolates confirmed with genomic identification and in a study of *P. freudenreichii* S141, 93% of the preliminary isolates were identified as S141 (Jan et al., 2002).

Conventional cultivation of fecal bacteria gives numbers of live cells but does not indicate metabolic activity of cells during the transit in GI tract. Besides, cultivation based methods bear several other discrepancies such as inability to detect the dormant or injured cells (Anderson et al., 2004). Therefore the relevance of the conventional cultivation of fecal samples is discussed. The recent development in quantitative PCR methods is notable, but due to the expensive investments needed, they are not commonly accessible. On the other hand, DNA-based techniques do not discriminate between living and dead cells and do not indicate the metabolic activity of the cells. Therefore bacterial mRNA has been considered as an indicative marker for the cell viability (Sheridan et al., 1998) and metabolic activity (Herve et al., 2007). Non-invasive molecular marker based on 5S subunit of transcarboxylase has been described for *P. freudenreichii* species (Herve et al., 2007). This 5S subunit of multienzyme complex of transcarboxylase is specific for *P. freudenreichii* species and as its expression occurs only during the propionic acid fermentation indicating the metabolic activity of the cells it offers an interesting option for the detection of metabolic activity. However, a successful *in situ* detection of mRNA requires that the cells are permeable to reverse transcriptase and RNA polymerase, that amplificon products do not already exist in the cells and that mRNA is present in sufficient concentration for its signal to be detectable (Walker et al., 2006).

The enumeration of LC705 was conducted on LAMVAB agar (Hartemink et al., 1997) and typical LC705 type colonies were selected for genotyping with randomly amplified polymorphic DNA (RAPD) with OPA-3 primer. For the confirmation of the genotyping, several other primers were used. Half of the subjects in the intervention study had isolates with typical colony morphology and with identical RAPD patterns for the strain LC705 already in the beginning of the study. The discriminatory power of the method is proven adequate (Alander et al., 1999), therefore it is assumed that those subjects were consumers of products containing the strain LC705 as a technological cheese starter. In this study, no restriction for diets during the intervention was recommended.

6. CONCLUSIONS

This study ensures the role of *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 as candidate probiotics in a combined culture. Besides, the study proves the *in vitro* probiotic potential and safety of *P. freudenreichii* ssp. *shermanii* JS and *in vivo* safety of the combined culture.

P. freudenreichii ssp. *shermanii* JS has been used for decades as a primary cheese starter and *L. rhamnosus* LC705 as an adjunct cheese starter in big eye cheeses in Finland. In this study the strains were applied in fermented products (yogurt and quark), in wheat bread, and in fruit juices (pH 3.8). The strains survived without any major loss in cell counts through the shelf lives of all products. The industrial scale production processes for both strains exist with high cell yields. These results and the good technological applicability indicate excellent fermentation, downstream processing and stability of the strains JS and LC705 in the products.

The studies with the combination of the strains JS and LC705 administered in fruit juices monitored primarily the survival of the strains JS and LC705 during the GI transit on healthy adults and secondly their effect on gut wellbeing properties measured as relief of constipation in institutionalized elderly and in healthy adults. An excellent survival and a transit colonization on the GI tract of healthy adults was proven for both strains. Especially the recovery rate of 70 to 80% of the JS strain was excellent even though the strain was administered in a demanding matrix of a whey-based fruit juice with a low pH of 3.8. An improvement in gut wellbeing measured as defecation frequency was reported in the institutionalized elderly and a trend of the increased defecation frequency in healthy adults. No side effects were reported in the human intervention trials and *in vitro* characterization of the strain JS ensured the safety by showing no deviation in the antibiotic resistance pattern compared to other PAB. Beside the phenotypic antibiotic resistance pattern, the presence of antibiotic resistance genes of the genome should be tested in order to confirm that the resistance is not plasmid encoded.

The biochemical identifications of the strains from the faecal samples were confirmed with modern molecular biological tools. The strain JS was identified to the species level by performing 23S rDNA restriction analysis and to the strain level by RAPD profiling. Similarly, the strain LC705 was identified to the strain level by RAPD profiling. Thus, strain specific identification methods were confirmed during the study.

Separately, the combination of *P. freudenreichii* ssp. s*hermanii* JS and *L. rhamnosus* LC705 was evaluated from the technological point of view as a bioprotective culture and the efficacy was proven against common spoilage yeasts in fermented milks and against ropiness causing *Bacillus* sp. in wheat bread. This natural bioprotective activity will be a valuable asset within the growing interest for mild food preservation tools.

In conclusion the combined culture of *P. freudenreichii* ssp. *shermanii* JS and *L. rhamnosus* LC705 has an encouraging role as a properly identified and safe probiotic culture with a limited but encouraging evidence for the efficacy in treating constipation in the elderly.

Moreover the combined culture may have an innovative dual role acting as a bioprotective and a probiotic culture even in demanding food matrixes.

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