Investigation into the influence of a bacteriocin-producing *Enterococcus* strain on the intestinal microflora

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1.0 INTRODUCTION

1.1. Lactic acid bacteria in food production and human health

The lactic acid bacteria (LAB) are a group of bacteria united by a constellation of morphological, metabolic and physiological characteristics. Generally, LAB are described as Gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid as a major end-product during fermentation of carbohydrates (Axelsson, 1998).

Historically, LAB have long been known to be involved in the production of fermented foods. Presently, these products constitute one-quarter of our diet and are characterised by a safe history, certain beneficial health effects, and an extended shelf life when compared to non-fermented, fresh foods (Hammes and Tichaczek, 1994). Lactic acid producing bacterial starter cultures are used in the production of fermented dairy, meat and plant products and the fermentation results in products which have improved shelf life, flavour, aroma and texture (Sofos, 1993). The major food commodities fermented by LAB are listed in Table 1. Fermentation ensures not only the microbial safety of the food product, but may also render some foods more digestible. In addition, fermentation may also serve to reduce the toxicity of the food substrate, as occurs for example in the fermentation of cassava tubers *(Manihot esculentum*) which contains toxic cyanogens (Caplice and Fitzgerald, 1999). Another beneficial aspect of fermentation is that growth of spoilage and pathogenic bacteria in fermented foods is usually inhibited due to competition for nutrients and the presence of starter-derived inhibitor compounds such as lactic acid, hydrogen peroxide and bacteriocins (Ray and Daeschel, 1995). Bacteriocins are extracellularly released peptides that are bactericidal to bacteria closely related to the producer microorganisms (Tagg et al., 1976). Bacteriocins produced by LAB may be considered as natural preservatives or 'biopreservatives', as these compounds have been produced by food-associated LAB and consequently have been associated with the human food supply for many years (Schillinger et al., 1996). LAB are generally considered as 'food grade' organisms. It is assumed that most representatives of this group do not pose any health risks to healthy humans. Most LAB are designated as 'GRAS' (**g**enerally **r**ecognised **a**s **s**afe) microorganisms in the USA based on a long history of safe use in foods. However, some species may act as opportunistic pathogens in rare cases (Aguire and Collins, 1993; Gasser, 1994; Holzapfel et al., 1995).

Of all lactic fermented foods, dairy products are the most important worldwide, both by production volume and value (Lücke, 1996). More than 50% of the total milk in Europe is processed into sourmilk, sour cream, butter and cheese. All dairy fermentations include an

initial lactic fermentation, only in rare cases accompanied by yeasts, as in the case of kefir and some traditional sourmilks. Desired sensory properties of some products are contributed by certain LAB, propionibacteria, brevibacteria, yeasts or moulds through secondary fermentation (Lücke, 1996).

Many studies have shown that fermentation with LAB improves the nutritional value of food products by increasing the quantity, availability, digestibility, and assimilation potential of nutrients. Yoghurt for example, contains higher levels of free amino acids as compared to milk as a result of proteolysis of the milk proteins by the yoghurt microflora (Gorbach, 1990). In addition, fermentation of foods with LAB can exert the following beneficial effects: (a) production of lactic acid and minor amounts of acetic and formic acids, which cause a drop in pH and thereby inhibit the growth of food spoilage or food poisoning bacteria, (b) detoxification by degradation of noxious compounds of plant origin such as cyanogens, (c) production of antimicrobial compounds (e.g. bacteriocins, hydrogen peroxide, fatty acids) which can also inhibit spoilage or food poisoning bacteria, and (d) probiotic effects as of LAB administered as live organisms in food (Hammes and Tichaczek, 1994).

Yoghurt and particularly probiotic fermented milk products are also reported to be effective in the treatment of a variety of disorders, including colitis, constipation, diarrhoea, prevention of recolonisation of the intestine with pathogens after antibiotic treatment, reduction of flatulence, prevention of gastroenteritis, gingivitis, hypercholesterolaemia, hepatic encelopathy and tumorigenesis. In addition, the lactose intolerance of affected persons can be alleviated by consuming yoghurt which contains bacterial β-galactosidase produced by lactobacilli during fermentation (Gorbach, 1990). Pouwels et al. (1998) studied the possibility for use of LAB in general and strains of *Lactobacillus* in particular, for oral vaccination purposes. This was done on the presumption that these bacteria have GRAS status, have adjuvant properties, mucosal adhesive properties and low intrinsic immunogenicity. However, a number of problems will have to be solved before the first LAB vaccine can be marketed. One such problem, for example, is that gene expression is difficult to be measured in the gut (Pouwels et al., 1998).

Table 1: Food commodities fermented by lactic acid bacteria (LAB) or LAB in combination with other microorganisms (Lücke, 1996).

Raw material	Product	Main fermentation agents	Additional fermentation agents in some products
Milk	sour milk	LAB	Yeasts
Milk	cheeses	LAB	*Propionibacterium, Brevibacterium, Penicilllium*
Meat	fermented sausages	LAB + *Staphylococcus* or *Micrococcus*	Yeasts, *Penicillium*
Doughs, grain	sourdough, sour	LAB + yeasts	
mashes	mash	LAB	
Vegetables	sauerkraut	LAB	
Olives	table olives	*Rhizopus,* LAB	
Soybeans, peanuts	tempeh, ontjom	Yeasts, LAB	
Soy mash	soy sauce	Yeasts	LAB
(Grape) musts	wine	Fish enzymes, LAB	
Fish	lightly salted fish products, fish sauces		

The currently recognised genera of LAB are *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissioccus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson, 1998). This classification is largely based on phenotypic characteristics such as morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance (Axelsson, 1998). As a group, the LAB are catalase-negative, non-sporeforming with coccoid, cocco-bacilli or rod-shaped morphology. They have less than 55% mol% G+C content in the DNA and therefore belong to the *Clostridium* branch of Gram-positive bacteria (Stiles and Holzapfel, 1997). Bifidobacteria, which were in the past considered to be a member of the LAB, have a mol% G+C content of more than 55% and therefore belong to 'Actinomycetes' branch of Gram-positive bacteria (Schleifer and Ludwig, 1995).

1.2.1. Technological and functional aspects of probiotic LAB strains

In recent years, the consumer has been increasingly confronted with functional food products which are claimed to promote his /her health and well-being. At the centre of these food products are the so-called pro- and prebiotics (Simmering and Blaut, 2001). The term 'probiotic' (Greek: 'for life') originally referred to a phenomenon observed when two organisms were cultured together, in which substances produced by one organism stimulated

the growth of the other organism. These substances were referred to as 'probiotics'. This term was subsequently used to describe living preparations of microbial cells that could be administered to animal and humans to promote the health of its consumer (Tannock, 1997). Fuller (1991) defined probiotics as 'a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance'. This definition was broadened by Reuter (1997) to 'any viable mono- or mixed culture of microorganisms which beneficially affects the host on the indigenous microflora'. Marketing of probiotics for human consumption relies heavily on this definition. In the case of farm animals, faster weight gain for the same amount of feed consumed has been the primary importance for probiotic application (Tannock, 1997). Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon (Gibson and Roberfroid, 1995; Simmering and Blaut, 2001). The concept of prebiotics arose from the observation that inulin and fructooligosaccharides (FOS) selectively stimulate the growth of bifidobacteria, which are considered to be beneficial for human health (Gibson and Roberfroid, 1995).

Traditionally, LAB are the basic components of probiotics as they best fit the functional and safety requirements by their definitions (Reuter, 1997). However, today a wide number of microbial species and genera also are considered to meet the requirement for probiotics (Table 2).

Table 2: Microorganisms used as probiotics (Holzapfel et al., 1998).

***Lactobacillus* species**	***Bifidobacterium* species**	**Other LAB**	**Non LAB**
L. acidophilus *L. amylovorus* *L. casei* *L. crispatus* *L. delbrueckii* subsp. *bulgaricus* *L. gallinarum*[1] *L. gasseri* *L. johnsonii* *L. paracasei* *L. plantarum* *L. reuteri* *L. rhamnosus*	*B. adolescentis* *B. animalis* *B. bifidum* *B. breve* *B. infantis* *B. lactis*[3] *B longum*	*Enterococcus faecalis*[1] *Enterococcus faecium* *Lactococcus lactis* *Leuconostoc mesenteroides* *Pediococcus acidilactici* *Sporolactobacillus inulinus*[1] *Streptococcus thermophilus*	*Bacillus cereus var*. toyoi[1,2] *Escherichia coli* strain nissle *Propionibacterium freudenreichii*[1,2] *Saccharomyces cerevisiae*[2] *Saccharomyces boulardii*[2]

[1]Main application for animals
[2]Applied mainly as pharmaceutical preparations
[3]Synonymous with *B. animalis*

Probiotic bacteria may be delivered to the consumer in a food system and they then begin their journey to the lower intestinal tract at uptake in the mouth. Therefore, physiologically important properties of probiotic LAB include their resistance to enzymes present in the oral cavity (e.g. lysozyme), tolerance to bile acids, and that they possibly attach to the epithelium of the small intestine and can grow and be metabolically active in the lower intestinal tract as to provide health benefits (Chou and Weimer, 1999).

Klaenhammer and Kullen (1999) compiled selection criteria for probiotic strains. These include: (1) appropriateness (accurate taxonomic identification, normal inhabitant of the host species targeted, i.e. human origin for human probiotics, nontoxic, nonpathogenic, GRAS status), (2) technological suitability (amenable to mass production and storage: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution; viability at high numbers; stability of desired characteristics during culture preparation, storage, and delivery); provides desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes; genetic stability; (3) survival competitiveness and establishment (capable of survival, proliferation, and metabolic activity at the target site *in vivo*, resistant to bile, resistant to acid, able to compete with normal microflora, including the same or closely related species: potentially resistant to bacteriocins, acid, and other antimicrobials produced by the residing microflora, and adherence and colonisation potential preferred), (4) performance and functionality (able to exert one or more clinically documented health benefits, antagonistic towards pathogenic/procarcinogenic bacteria, production of antimicrobial substances such as bacteriocins, immunostimulatory, antimutagenic and anticarcinogenic activity, as well as production of bioactive compounds such as enzymes, vaccines or peptides). Bacteriocin production thus is considered as one of the performance or functional characteristics of probiotic strains and is discussed in more detail below.

Because of the potential health benefit of probiotic bacteria, these organisms are increasingly being incorporated into dairy and other foods. Claimed health benefits include prevention or alleviation of diarrhoea, antimicrobial, antimutagenic and anticarcinogenic properties, reduction of serum cholesterol levels, and improvement of lactose tolerance (Shah, 2000). Therapeutic properties of probiotic *Lactobacillus acidophilus* and bifidobacteria are summarised in Table 3.

Table 3: Therapeutic (probiotic) properties of *Lactobacillus acidophilus* and Bifidobacteria (Shah, 2000).

Therapeutic (probiotic) properties	Possible causes and mechanisms
Colonisation of gut and inhibition of pathogenic microorganisms	Survive gastric acid, resist lysozyme, tolerate high bile salt concentration, adhere to intestinal surface and production on inhibitory compounds i.e. acids, H_2O_2 and bacteriocins.
Improved digestibility of food and enhanced growth of host	Partial breakdown of protein, fat, carbohydrate and improved bioavailability of nutrients.
alleviation of lactose intolerance	Reduced lactose in the product and further availability on bacterial lactase enzymes for lactose hydrolysis.
Hypocholesterolaemic effect	Production of inhibitors of cholesterol synthesis, deconjugation of bile salt, assimilation of cholesterol.
Anticarcinogenic effect	Inhibition of carcinogens and enzymes involved in converting procarcinogens to carcinogens, inhibition of growth of putrefying organisms and stimulation of host immune system.
Stimulation of the host immunological system	Enhancement of macrophage formation, stimulation of T supressor cells and production of interferon.
Increased vitamin availability to host	Synthesis of group B (folate) vitamins.

Dunne et al. (1999) defined criteria by which a microorganism may be considered as a probiotic. Thereby, a probiotic microorganism should:

1. Be of human origin.
2. Demonstrate non-pathogenic behaviour.
3. Exhibit resistance to technological production processes.
4. Prove resistance to gastric acid and bile.
5. Adhere to gut epithelial tissue.
6. Be able to persist, albeit for short periods, in the gastrointestinal tract.

7. Produce antimicrobial substances.
8. Modulate immune responses.
9. Have the ability to influence metabolic activities (e.g. cholesterol assimilation, lactase activity, vitamin production).

LAB used in probiotic preparations usually are derived from human sources and include strains which are typical of the human gastrointestinal tract (Holzapfel et al., 2001). These are represented by three groups: 1) the *L. acidophilus* group involving strains that are recognised today as *L. acidophilus*, *L. gasseri*, *L. crispatus* and *L. johnsonii* 2) *Lactobacillus salivarius* and 3) *Lactobacillus casei* group involving strains of *L. casei*, L. *paracasei* and *L. rhamnosu*s (Holzapfel et al., 2001). Some probiotic lactobacilli strains which have been utilised in commercial products include *L. paracasei* Shirota from Yakult, *L. casei* GG (although this strains was first classified as a *L. casei* strain, it actually is a *L. rhamnosus* strain) from Emmi and *L. acidophilus* LA-1 (this strain was later shown to be a *L. johnsonii* strain) from Nestle. *L. paracasei* Shirota and *L. casei* GG are marketed in yoghurt drinks, whilst *L. acidophilus* LA-1 is marketed in yoghurt –products (Table 4).

Table 4: *Lactobacillus* strains and their used in probiotic yoghurts or yoghurts-like products and levels in which they are employed (Holzapfel et al., 2001).

Probiotic strain	**Type of product**	**Identification on the basis of DNA-homology analysis**	**Viable counts (Log_{10}CFU/g)**
L. acidophilus LA-1	Yoghurt	*L. johnsonii*	7.1-8.0
L. acidophilus LA-7	Yoghurt	*L. acidophilus*	3.9-6.1
L. acidophilus LA-H3	Dietetic yoghurt	*L. acidophilus*	5.8-8.4
L. acidophilus	Yoghurt	*L. crispatus*	6.8-8.2
L. acidophilus	Yoghurt	*L. acidophilus*	5.5-6.8
L. casei Actimel	Yoghurt drink	*L. paracasei*	7.4-8.4
L. casei Shirota	Probiotic drink	*L. paracasei*	7.9-8.9
L. casei GG	Yoghurt drink	*L. rhamnosus*	8.0
L. casei LC-H2	Dietetic yoghurt	*L. casei*	4.7-5.3
L. casei	Yoghurt	*L. paracasei*	6.2-7.8
L. casei	Yoghurt	*L. paracasei*	8.6-8.7

Not only lactobacilli strains have been extensively studied regarding their functional properties as probiotics, but a great amount of research has also been performed on enterococci as probiotics. One such strain, i.e. *Enterococcus* (*E.*) *faecium* strain SF68 in particular, has been studied in detail for use as a human probiotic, especially in the treatment of diarrhoea. The strain was originally isolated in Sweden and patented in Switzerland and other countries (Franz et al., 1999). Its effectiveness for treatment of intestinal disorders can probably be attributed to the fact that it has a short lag phase and generation time (ca. 20 min under optimal conditions) and thus may outcompete gastrointestinal pathogens. This strain is sensitive to clinically relevant antibiotics and has an inhibitory effect *in vitro* to the growth of *E. coli, Salmonella* spp., *Shigella* spp., and *Enterobacter* spp. It is resistant to low pH and insensitive to bile salts (Canganella et al., 1996) which enables the strain to survive transit through the stomach and the conditions encountered in the small intestine. Use of *E. faecium* SF68 for treatment of diarrhoea is considered an alternative to antibiotic treatment (Bellomo et al., 1980). However, some enterococcal probiotic preparations for which the strains that are included are not well defined are sold in 'health food' stores as nutrition supplements, with vague claims to improve the gastrointestinal balance. Such preparations containing strains of *E. faecium* are also available on the market (Franz et al., 1999).

The functional properties of probiotic LAB strains (e.g., stimulation of immune response, anticarcinogenis/antimutagenic activity, cholesterol reduction, antimicrobial activity by bacteriocin production, and alleviation of lactose intolerance) will be discussed in more detail below.

1.2.1.1. Immune response

The immune system consists of a number of organs and different cell types that recognise foreign antigens of intruder microorganisms. The leukocytes are the most dominant in the immune system and they comprise two cell types, i.e. (1) phagocytes, which provide non-specific immunity, and (2) the lymphocytes, which provide specific immunity. It has been suggested that LAB might thus be responsible for immune activation and thus prevent and stabilise enteric infections.

Perdigon and Alvarez (1992) studied the effect of *L. acidophilus, L. casei, L. delbrueckii* subsp. *bulgaricus* and yoghurt on non specific immune response (mediated by macrophages). They concluded that *L. acidophilus, L. casei* and *L. delbrueckii* subsp. *bulgaricus* strains could activate the cells involved in the non-specific immune response and that oral administration was as effective as intraperitoneal administration. *L. casei* was also

shown to stimulate the increase of the mucosal intestinal IgA levels against *Salmonella typhimurium* in mice, indicating its potential as probiotic oral adjuvant to prevent enteric functions (Perdigon et al., 1990; Perdigon et al., 1995). Tomioka et al. (1992) found that *L. casei* protected mice against *Listeria monocytogenes* infection when pre-treated with *L. casei* 2 or 13 days before infection. This was due to immune stimulation of the probiotic resulting in higher levels of macrophage.

1.2.1.2. Anticarcinogenic/antimutagenic activity

Consumption of fermented milk products or the intake of live probiotics have been shown to exert anticarcinogenic and antimutagenic effects. Several studies with animals or humans have indicated the effect of fermented milk products or supplementation with *Lactobacillus* and *Bifidobacterium* strains on enzyme activity in the gastrointestinal tract (Mital and Garg, 1992; Kulkarni and Reddy, 1994; Rafter, 1995).

Goldin et al. (1992) showed that *Lactobacillus* GG could survive and colonise the human gastrointestinal tract (GIT) and simultaneously lower the faecal bacterial β-glucuronidase activity. β-Glucuronidase is one of the bacterial enzymes associated with the generation of mutagens, carcinogens and tumour promoters. For example, β-glucuronidase can release from conjugated forms a number of dietary carcinogens such as polycyclic aromatic hydrocarbons. Other such enzymes include β-galactosidase, nitroreductase, azoreductase and 7-α-hydroxy-steroid dehydrogenase (Goldin, 1990) and are involved in the generation of mutagens or carcinogens including *N*-nitroso compounds, ammonia, phenols, cresols and deoxycholic acid (Burns and Rowland, 2000). When healthy volunteers were fed fermented dairy products containing *L. acidophilus* and *Bifidobacterium bifidum,* no changes were detected in the levels of β-glucuronidase and azoreductase, whilst nitroreductase was reduced (Marteau et al., 1990). McConnell and Tannock (1991) showed that the azoreductase activity was 31% lower in the cecae of mice colonised with *L. delbrueckii* and *L. fermentum* when compared to *Lactobacillus*-free mice.

LAB may also retard or prevent the initiation and promotion of tumours. Tumour suppression is associated with intact viable cells, intact dead cells, as well as cell wall fragments of lactobacilli and bifidobacteria (Fernandez and Sahani, 1990; Modler et al., 1990; Zhang and Ohta, 1991). Feeding fermented milk or live bacteria such as *L. acidophilus* and *L. bulgaricus* and/or *L. casei* was shown to suppress Ehrlich ascites tumour or sarcoma 180 in mice (Fernandez and Sahani, 1990; Goldin, 1990).

The antimutagenic effects of fermented milk have been widely demonstrated (Bodana and Rao, 1990; Nadathur et al., 1994; Abdelali et al., 1995; Hosoda et al., 1996). These were suggested as a result of the presence of viable LAB (Kulkarni and Reddy, 1994; Bouberki and Ohta, 1996). Orally administered strains of *L. casei* were effective in preventing recurrence of superficial bladder cancer (Aso et al., 1995). *B. longum* and lactulose exerted an anticarcinogenic effect against azoxymethane-induced colonic aberrant crypt foci in rats (Challa et al., 1997).

The mechanisms of anticarcinogenicity and antigenotoxicity are not well understood. However, a large number of reports describe the adsorption or binding *in vitro* by LAB of a variety of foodborne carcinogens including heterocyclic amines formed during cooking of meat, the fungal toxin aflatoxin B1, benzo(a)pyrene and the food contaminant AF2 (Burns and Rowland, 2000). The adsorption appears to be a physical phenomenon, mostly due to a cation exchange mechanism. However, the effectiveness of adsorption in vivo has been questioned especially since the adsorption appears to take place in vitro at pH values lower than encountered in the small or large intestine (Burns and Rowland, 2000). Other mechanisms for anticarcinogenic and antigenotoxic activity may be based on the fact that LAB when given as a probiotic increase in number and suppress other bacteria of the colonic microflora. LAB themselves do not appear to form appreciable amounts of enzymes which may convert procarcinogens into carcinogens. In addition, the LAB may stimulate the immune response and thus the anticarcinogenic activity of the human immune system (Perdigon et al., 1998; Burns and Rowland, 2000).

1.2.1.3. Cholesterol reduction

Heart diseases, especially atherosclerosis, are one of the major causes of death in humans. Hypercholesterolaemia is considered the most common risk factor associated with atherosclerosis leading to cardiovascular disease in modern industrialised countries. Cholesterol accumulation in the wall of the arteries causes atherosclerosis, which is a condition resulting from plaques forming in the vessels that inhibit blood flow. This leads to clot formation and heart attack. Low-density lipoproteins (LDL) are usually responsible for plaque formation in the aetiology of atherosclerosis, whilst low levels of coronary heart disease may also be as a result of plaque formation by high-density lipoprotein (HDL). Cholesterol is usually synthesised in the liver at a rate the body requires; however, alteration of the diet might have a significant effect on the serum cholesterol level.

Zacconi et al. (1992) showed that serum cholesterol levels were lower in axenic mice colonised with *E. faecium* and *L. acidophilus.* Serum cholesterol levels of rabbits fed on a diet supplemented with yoghurt decreased significantly (Kiyosawa et al., 1984). Feeding *L. acidophilus* or a commercial probiotic preparation (consisting of a mixture of *L. acidophilus, L. casei, B. bifidum,* and *Aspergillus oryzae*) decreased serum cholesterol levels in chickens and in egg yolk (Abdulrahim et al., 1996; Mohan et al., 1996). In humans, GAIO, a fermented milk product containing a strain of *E. faecium* of human origin and two *Streptococcus thermophilus* strains, decreased serum cholesterol and LDL levels after six weeks of consumption in a group of 44-year old healthy men (Hølund, 1993).

Klaver and van der Meer (1993) suggested that the assimilation of cholesterol, associated with the cholesterol reducing activity of probiotic bacteria, was not due to a direct interaction between the bacteria and cholesterol, but rather due to the co-operation of cholesterol with deconjugated bile salt at pH values below 6.0. This would not explain reduction of cholesterol *in vivo,* as the pH of the lower GIT is neutral to alkaline. Marshall and Taylor (1995) also observed co-precipitation of cholesterol with deconjugated bile salts, but also reported cholesterol removal in the absence of bile. They suggested a physical association between cholesterol and the cell surface. Cholesterol lowering effects have more recently been linked to bacterial bile salt hydrolase (BSH) activity. BSH activity is found among various Gram-positive intestinal bacteria, including members of the genera *Enterococcus, Peptostreptococcus, Bifidobacterium, Clostridium, Bacteroides* and *Lactobacillus* (Hylemon 1985, Chikai et al., 1987). The conjugated BSH enzyme (E.C. 3.5.1.24) deconjugates bile salts by liberating the glycine and/or taurine moiety from the side chain of the steroid core (Chikai et al., 1987). The two predominant bacterial modifications of bile salts include: (1) hydrolysis of glycocholic acid into cholic acid and (2) dehydroxylation of cholic acid into deoxycholic acid acid (de Boever and Verstraete, 1999).

Although BSH activity is a commonly observed phenomenon, its ecological significance for the bacteria is not well understood. Certain BSH-active clostridia were proposed to use the released taurine as an electronacceptor, which was argued to result in a higher growth rate. On the other hand, deconjugation might be a detoxification reaction of vital importance to the microbial cell (de Boever and Verstraete, 1999). It has also been suggested that the BSH enzyme might be a detergent shock protein that enables lactobacilli to survive the intestinal bile stress. As such, BSH active lactobacilli may have a competitive advantage over other bacteria for surviving in the small intestine (de Smet et al., 1995). However, Moser and Savage (2001) tested a wide spectrum of BSH^+ lactobacilli for their

resistance against bile salt toxicity, and could not support this hypothesis. This led Moser and Savage (2001) to suggest that instead of protecting lactobacilli from toxicity of conjugated bile salts, BSH activity may rather be important for the bacteria to grow in and colonise the intestine.

At hte human/animal physiological level, the conversion of glycocholic acid to cholic acid may contribute to lower cholesterol levels, because free bile acids may be excreted more likely from the gastrointestinal tract than conjugated bile salts (Chikai et al., 1987). If enhanced faecal loss of bile acids occurs as a result of bacterial BSH activity, it may increase the demand for cholesterol as a precursor for *de novo* synthesis of bile salts, which in turn may lower cholesterol levels (de Rodas et al., 1996; Driessen and de Boer, 1989).

So far, the *bsh* genes from *Lactobacillus (L.) plantarum* (Christiaens et al., 1992), *L. gasseri* (Russell et al., 2001), *L. johnsonii* (Elkins and Savage, 1998), *Bifidobacterium longum* (Tanaka et al., 2000) and *Clostridium perfringens* (Coleman and Hudson, 1995) have been cloned and characterised. To our knowledge, a BSH gene from an *Enterococcus* strain has not been cloned and characterised yet.

1.2.1.4. Improvement of lactose digestion

Lactose can not be utilised by a large part of the human population, especially of Asian and African origin, due to a lack of the intestinal mucosal enzyme β-galactosidase (lactase) or by a reduction in lactase activity caused by intestinal infection (e.g. rotaviral gastroenteritis). The enzyme β-galactosidase hydrolyses milk lactose to glucose and galactose. The symptoms of lactose maldigestion are flatulence, abdominal pain and diarrhoea. However, in fermented dairy products, the starter cultures degrade lactose or produce β-galactosidase, which is secreted in the intestinal tract (Fuller, 1991; O'Sullivan et al., 1992). Intracellular lactase will survive passage through the stomach and reach the small intestines (Daly, 1991). Thus, for aiding human lactose digestion, either the LAB culture degrades lactose in the milk product, or the enzyme is supplied by the microbe and delivered to the gastrointestinal tract of the human, where it can reduce lactose levels.

In one study, milk fermented with *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* was much more effective than *L. acidophilus* strains in improving lactose tolerance (Noh and Gilliland, 1993). The yoghurt starter cultures *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were shown to possess high levels of β-galactosidase activity. Both species are, however, sensitive to bile salts, which causes the microorganisms to die and cells to disintegrate, thus leading to the release of high levels of β-galactosidase in the

gastrointestinal tract (Noh and Gilliland, 1993). *L. acidophilus*, on the other hand, is more bile resistant and has lower levels of β-galactosidase than the yoghurt starter cultures. Therefore, strains of this species may be able to grow in the gastrointestinal tract and thus may increase β-galactosidase levels for longer periods (Sanders. 1993).

1.2.1.5 Bacteriocin production

A bacteriocin is defined as a proteinaceous compound (protein or peptide) that has bactericidal or bacteriostatic action against a limited range of organisms, which are usually closely related to the producer organism (Barnby-Smith, 1992). The inhibitory activity of these substances produced by LAB is generally confined to Gram-positive bacteria. Bacteriocin inhibition of Gram-negative bacteria has not been demonstrated unless chelating agents (e.g. EDTA) were added to the test system, which make the Gram-negative outer membrane leaky for these compounds (Abee et al., 1995). Bacteriocins are believed to be important for the ability of LAB to compete in non-fermentative ecosystems such as the gastrointestinal tract (Lindgren and Dobrogosz, 1990).

The major classes of bacteriocins produced by LAB include: Class I, lantibiotics, which are small, heat stable, and contains unusual amino acids such as lanthionine and β-methyllanthionine; Class II, small heat stable peptides (30-100 amino acids), which are further divided into the following subclasses: Subclass IIa, the pediocin-like bacteriocins that are characterised by anti-*Listeria* activity; Subclass IIb, two peptide bacteriocins for which the antimicrobial activity depends on the presence of two peptides; and subclass IIc, the *sec*-dependent bacteriocins which are secreted via the general secretory (*sec*) pathway; and finally Class III, large (>30 kDa) heat labile proteins (Nes et al. (1996). The lantibiotic nisin is the only bacteriocin that is currently commercially exploited and which is used in food preservation in more than 40 countries. Examples of bacteriocins are shown in Table 5.

Table 5: Examples of bacteriocins produced by lactic acid bacteria (Barnby-Smith, 1992)

Microorganisms	Bacteriocin	Inhibitory spectrum
Lactococcus lactis subsp. *lactis*	Nisin	Many Gram-positive bacteria
Lactococcus lactis subsp. *lactis* CNRZ	Lacticin 481	LAB, clostridia
Pediococcus pentosaceus FBB61	Pediocin A	LAB, clostridia, listeria, staphylococci
Pediococcus acidilactici	Pediocin PA1	Some LAB, listeria
Lactobacillus sake	Sakacin A	Some LAB, listeria
Leuconostoc mesenteroides UL5	Mesentericin 5	Some LAB, listeria

Bacteriocins are usually formed as prepeptides in the cell and mature bacteriocins are generated by processing during export (Nes et al., 1996; Garneau et al., 2002). This is generally accomplished by removal of a N-terminal leader peptide either by signal peptidase in sec-pathway secreted bacteriocins, or more often at a specific double glycine position (-2,-1) in the prepeptide, concomitant with externalisation by a dedicated ABC-transporter protein and an accessory protein (Garneau et al., 2002). With the exception of disulfide bridge formations, posttranslational modifications are rare among the non-lantibiotic bacteriocins (Nes et al., 1996; Ennahar et al., 1999; Garneau et al., 2002).

At least four different genes are required to achieve the production of bacteriocins by Gram-positive bacteria: 1) a structural gene (or two structural genes in two-peptide bacteriocins) encoding the prepeptide; 2) dedicated immunity gene; 3) a gene encoding a dedicated ABC transporter; and 4) a gene encoding an accessory protein which is necessary for bacteriocin transport (Nes et al., 1996; Ennahar et al., 1999). Usually, the bacteriocin structural and immunity genes occur in an operon, as do the transport genes. For most nonlantibiotics, the bactericidal effect of bacteriocins occurs as a result of permeabilisation of the cell membrane (Nes and Holo, 2000). This pore forming ability leads to cell leakage and efflux of K^+ ions, which in turn leads to dissipation of membrane potential and inhibition of amino acid uptake. Cell death is finally effected by futile cycle of ATP-driven potassium uptake and bacteriocin-mediated potassium release in combination with increased ATP hydrolysis by an ATPase (Jack et al., 1995; Garneau et al., 2002, Fig. 2).

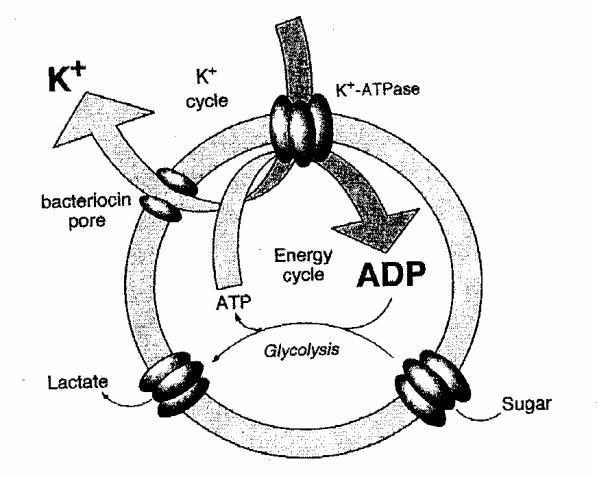


Fig. 1: Bactericidal action of bacteriocin by potassium ion efflux and increased ATP demand from K^+ ATPase (adapted from Garneau et al., 2002)

Mature bacteriocins are generally cationic, amphiphilic species and the environment in which they are found can strongly dictate their secondary structure. In cases where the structure has been examined, they usually exist as random coils under aqueous conditions, but in charged, weakly ionic solution they exist in ordered conformation (β-sheet and α-helices) (Garneau et al., 2002). It has been shown that upon contact with the membranes, the otherwise random-coiled peptides adopt ordered helical structures. The formation of such amphipathic helices is the critical conformational change required for pore formation by the 'barrel-stave' or 'wormhole' mechanism. This mechanism relies upon stabilising interactions between membrane phospholipids and the cationic residues of the peptide allowing for insertion of hydrophobic regions into the outer leaflet of the membrane. Once associated with the membrane surface, a number of the ordered bacteriocins can potentially aggregate and the complex can completely span the membrane, thereby forming a transient pore (Fig. 3) (Moll et al., 1996; Abee, 1995; Garneau et al., 2002).

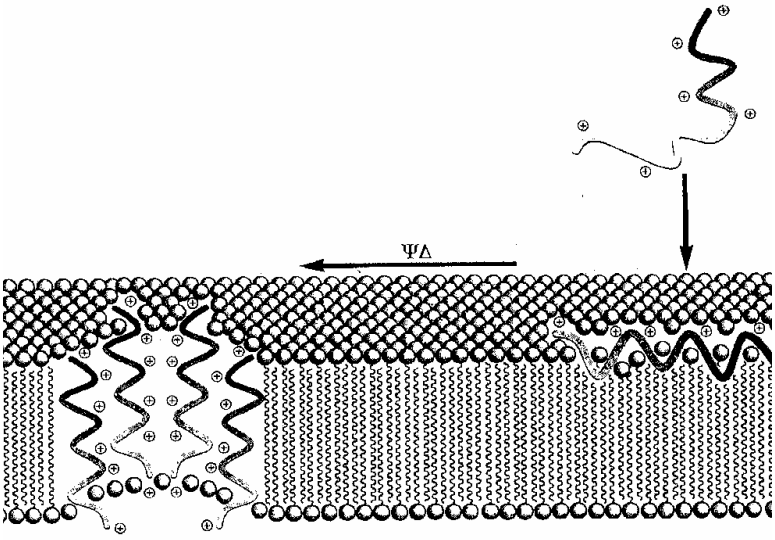


Fig. 2: Barrel-stave (wormhole) mechanism of pore formation by cationic bacteriocin peptides (adapted from Garneau et al., 2002).

Intestinal LAB are known to produce bacteriocins which can inhibit pathogenic bacteria, for example the intestinal isolates *Lactobacillus acidophilus* 30SC (Oh et al., 2000), *Lactobacillus delbrueckii* subsp. *lactis* UO004 (Boris et al., 2001), *Lactobacillus salivarius* subsp. *salivarius* UCC118 (Flynn et al., 2002), as well as a vaginal isolate of *Lactobacillus salivarius* CRL 1328 (Ocana et al., 1999). *L. acidophilus* LF221, isolated from infant faeces was shown to produce at least two bacteriocins (designed as acidocin LF221 A and acidocin LF221 B) which exhibited activity against different bacteria including some pathogenic species such as *Bacillus cereus, Clostridium difficile*, *Listeria innocua, Staphylococcus aureus* and group D streptococci. Bogovič-Matijašić et al. (1998) proposed *L. acidophilus* LF221 as a probiotic strain on the basis of its human origin and bacteriocin-inhibition of pathogenic bacteria. Gusils et al. (1999) showed that *Salmonella* Gallinarum could be inhibited by *Lactobacillus animalis* isolated from the chicken intestine in a competition experiment in which 1 x 10^7 CFU/ml *L. animalis* and 10^6 CFU/ml *Salmonella* Gallinarum were inoculated in co-culture *in vitro*. The growth of the mixed culture was followed by measuring the OD_{560} and measuring the pH at different times, as well as determining total counts of the different strains. Moreover, Audisio et al. (2000) showed that *Enterococcus faecium* J96, isolated from a healthy free-range chicken, exhibited a protective effect on chicks infected with *Salmonella* Pullorum. In a former experiment, Audisio et al. (1999)

found that the protective effect exerted by *L. animalis* was caused by production of both lactic acid and bacteriocin.

The effect in the intestinal tract of bacteriocin-producing *Lactobacillus sakei* MI401 against *Listeria monocytogenes* EP2 was examined *in vivo* in 20Mol:Wistar SPF rats by Saadbye et al. (1999). The rats were treated with streptomycin to facilitate the introduction of streptomycin-resistant *L. monocytogenes* and *L. sakei* into the gastrointestinal tract. One group (10 rats) was orally inoculated with 10^9 CFU *L. sakei* and 7 days later orally inoculated with 10^9 CFU *L. monocytogenes* EP2. This group reached a maximum *Listeria* concentration of 10^4 CFU/g feces whereas the control group reached a maximum of 10^8 CFU/g feces. The control group did not receive *L. sakei* but otherwise treated identically. The numbers of *L. monocytogenes* were significantly lower ($p < 0.001$) in the group which received *L. sakei* compared to the control group.

Du Toit et al. (1998, 2000) showed that enterococci isolated from the faeces of Göttingen mini-pigs had bacteriocin activity against many enterococci species, *Lactobacillus salivarius* which is often associated with the human gastrointestinal tract, as well as *Clostridium* and *Listeria* spp. One of the bacteriocin, produced by *E. faecalis* BFE 1071, was characterised at the chemical and genetic level in detail in a follow-up study (Balla et al., 2000). This bacteriocin, termed enterocin 1071A&B was shown to be a two-peptide bacteriocin and thus can be classified as a class IIb bacteriocin according to the bacteriocin classification scheme of Nes et al. (1996). This bacteriocin will be discussed in more detail, as the producer strain *E. faecalis* BFE 1071 and a bacteriocin-negative mutant were used in this study to determine the effect of the bacteriocin-producer on the gastrointestinal microflora of the rat.

Clearly therefore, bacteriocin production does occur also from gastrointestinal isolates of LAB. There are, however, little data on the *in vivo* effect of bacteriocin production on the intestinal microflora. It is thought that bacteriocin production serves to increase the competitiveness of bacteria in their relative ecological niche (Dykes, 1995). Thus bacteriocin production may change the composition of the microflora of a particular ecosystem, in that sensitive organisms are suppressed at the expense of the bacteriocin producer. However, such investigations of interaction between bacteriocin producer and the dynamics of the microflora of complex ecosystems have not been conducted yet.

1.3 The enterococci

1.3.1 Phylogeny and taxonomy of enterococci

Enterococci were described for the first time by Thiercelin in 1899. They were thought to be a new Gram-positive diplococcus and were ater included in the new genus *Enterococcus,* proposed by Thiercelin and Jouhaud in 1903, with the type species *Enterococcus proteiformis*. Andrewes and Horder (1906), however, renamed Thiercelin's enterococci as *Streptococcus faecalis* based on their ability to form short or long chains. The species epithet 'faecalis' was suggested because of their close resemblance to strains isolated from the human intestine. This explains why the history of enterococci cannot be separated from that of the genus *Streptococcus* (Devriese and Pot, 1995; Franz et al., 1999).

A serological typing system for streptococci was developed by Lancefield in 1933 in which those of 'faecal origin' possessed the group D antigen. This correlated with the grouping of Sherman in 1937, who proposed a new classification scheme for the genus *Streptococcus* that separated it into four divisions designated pyogenic, viridans, lactic and 'enterococcus'. The 'enterococcus' group included *Streptococcus faecalis, Streptococcus faecium, Streptococcus bovis* and *Streptococcus equinus* as the 'enterococcal' or group D strains.

The genus *Enterococcus* was finally described by Schleifer and Kilpper-Bälz (1984), who used DNA:DNA hybridisation to demonstrate that *Streptococcus faecalis* and *Streptococcus faecium* were sufficiently distinct from other streptococci to warrant their transfer to a separate genus. Based on 16S rRNA cataloguing, DNA:DNA and DNA:rRNA hybridisation and serological studies with superoxide dismutase antisera, the streptococci *sensu lato* were subdivided into three genera: *Streptococcus sensu stricto, Enterococcus* and *Lactococcus* (Devriese et al., 1993).

Enterococci belong to the clostridial subdivision of the Gram-positive bacteria, together with other genera of LAB (Devriese et al., 1993; Devriese and Pot, 1995). Based on the 16S rDNA sequence data, the enterococci form a distinct cluster within this subdivision with *Vagococcus, Tetragenococcus,* and *Carnobacterium* as their closest neighbours (Devriese and Pot, 1995).

1.3.2 Phenotypic and biochemical characteristics of enterococci

The classical taxonomy of the enterococci is vague because there are no phenotypic characteristics that unequivocally distinguish them from other Gram-positive, catalase-negative, ovoid coccus-shaped bacteria (Devriese et al., 1993). However, the majority of

Enterococcus species can be distinguished from coccus-shaped bacteria by their group D antigen, grow at 10 and 45 °C, in 6.5% sodium chloride, at pH 9.6 and to survive heating at 60 °C for 30 min (Hardie and Whiley, 1997). However, not all *Enterococcus* species possess the group D antigen. Some *Streptococcus* species such as *S. bovis, S. suis,* and *S. alactolyticus,* as well as pediococci and certain *Leuconostoc* strains also react with Lancefield's group D antiserum. Some strains of lactococci, pediococci, aerococci and leuconostocs can also grow in the presence of 6.5% sodium chloride, but *E. avium, E. cecorum* and *E. columbae* do not (Devriese et al., 1993). On the other hand, pediococci and some lactococci can grow at 45°C, while most lactococci, leuconostocs and some streptococci grow at 10°C, but *E. avium* generally does not (Murray, 1990; Devriese et al., 1993). Thus, it can be difficult to distinguish these bacteria from other Gram-positive, catalase-negative cocci on the basis of phenotypic characteristics alone, without the help of genotypic methods such as DNA:DNA hybridisation or 16S rDNA sequencing. Nevertheless, Klein (1998) proposed a scheme based on phenotypic characteristics to separate enterococci from other Gram-positive cocci, and to presumptively identify the most common enterococcal species (Figure 1).

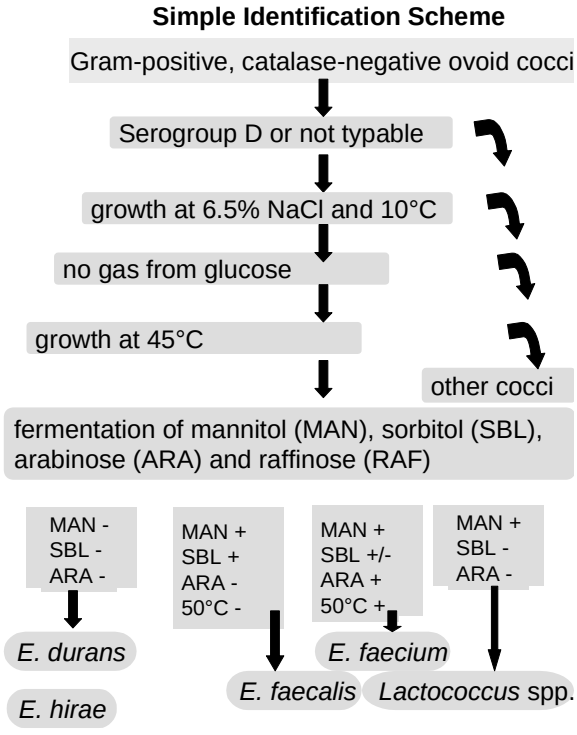


Figure 3: Simple scheme for presumptive identification of enterococci based on phenotypic characteristics according to Reuter (1995) and Klein (1998).

Schleifer and Kilpper-Bälz (1984) described certain characteristics of the genus *Enterococcus* that are valid for all described species. According to this description, the cells are ovoid and Gram-positive, occur singly, in pairs or in short chains. Within the chains, the cells are frequently arranged in pairs and are elongated in the direction of the chain. Endospores are absent and cells may be motile. Enterococci are facultatively anaerobic chemo-organotrophs with a fermentative metabolism, and a homofermentative. The predominant end product of glucose fermentation is L(+)-lactic acid. The peptidoglycan type is lysine-D-asparagine (with D-isoasparagine as cross-bridge) in all species described, except in *E. faecalis,* which has a peptidoglycan of the lysine-alanine$_{2-3}$ type. The minimum nutritional requirements are generally complex. They are benzidine-negative and usually catalase-negative, but some strains may produce pseudo-catalase.

Thus far, more than 20 enterococcal species have been described and include *E. faecalis, E. faecium, E. durans, E. hirae, E. mundtii, E. avium, E. pseudoavium, E. malodoratus, E. raffinosus, E. gallinarum, E. casseliflavus, E. flavescens, E. cecorum, E. columbae, E. dispar, E. sulfureus, E. saccharolyticus*, *E. haemoperoxidus, E. asini*, *E. moraviensis, E. villorum,* and *E. porcinus* (Franz et al., 2003).

1.3.3 Infections caused by enterococci

Enterococci constitute an important part of the autochthonous bacteria associated with the mammalian gastrointestinal tract (Leclerc et al., 1996). With the exception of *E. faecium* and *E. faecalis,* the enterococci are rarely reported to be involved in human pathogenesis (Franz et al., 1999). Formerly, enterococci were indeed viewed as organisms of little clinical impact; however, over the last two decades enterococci, especially strains belonging to *E. faecalis* and *E. faecium,* have emerged as important nosocomial pathogens in immunocompromised patients and intensive care units. The enterococci do not possess potent virulence factors compared to those found in many other bacteria, but they have a number of other characteristics such as subtle virulence determinants and resistance to antimicrobial agents which may contribute to their virulence and make them effective opportunistic pathogens (Adams, 1999). They are considered the second most common cause of hospital-acquired infections after *Escherichia coli* in the USA (Schaberg and Gaynes, 1991).

About 90% of the human enterococcal infections are caused by *E. faecalis* and the remaining 10% by *E. faecium*. Enterococci cause about 5% of all urinary tract infections (UTI) and this incidence is even higher for patients with functional anomalies of the urinary tract (Morrison and Wenzel, 1986). Furthermore, enterococci constitute about 12% of

bacterial pathogens associated with septicaemia, and 5 to 15% of cases of bacterial endocarditis are caused by enterococci (Schaberg and Gaynes, 1991).

1.3.4 Virulence of enterococci

For enterococci to cause infection, they must colonise host tissue, resist host specific and unspecific defence mechanisms, and produce pathological changes (Franz et al., 1999). Virulence traits of enterococci include adherence to host tissues by a variety of adhesins, e.g. the 'enterococcal surface protein' (Esp), 'aggregation substance' (AS), the 'enterococcal endocarditis antigens' ($EfaA_{fm}$ or $EfaA_{fs}$ from *E. faecium* or *E. faecalis*, respectively) or 'adhesin to collagen from *E. faecalis'* (Ace) (Franz et al., 2003). Furthermore, enterococcal virulence is based on invasion and abscess formation, resistance to and modulation of host defence mechanisms, secretion of toxin such as the plasmid-encoded cytolysin, as well as secretion of hyaluronidase, and gelatinase (Witte et al., 1999; Franz et al., 2003).

Some enterococci possess a plasmid collection mechanism which is based on production of chromosomally encoded 'sex pheromones'. Sex pheromones are small, linear peptides of 7 or 8 amino acids that are excreted by *Enterococcus faecalis* strains which promote the acquisition of plasmid DNA. When pheromones bind to receptors on the cell surface of strains that contain plasmid DNA, this signal is transduced and leads to induction of the aggregation substance gene. When expressed, AS mediates the formation of cell clumps by binding to a complementary receptor termed ‚binding substance' that allows the highly efficient transfer of the pheromone plasmid on which the AS gene is encoded (Clewell, 1993; Dunny et al., 1995). The pheromones, however, not only have a role in transfer of plasmid DNA, they also serve as chemo-attractive substances for human neutrophils and induce inflammation and superoxide production (Johnson, 1994; Witte et al., 1999).

Gelatinase is a proteolytic enzyme that acts on collagenous material present in tissues (Franz et al., 2002). The production of gelatinase was shown to increase pathogenicity in an animal model (Singh et al., 1998), which confirms its role in virulence. Enterococcal lipoteichoic acid from the cell wall was shown to induce production of interleukin-1β, interleukin-6 and TNF-α *in vitro* and may, therefore, contribute to local tissue damage (Witte et al., 1999). Hyaluronidase is a cell surface-associated enzyme which cleaves the mucopolysaccharide moiety of connecting tissues or cartilage. This enzyme has been implicated to act as 'spreading factor' for dissemination of some microorganisms (Witte et al., 1999).

Virulence of enterococci is strongly enhanced by their frequent resistance to commonly used antibiotics, which makes them effective opportunists in nosocomial infections

(Giraffa, 2002). The antibiotics against which enterococci show constitutive resistance include cephalosporins, β-lactams, sulphonamides, and low levels of clindamycins and aminoglycosides, while acquired resistances are based on acquisition of plasmids and transposons including resistance to chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycycline, β-lactams, fluoroquinolons and glycopeptides (Murray, 1990; Leclercq, 1997; Franz et al., 1999).

1.3.5 Bacteriocin production by enterococci.

Many enterococcal strains produce bacteriocins. These are called enterocins, and they generally belong to the class II bacteriocins (Franz et al., 1999). The enterocins are generally active against other enterococci, as well as strains of *Listeria monocytogenes* (Giraffa, 1995). Currently, the only enterocin that can be grouped into class IIa (see above) is the enterocin A produced by *E. faecium* strains CTC492 and BFE 900 (Aymerich et al., 1996; Franz et al., 1999). It has antimicrobial activity against *Enterococcus, Lactobacillus* and *Listeria* spp., including *Listeria monocytogenes.* Enterocin (Ent) A consists of 47 amino acids with a theoretical molecular weight of 4829 Da. Mundticin (Bennik et al., 1998) is a class II bacteriocin consisting of 43 amino acids with activity against *Listeria*, *Enterococcus*, *Lactobacillus*, *Pediococcus* and *Pediococcus* spp., as well as *Clostridium botulinum.* However, the genes for mundticin have not been cloned and it is not known whether this bacteriocin belongs to class IIa or to the *sec*-secretion-dependent class IIc enterocins. Enterocin P is a bacteriocin produced by *E. faecium* strains P13 and L50 that were isolated from Spanish sausages (Cintas et al., 1997; Cintas et al., 2000). It also has activity against other *Enterococcus* spp., as well as *Lactobacillus* and *Pediococcus* spp. In addition, the bacteriocin inactivates *B. cereus, S. aureus, C. perfringens* and *C. botulinum* (Cintas et al., 1997). The mature bacteriocin consist of 44 amino acids and has a theoretical molecular weight of 4493 Da. Enterocin P is produced as a prepeptide of 71 amino acids, bearing a signal peptide of 27 amino acids. Secretion of enterocin P, therefore, occurs via the *sec*-pathway and the bacteriocin can thus be classified as a class IIc bacteriocin (Cintas et al., 1997).

Some enterocins can not be readily grouped into one of the existing bacteriocin classes. For example, enterocin L50A and B is a two-peptide bacteriocin consisting of the 44 amino acid EntL50A and the 43 amino acid EntL50B (Cintas et al., 1998). These peptides are produced without a leader peptide and thus a distinction between class IIa bacteirocins, which are secreted by a dedicated ABC transporter, and class IIc bacteriocins, which are secreted via

the *sec*-pathway, can not be made. Enterocin L50A and B share sequence homology to staphylococcal peptide toxins which include δ-lysin, SLUSH A to C and AGS1 to 3 (Cintas et al., 1998). The 34 amino acid bacteriocin enterocin Q also produced by *E. faecium* L50 also does not contain a N-terminal extension, i.e. it is not produced as a prepeptide (Cintas et al., 2000). Enterocin B produced by *E. faecium* strains CTC 492 and BFE 900 (Casaus et al., 1997, Franz et al., 1999) is unusual in that it does not contain a conserved YGNGV motif at the N-terminal end of the mature peptide. This amino acid motif is conserved in all other class IIb bacteriocins and was shown to be important for activity of some bacteriocins (Nes et al., 1996). Enterocin AS-48 on the other hand is a unique bacteriocin as it is a cyclic molecule that results from head-tail linkage of the N-terminal methionine (M^{+1}) with the C-terminal tryptophane (W^{+70}) (Martinez-Bueno et al., 1994). AS-48 also is unique in that the bacteriocin has a broad range of activity, including Gram-negative bacteria (Martinez-Bueno et al., 1994)

Enterococcus faecalis BFE 1071 isolated from the faeces of Göttingen mini-pigs produces a two peptide bacteriocin, designed as enterocin 1071A&B. The bacteriocin is active against many Gram-positive bacteria such as *E. faecalis*, *E. faecium, E. durans, Bifidobacterium gallicum, Clostridium tyrobutyricum, Staphylococcus aureus, Listeria innocua, Leuconostoc mesenteroides* subsp. *cremoris, L. acidophilus, L. casei, L. fermentum, L. curvatus, L. helveticus, L. plantarum, L. reuteri, L. sakei, L. salivarius* subsp. *salivarius, Lactococcus lactis* subsp. *lactis, Propionibacterium acidipropionici*, and *Pediococcus pentosaceus* (Balla et al., 2000). Thus, the activity spectrum includes strains of lactobacilli typical of in the human intestine, i.e. *L. acidophilus* and *L. salivarius*. This one reason for using this bacteriocin-producer in this study to evaluate the effect of the strain on the microflora composition of the rat gastrointestinal tract.

The bacteriocin enterocin 1071 was studied in detail both at a chemical and genetic level (Balla et al., 2000; Franz et al., 2002). The molecular masses of the purified bacteriocins were determined by electron spray mass spectrometry to be 4285 and 3899 Da for the enterocins 1071A and 1071B, respectively (Balla et al., 2000). The genetic determinants for this two-peptide bacteriocin were located on plasmid DNA (Balla et al., 2000; Franz et al., 2002) and DNA sequencing revealed two open reading frames (ORFs) encoding the 39 and 34 amino acid enterocin 1071A and 1071B peptides, respectively (Balla et al., 2000; Franz et al., 2002). These bacteriocins were encoded as prepeptides, each bearing a double-glycine-type leader peptide of either 18 amino acids (Ent1071A) or 24 amino acids (Ent 1071B) (Franz et al., 2002). In addition to the bacteriocin structural genes, a gene (*eni1071*) encoding a protein of 110 amino acids with an isoelectric point of 9.278, was found downstream of

ent1071B. This gene was cloned and expressed in a heterologous host, where it was shown to confer immunity to enterocin 1071 (Franz et al., 2002). A truncated ORF (*entT*) was found upstream of the ent1071A and B genes on a 3.2-kb plasmid fragment cloned by Franz et al. (2002). This ORF showed homology to ABC transporter proteins of other bacteriocin systems, and was therefore speculated to be involved in transport of enterocin 1071 (Franz et al., 2002). Although an accessory gene is generally associated with bacteriocin transport, the fragment cloned in the study of Franz et al. (2002) did not contain a gene for this protein. Thus to date, this gene remains undetected , probably downstream of the ABC transporter gene on a region of the plasmid that was not cloned or sequenced (Franz et. al., 2002).

1.3.6. Enterococci as probiotics

Specific strains of enterococci are being used as probiotics for humans and animals. One of the best studied enterococci used as a human probiotic, especially in the treatment of diarrhoea, is *Enterococcus faecium* SF 68. especially in the treatment of diarrhoea. The strain was originally isolated in Sweden and was patented in Switzerland and other countries (Lewenstein et al., 1979). Its effectiveness for treatment of intestinal disorders can probably be attributed to the fact that it is a commensal of the intestine and that it has a short lag phase and generation time (*ca*. 20 min generation time under optimal conditions). It is moderately resistant to antibiotics and has an inhibitory effect *in vitro* to growth of *E. coli, Salmonella* spp.*, Shigella* spp. and *Enterobacter* spp. In addition, this strain is resistant to low pH, insensitive to bile salts, and individuals show a high tolerance to it with no side effects (Lewenstein et al., 1979; Bellomo et al., 1980; Canganella et al., 1996). *E. faecium* SF 68 is considered as an alternative to antibiotics for treatment of diarrhoea (Lewenstein et al., 1979; Bellomo et al., 1980). Several controlled, 'double blind'-type clinical studies have shown that treatment of enteritis with *E. faecium* SF68 was successful for both adults and children. It decreased the duration of diarrhoeal symptoms and the time for normalisation of patient's stools (Bellomo et al., 1980, Bruno and Frigerio, 1981; D'Apuzzo and Salzberg, 1982).

Another probiotic *Enterococcus* is the Causido® culture that consists of two strains of *S. thermophilus* and one strain of *E. faecium*. This probiotic has been claimed to be hypocholesterolaemic in the short-term (Agerholm-Larsen et al., 2000), but long-term reduction of LDL-cholesterol levels was not demonstrated (Richelsen et al., 1996; Sessions et al., 1997); hence the clinical relevance of this effect is uncertain (Lund et al., 2002).

However, the use of enterococci as probiotics remains a controversial issue. While the probiotic benefits of some strains are well established, the emergence of antibiotic resistant

strains of enterococci and the increased association of enterococci with human disease (see below), has raised concern regarding their use as probiotics. Therefore, should an *Enterococcus* strain be considered as a human probiotic it should be determined whether the strain is antibiotic resitant and whether it contains any virulence determinants.

1.4 Gastrointestinal microbiology of the human and rat

The bacterial flora of the gastrointestinal system represents an ecosystem of the highest complexity and our understanding of this system and its interactions is still limited (Berg, 1996). The gastrointestinal tract (GIT) of an adult human is estimated to harbour about 10^{14} viable bacteria, i.e. ten times the total number of eukaryotic cells in all the tissues of a humans' body. The importance of these bacteria in the GIT was neglected for a long time, while the focus was merely placed on enteric pathogens and other factors leading to gastrointestinal disorders (Holzapfel et al., 1998). Both the variety and the overall sum of microbial numbers are determined by an array of complex factors, intrinsic to the respective gastrointestinal tract sections, and 'extrinsic' related to, for example, diet, stress and drugs. After the more or less neutral pH of the oral cavity, the low pH of the stomach (ranging from 2.5 to 3.5) is destructive to most microbes. The population averages $\log_{10}$ 3.0 bacteria g^{-1} and is dominated by Gram-positive bacteria such as streptococci and lactobacilli, and by yeast (Holzapfel et al., 1998). The duodenum also represents a hostile environment due to the aggressive intestinal fluid (e.g. bile, pancreatic juices) and the short transit time and thus contains relatively low numbers of microbes (Holzapfel et al. 1998). Nevertheless Reuter (2001) showed that lactobacilli can occur at numbers of $\log_{10}$ 5.6-9.4 CFU/g or $\log_{10}$ 6.0 –8.4 CFU/g in the jejunum and ileum, respectively. Other bacteria occurring in these sites are from the *Enterobacteriaceae*, and the genera of *Lactobacillus*, *Streptococcus*, *Bacteroides*, *Bifidobacterium* and *Fusobacterium* (Simon and Gorbach, 1982).

It is estimated that the colon of healthy adults harbours about 300-400 different cultivable species belonging to more than 190 genera. Among the known colonic microflora, only a few major groups dominate at levels around 10^{10}-10^{11} g^{-1}. They are all strict anaerobes such as *Bacteroides, Eubacterium, Bifidobacterium,* and *Peptostreptococcus.* Facultative aerobes are considered to belong to the sub-dominant flora, and are constituted of *Enterobacteriaceae*, streptococci and lactobacilli (Gedek, 1993).

Compared to the colonic flora, the fecal flora undergoes distinct quantitative variations but seems to be a good qualitative indicator of the distal colonic microflora. However, it does not accurately reflect the intestinal flora and most definitely not those of the small intestine.

Furthermore, our knowledge on the stability of the strains, species and even genera relationship is still extremely limited (Gedek, 1993).

Various species of laboratory animals are used for metabolic and toxicological studies and the extrapolation of results from animals to man has always taxed experimenters. Animals, particularly rodents and more recently pigs, have been used for gut flora studies, both to investigate ecological mechanisms in the gut and to study microbial metabolisms (Rumney and Rowland, 1992). Research has mainly concentrated on the easily accessible microflora of the faeces (Vaughan et al., 2000). Although animals are very convenient for investigating ecological mechanisms in the gut, to study microbial metabolism and enable monitoring of the flora in different gut regions as well as faeces, there are major differences between animals and humans in the microflora composition and metabolism (Rumney and Rowland, 1992). The differences in gut microflora between laboratory animals and man limit the relevance of studies on colonization resistance in animals to the human situation, but ethical considerations do not allow human volunteers to be challenged with live, potentially pathogenic organisms. An acceptable alternative for such studies is provided by human flora-associated animals (Rumney and Rowland, 1992). Rumney and Rowland (1992) studied metabolism of the colon carcinogen 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (IQ) by suspensions of fecal organisms from rodents (rat and mouse) and humans. They found that the rate of the reaction was fastest in suspensions of microorganisms from rat and mouse compared with those from humans. In the study of dietary modification of the human gut microflora, Rumney and Rowland (1992) found that conventional microflora rats fed with a diet containing 5% pectin showed large increases in the activity of nitrate reductase, ß-glucuronidase, and ß-glycosidase. In contrast, the feeding of pectin (18 g/d) to human volunteers elicits no such increase in the activity of microbial enzymes. These studies exemplify that direct comparisons in gastrointestinal biology between the animal model and the human appear not to correlate well. In our animal experiment to test the influence of a bacteriocin-producing potentially probiotic LAB strain on the composition of the microflora, we nevertheless used healthy male Sprague-Dawley rats, because human experimentation with enterococci would be difficult to do because of safety concerns (see above). In addition, it would be more difficult to control and more costly. Nevertheless the animal model can point towards whether a bacteriocin-producing strain can in principle modify the gastrointestinal flora and if such changes occur, which bacterial groups would be affected.

Campbell et al. (1997) analysed microflora in male Sprague-Dawley rats after being fed with control diet containing (in g/100 g dry matter) protein (19.7), L-Cystine (0.3),

carbohydrate (64.75), fat (10.5), choline (0.25), vitamins (1), minerals (3.5) and gross energy (18.43 MJ/kg diet). It was shown that cell counts of bifidobacteria, lactobacilli, total aerobes and total anaerobes were (log_{10} CFU/g wet stool) 9.1, 7.2, 8.2, and 8.8, respectively. The cecal bacteria in male Wistar rats after being fed with fiber-free control diet containing (g/kg diet) casein (200), wheat starch (650), sunflower oil (50), mineral mixture (50), vitamin mixture (30), microcrystaline cellulose (20) and 16.53 MJ/kg diet gross energy, were analysed by Noack et al. (1998). They found that dominant cecal bacteria consisted of (log_{10} CFU/g dry cecal contents) bifidobacteria (<5.3), eubacteria (8.9), Gram-positive anaerobic cocci (8.3), clostridia (5.4), *Bacteroides* and *Fusobacterium* (9.4), lactobacilli (9.6), enterococci 7.5), and enterobacteria (7.6), with total counts of 9.9 log_{10} CFU/g. Henderson et al. (1997) found that long-term, i.e. 20 weeks, food restriction had little effect on the microflora of female Fischer 344 rats.

1.5 Research motivation

Although a great deal of research has been dedicated to elucidate aspects of bacteriocin activity such as genetics of production, immunity and transport, as well as toxicological aspects and application of bacteriocins in foods, there are almost no data available on the effect of bacteriocins or bacteriocin-producing probiotic lactic acid bacteria on the hosts autochthonous flora. Because bacteriocin activity has been viewed as a functional characteristic of probiotic lactic acid bacteria, some research efforts have been dedicated to evaluate the effect of purified bacteriocins or bacteriocin producers on foodborne pathogens *in vitro* or in seldom cases also *in vivo*. However, no studies have yet addressed the question about the effect on the host autochthonous flora, which is surprising, considering that bacteriocins are active usually against closely related species and may thus interfere with the hosts established microflora. The hosts' own microflora is considered to have an important function itself in protecting the gastrointestinal tract from invading pathogens. Such a potential interference with the protective activity of the hosts autochthonous flora has raised concerns on whether bacteriocin-production by probiotic bacteria is actually a desirable functional characteristic. This study, therefore, serves as a novel investigation, which addresses the question whether bacteriocin-producing, probiotic bacteria can interfere with the hosts own microflora, by using the rat as an experimental animal model.

1.6 Study objective and aims

The objective of this study was to determine the influence of a potentially probiotic, bacteriocin-producing *Enterococcus faecalis* strain BFE 1071 and its bacteriocin-negative mutant on the composition of the intestinal microflora of male Sprague-Dawley rats in an *in vivo* experimental model.

To reach this objective the study aimed to:

1) Investigate the suitability of various enterococci strains isolated from food or gastrointestinal sources for use as a probiotic on the basis of their technological and functional properties. The technological properties included resistance to acid and tolerance to bile and duodenum secrete in an *in vitro* model. The functional, probiotic properties included investigations into adherance potential using a test for hydrophobicity and adherance to HT-29 MTX cells in vitro as well as studies for bacteriocin production and bile salt hydrolase activity.
2) To study bile salt hydrolase activity of enterococci, for which no information is available at the genetic level, using a molecular biological approach which included cloning and genetic characterisation of the bile salt hydrolase gene of one of the enterococci strains.
3) To evaluate and compare the probiotic potential of the selected *Enterococcus* strains using a commercial *Enterococcus faecium* SF68 probiotic culture, and to select a bacteriocin-producing strain and its bacteriocin-negative mutant for use in an *in vivo* experiment to determine their effects on the hosts autochthonous intestinal flora.
4) To investigate the potentially probiotic enterococci strains for virulence determinants and antibiotic resistances in a safety evaluation for possible future probiotic research and application of the strain.
5) To determine the effect of the bacteriocin and the effect of the bacteriocin producer on the intestinal microflora of the rat by recovering predominant gastrointestinal bacteria from the faeces on selective media, and specifically by phenotypic and genotypic identification of the lactobacilli and enterococci isolates.

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. Microorganisms used in this study

The strains investigated for their probiotic potential in this study are listed in Table 4. The probiotic *Enterococcus faecium* strain SF68 was supplied by the manufacturers of this probiotic strian (Cerbios Pharma S.A., Lugano, Switzerland). *Enterococus faecalis* BFE 1071 was isolated from the faeces of Göttingen-minipigs (Du Toit et al., 2000). The genes associated with bacteriocin-production of this microorganism were cloned and characterised in a previous study (Balla et al., 2000; Franz et al., 2002) and the strain was shown to produce a two-component bacteriocin termend enterocin 1071A&B (Balla et al., 2000). The plasmid-cured, bacteriocin-negative mutant *E. faecalis* 1071/79(-) used in this study (Table 4) was also previously created (Balla et al., 2000). The other enterococci were obtained from the FAIR-E culture collection of the European Union (EU) project entitled *Enterococci in Food Fermentations: Functional and Safety Aspects* (FAIR-CT97-3078) and were all isolated from food. These bacteria were selected as potentially probiotic candidates on the basis of limited safety investigations (gelatinase-negative, no beta-haemolysis on blood agar, vancomycin-sensitive) and absence of plasmid DNA done in the FAIR-CT97-3078 study. *Enterococcus faecium* BFE 900 produces the bacteriocins entrocin A and B and the genetics of bacteriocin-production have been studied in detail (Franz et al., 1999). This strain was isolated from Spanish olives and was used in this study as a potential probiotic candidate based on it's ability to produce two chromosomally-encoded bacteriocins. *Lactobacillus sakei* Lb706 isolated from meat was included as a further potentially probiotic strain, for which bacteriocin-production was well studied at the genetic level (Axelsson et al., 1993; Axelsson and Holck, 1995). This strain produces the plasmid-encoded bacteriocin sakacin A and a bacteriocin-negative mutant of this strain was also created previously by mutation with ethidium bromide (Axelsson et al., 1993). All the strains were grown aerobically in MRS (de Man, Rogosa and Sharpe) broth (Merck, Darmstadt, Germany) adjusted to pH 6.5 at 37 ºC, and stock cultures were kept in the same medium at -80 ºC with 15% (vol./vol.) glycerol added.

Table 4: Strains and relevant geno- or phenotype of potentially probiotic lactic acid bacteria used in this study.

Strain	**Relative genotype/phenotype**
Enterococcus faecium SF68	Commercial probiotic strain
Enterococcus faecalis BFE 1071	Enterocin 1071 producer. The *ent1071*, bacteriocin gene is located on a 50-kbp plasmid pEF1071
Enterococcus faecalis BFE 1071 /79(-)	Bacteriocin-negative, plasmid pEF1071 cured derivative of *E. faecalis* BFE 1071.
Enterococcus faecium BFE 900	Plasmidless. Enterocin A and Enterocin B producer. The *entA*$^+$ and *entB*$^+$ bacteriocin genes are located on the chromosome.
Lactobacillus sakei Lb706	Sakacin A producer. The *sakA* bacteriocin gene is located on a 60-kbp plasmid
Enterococcus faecium FAIR-E 6	Food isolate
Enterococcus faecium FAIR-E 9	Food isolate
Enterococcus faecium FAIR-E 15	Food isolate
Enterococcus faecium FAIR-E 210	Food isolate
Enterococcus faecium FAIR-E 243	Food isolate
Enterococcus faecium FAIR-E 345	Food isolate
Enterococcus faecium FAIR-E 349	Food isolate
Enterococcus faecium FAIR-E 365	Food isolate

2.1.2. Media, solutions and chemicals

The bacteriological media were prepared by sterilizing at 121 °C for 15 min. For the production of solid agar plate media, agar-agar was added at a concentration of 15 g/L. Bacteriological media were used to propagate potentially probiotic strains or to isolate strains from rat faeces in the animal experiment.

2.1.2.1 **MRS medium** (de Man et al., 1960; Merck) (g/L):

D(+) Glucose	20
Universal peptone	10
Meat extract	5
Sodium acetate	5
$K_2HPO_4.3H_2O$	2
di-Ammonium hydrogen citrate	2
Tween 80	1
$MgSO_4.7H_2O$	0.1
$MnSO_4.4H_2O$	0.05
pH	6.5

(pH was adjusted to pH 6.5 with 1 N NaOH).

For MRS soft agar, 8 g/L of bacteriological agar was added to MRS broth before sterilisation.

2.1.2.2 **Rogosa agar** (Merck, Darmstadt, Germany) (g/L):

Peptone from casein	10.0
Yeast extract	5.0
D(+)-Glucose	20.0
Potassium dihydrogen phosphate	6.0
Ammonium citrate	2.0
Tween 80	1.0
Sodium acetate	15.0
$MgSO_4.7H_2O$	0.575
Iron (II) sulfate	0.034
Manganese sulfate	0.12
Agar-agar	15.0
Neutral red	0.03
Crystal violet	0.002
Agar-agar	13.0

2.1.2.3 **DRCM Agar (Differential Reinforced Clostridia Medium)** (Merck, Darmstadt, Germany) (g/L):

Peptone from casein	5.0
Peptone from meat	5.0

Meat extract	8.0
Yeast extract	1.0
Starch	1.0
D(+)-Glucose	1.0
L-cysteine chloride	0.5
Sodium acetate	5.0
Sodium disulfide	0.5
Ammonium iron (III) citrate	0.5
Resazurin sodium	0.002

2.1.2.4 **Azide blood agar base** (Oxoid, Hampshire, England) (g/L):

Tryptose	10.0
'Lab-lemco' powder	3.0
Sodium chloride	5.0
Sodium azide	0.2
Agar-agar	12.0

2.1.2.5 **Columbia agar** (Merck, Darmstadt, Germany) (g/L):

Special nutrient substrate	23.0
Starch	1.0
Sodium chloride	5.0
Agar-agar	13.0

2.1.2.6 **Kanamycin aesculin azide agar** (Merck, Darmstadt, Germany) (g/L):

Peptone from casein	20.0
Yeast extract	5.0
Sodium chloride	5.0
Sodium citrate	1.0
Sodium azide	0.15
Kanamycin sulfate	0.02
Esculin	1.0
Ammonium iron (III) citrate	0.5
Agar-agar	15.0

2.1.2.7 Bifidobacterium agar (g/L):

Trypticase	10.0
Phytone	5.0
D(+)-Glucose	5.0
Yeast extract	2.5
Tween 80	1.0 ml
Cysteine	0.5
$K_2HPO_4. H_2O$	2.0
$MgCl_2. H_2O$	0.5
$ZnSO_4. H_2O$	0.25
$CaCl_2. H_2O$	0.15
$FeCl_3$	0.01
Selective agent LiCl	3.0
Agar-agar	15.0

2.1.2.8 Todd-Hewitt agar (Becton and Dickinson, Heidelberg, Germany) (g/L):

Infusion from beef heart	500.0
Bacto neopeptone	20.0
Bacto dextrose	2.0
Sodium chloride	2.0
Disodium phosphate	0.4
Sodium carbonate	2.5
Agar-agar	15.0

Todd-Hewitt broth consisted of the same formulation, but without the agar-agar added.

2.1.2.9 Dulbecco's Modified Eagles medium (Life Technologies, Karlsruhe, Germany) (mg/L):

$CaCl_2.2H_2O$	264.0
$Fe(NO_3).9H_2O$	0.10
KCl	400.0
$MgSO_4.7H_2O$	200.0
NaCl	6 400.0
$NaHCO_3$	3 700.0

$NaH_2PO_4.2H_2O$	141.0
D(+)Glucose	4 500.0
Phenol red	15.0
Aminopterin	0.176
Hypoxanthin	13.60
Thymidin	3.88
L-Arginine.HCl	84.00
L-Cystine	48.0
L-Glutamine	580.0
Glycine	30.0
L-Histidine.HCl.H_2O	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-Lysine.HCl	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Serin	42.0
L-Threonin	95.0
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	94.0
D-Ca-Panthothenate	4.0
Cholinchloride	4.0
Folic acid	4.0
i-Inositol	7.2
Nicotinamide	4.0
Riboflavine	0.4
Thiamine HCl	4.0
Pyridoxal HCl	4.0

2.1.2.10 Mueller-Hinton II agar (g/L)

Beef Infusion	300.0
Casaminoacids	17.5

Starch	5
Agar-agar	17.0
pH	7.3

2.1.2.11 Reduced Brain Heart Infusion medium (g/L):

Infusion from calf brains	200.0
Infusion from beef heart	250.0
Proteose peptone	10.0
Dextrose	2.0
Sodium Chloride	5.0
di-Sodium phosphate	2.5
Cysteine monohydrochloride	1.0
pH	7.4

2.1.3 Media used for testing technological properties of potentially probiotic lactic acid bacteria strains

2.1.3.1 Acid and bile tolerance

MRS broth (see 2.1.2.1) adjusted to pH 3.0 was used as artificial gastric juice to test for acid resistance of potentially probiotic strains. MRS broth with a pH of 3.0 was prepared by dissolving 52.5 g MRS dry medium in 1 L destilled water and adjusting the pH to 3.0 using 5 N HCl. An Oxgall (Difco, Detroit, USA) solution was made by dissolving 10 g of Oxgall powder in 100 ml volume of aqua bidest. This oxgall solution was used to simulate bile salt and to assess the tolerance of potentially probiotic strains. A bicarbonate buffer was used as artificial duodenum juice to evaluate the ability of potentially probiotic strains to tolerate gastric juice. This artificial duodenum juice was made by dissolving 6.4 g $NaHCO_3$, 0.239 g KCL, and 1.28 g NaCl in 100 ml aqua bidest. The pH was adjusted to 7.4 with 5 N HCl.

2.1.4 Media and solutions used for testing functional properties of potentially probiotic lactic acid bacteria strains

2.1.4.1 Bacteriocin test

MRS plate agar and MRS soft agar (see 2.1.2.1) were used for testing bacteriocin activity.

2.1.4.2 Bile salt hydrolase activity

Bile salt hydrolase activity was detected on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (Sigma, Deisenhofen, Germany) and 0.37 g/l of $CaCl_2$ (Merck, Darmstadt, Germany).

2.1.4.3 Adhesion to mucus-secreting HT-29-MTX cells and Extracellular Matrix Proteins

Dulbecco's Modified Eagle's medium (DMEM) (see 2.1.2.11) containing 2% foetal calf serum (Life Technologies, Karlsruhe, Germany) was used to grow test bacteria. HT-29 cells were cultivated in 24 well Tissue culture plates in the absence of antibiotics in glucose-free DMEM supplemented with 15% dialysed fetal bovine serum, 4 mmol L-glutamine/L, and 5 mmol galactose/L.

2.1.4.4 Hydrophobicity

For hydrophobicity testing, bacteria were grown in 9 ml of MRS broth (see 2.1.2.1) and hydrophobicity was tested in qurter-strength Ringer‘s solution and n-hexadecane (Merck).

2.1.5 Media used for testing virulence and antibiotic resistance of potentially probiotic enterococci strains

2.1.5.1 DNAse production

DNAse test agar (Becton Dickinson, Heidelberg, Germany) with methyl green was used for testing for DNAse activity, and contained (g/L):

Bacto tryptose	20.0
Deoxyribonucleic acid	2.0
Sodium chloride	5.0
Bacto agar	15.0
Methyl green	0.05

2.1.5.2 Production of gelatinase

Production of gelatinase was detected on Todd-Hewitt agar (see section 2.1.2.10) containing 30 g/l gelatine (Merck, Darmstadt, Germany).

2.1.5.3 Clumping assay for presence of aggregation substance

Todd-Hewitt broth (see section 2.1.2.10) was used to test for clumping of enterococci in presence of pheromone.

2.1.5.4 Haemolysin production

All 4 human blood types were collected, defribinated and added (5%) to Columbia agar (see 2.1.2.6).

2.1.5.5 Mucin degradation assay

Mucin degradation was tested using medium B of Zhou et al. (2001) in a Petri dish. Mucin degradation was tested three times in medium B containing either 3 or 5 g/L of purified hog gastric mucin and either 10 or 30 g/L of D(+) glucose.

Medium B (g/L):

Tryptone	7.5
Casitone	7.5
Yeast extract	3.0
Meat extract	5.0
NaCl	5.0
$K_2HPO_4.3H_2O$	3.0
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.5
Cysteine HCl	0.5
Resazurin	0.002
D(+)-glucose	10.0 or 30.0
Purified hog gastric mucin	3.0 or 5.0
Agarose	15.0
pH	7.2

2.1.5.6 Staining and destaining solution for detection of mucin degradation

The staining agent amido black was solubilized in 3.5 N acetic acid at an end concentration of 0.1%. Acetic acid (1.2 N) was used as destaining solution.

2.1.5.7 Antibiotic resistance determination

Antibiotic resistances of potentially probiotic enterococci was tested on Mueller-Hinton agar (see 2.1.2.10) (Becton Dickinson, Heidelberg, Germany):

2.1.5.8 Solutions used for total genomic DNA isolation of potentially probiotic enterococci for PCR amplification of virulence determinants

Total genomic DNA of enterococci for PCR amplification of virulence determinants was isolated according to the guanidium thiocyanate method Pitcher et al. (1989). The composition of the TERMLS solution used for degradation of the bacterial cell wall, the GES solution to lyse cells and to degrade protein and the TE buffer (1 X TE solution) used to store DNA are given below:

2.1.5.8.1 TE buffer

TE Buffer (1 X TE solution) contains 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0).

2.1.5.8.2 TERLMS solution

TERLMS solution consisted of 25 µg/ml RNAse, 25 mg/ml lysozyme, 10 U/ml mutanolysin, and 0.2 g/ml sucrose in 1X TE solution (see 2.1.4.8.1).

2.1.5.8.3 GES solution

This reagent consisted of 5 M guanidium thiocyanate, 100 mM EDTA, and 0.5% Sarkosyl and was filter-sterilised and stored at room temperature.

2.1.6 Solutions used for cloning of the bile salt hydrolase gene

For cloning of the bile salt hydrolase gene, total genomic DNA was isolated from *E. faecalis* FAIR-E 345 according to the method of Pitcher et al. (1989) using the TE buffer, TERMLS and GES solutions described above (see 2.1.5.8.1, 2.1.5.8.2 and 2.1.5.8.3). In addition, small-scale plasmid DNA was isolated by the method of van Belkum et al. (1995). Solutions for plasmid DNA isolation include TELS solution to degrade the bacterial cell wall and lysis solution to lyse cells as described below.

2.1.6.1 TELMS solution

TELMS solution consisted of 25 mM Tris, 10 mM EDTA, 20% sucrose, 20 mg/ml lysozyme and 1000 U/ml mutanolysin in bidest water.

2.1.6.2 Lysis solution

Lysis solution consisted of 1% SDS and 0.2 N NaOH in bidest water.

Restriction enzymes and buffers used for cloning of the bile salt hydrolase gene were obtained from New England Biolabs (Frankfurt am Main, Germany).

2.1.7 Buffers used in Southern hybridization of plasmid DNA with bile salt hydrolase gene probe

Plasmid DNA was run on 1.2 % agarose gels, transferred to nylon membrane (see methods section below) and hybridised with a bile salt hydrolase gene probe (see methods below) according to Sambrook et al. (1989).

2.1.7.1. Buffer 1

Buffer 1 consisted of 100 mM Tris (pH8.0) and 150 mM NaCl in bidest water.

2.1.7.2 Buffer 2

Consisted of blocking reagent (Hering sperm DNA, commercially supplied with the DIG-labelling and detection kit; Roche, Mannheim Germany) at a concentration of 0.5% and is dissolved in buffer 1 while heating at 50 to 70 °C. This buffer is kept frozen at –20 °C until use.

2.1.7.3 Buffer 3

Buffer 3 consisted of 100 mM Tris (pH 8.0), 100 mM NaCl and 50 mM $MgCl_2$ in bidest water. The pH of buffer 3 was adjusted to pH 9.5 with 1 N NaOH.

2.1.7.4 Prehybridization solution

This solution consisted of 5X SSC (5X SSC solution was obtained by diluting 20X SSC solution containing 0.3 M sodium citrate, pH approximately 7.0 containing 3 M NaCl), 0.1% (weight/vol.) N-laurosylsacrosine, 0.02% (weight/vol.) SDS, 1% Blocking reagent (Roche) in bidest water.

2.1.7.5 Denaturing solution

Denaturing solution consisted of 1.5 M NaCl and 0.5 M NaOH in bidest water.

2.1.7.6 Transferring solution

Transferring solution consisted of 1 M ammonium acetate and 20 mM NaOH which were solubilised in bidest water.

2.1.7.7 DIG-Labelling and detection

A commercial DIG-labelling and detection kit (Roche) was obtained, containing solutions for labelling the bile salt hydrolase gene probe and detection of hybridization of the probe to plasmid DNA according to the manufacturer's specifications.

2.1.8 Animal experiments

Thirty six male Sprague-Dawley rats (weight between 125 and 150 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). The basal diet Altromin C 1000 (Altromin GmbH, Lage, Germany, see appendix A) was ordered in powder form.

Biomass of probiotic bacteria was grown in MRS broth, collected by centrifugation, washed in quarter-strength Ringers's solution (see methods below). A 10% skim milk solution (Merck, Darmstadt, Germany; sterilised at 120 °C for 15 min by autoclaving) was added to the probiotic cell biomass as protective agent during freeze-drying.

2.1.8.1 Identification of the bacteria isolated from fecal samples of rats

Rat faeces was collected and diluted ten times in reduced brain heart broth (see 2.1.2.11).
Seven groups of bacteria were selected with selective media as described above. Rat faeces was serially diluted using a ten-fold dilution series in quarter-strength Ringer's solution (see 1.2.1.12) and plated out onto the (semi)selective media for: lactobacilli (Rogosa agar), enterococci (Kanamycin aesculin azide agar), Gram-positive anaerobic cocci (Bacto azide blood agar base), clostridia (DRCM agar), bifidobacteria (Bifidobacteria agar), and total count (Columbia agar).

2.2 METHODS

2.2.1 Investigations into technological traits of potentially probiotic enterococci

2.2.1.1 Acid and bile tolerance

For determinations of bile resistance and acid tolerance of potentially probiotic lactic acid bacteria strains (enterococci and other bacteriocin producer, i.e. *L. sakei* Lb706) 3 ml of MRS broth (pH 3.0) were seperately inoculated at a level of 2 x 10^8 CFU/ml. A pH of 3.0 was chosen to represent the pH of the stomach after swallowing a meal. The culture was placed in an aerobic chamber (atmosphere of 80% N_2, 10% CO_2, and 10% H_2) in an anaerobic work chamber (MK3 anaerobic chamber work station, dw Scientific, Shipley, England). After one hour, a 1 ml sample was withdrawn under anaerobic conditions and then removed from the anaerobic chamber for determination of the viable cell count and, consequently acid resistance of the test microorganism. Aliquots of 1.2 ml of Oxgall solution (10%) and 5.1 ml bicarbonate buffer (artificial duodenum juice) were added after removal of the sample for cell counts, again under anaerobic conditions. Thus the test set up simulated conditions as bacteria would encounter when reaching the small intestine after a one-hour residency time in the stomach. Further samples were withdrawn under anaerobic conditions after one and two hours anaerobic incubation after addition of the Oxgall and artificial duodenum juice to determine the test organisms bile resistance and resistance to simulated small intestinal conditions. For viable count determination, samples were serially diluted using ten-fold dilution steps in quarter-strength Ringer's solution and spread-plated onto MRS agar medium (pH 6.5). All plates were then incubated anaerobically at 37 ºC for 48 hours. The test was done in duplicate and duplicate counts were meaned. The test was repeated three times in separated days.

2.2.2 Investigations into probiotic properties of potentially probiotic enterococci

2.2.2.1 Bacteriocin activity

A qualitative bacteriocin test, i.e. the 'sandwich overlay method' of Mayr-Harting et al. (1972) was used to test the antimicrobial spectrum of the bacteriocin-producing, potentially probiotic lactic acid bacteria. Three µl of overnight culture of bacteriocin-producing strain was spotted onto MRS agar plates and plates were incubated at 37 °C overnight. MRS soft agar was inoculated with 100 µl of an overnight culture of indicator bacteria (approx. 1 x 10^6 CFU/ml) and overlayered onto MRS plates onto which the bacteriocin producer was spotted. The plate was again incubated overnight at 37 °C. A clear zone surrounding the bacteriocin

producer colony after the growth of the indicator strain was considered as bacteriocin-positive.

A quantitative bacteriocin test expressed in arbitrary unit (AU) of activity, developed by Mayr-Harting et al. (1972), was also used. For this, an overnight culture of the bacteriocin producing strain was centrifuged (7 500 rpm, 6 min, 4 °C) to obtain culture supernatant. This supernatant was heated at 95 °C for 10 min or filter-sterilized to remove or inactivate remaining cells. A 100 µl volume of supernatant was then diluted in a doubling dilution series in a microtitre plate, using sterile MRS broth as diluent (pH 6.5). Ten µl of each dilution was spotted onto MRS soft agar that was seeded with 1% indicator bacteria and allowed to solidify. Plates were incubated anaerobically at 37 °C. Arbitrary activity was determined as the reciprocal of the highest dilution that gave a clear inhibition zone, multiplied by a factor of 100 to obtain AU/ ml.

2.2.2.2 Bile salt hydrolase activity

The ability of potentially probiotic enterococci (food and human isolates) and also *L. sakei* Lb 706 to hydrolyse bile salt was assayed using a plate test. For this, 10 µl of an overnight culture was spotted onto MRS agar plates (pH 6.5) containing 0.5% Na-TDCA (taurodeoxycholic acid) and 0.37 g/l $CaCl_2$ (Franz et al., 2001). Plates were incubated anaerobically at 37 ºC for 72 hours. A strain was considered bile salt hydrolase positive if a white zone of precipitation occurred which surrounded the colony.

2.2.2.3 Adhesion to mucus-secreting HT-29-MTX cells

HT-29 MTX cells were cultivated according to Huet et al. (1987) in the absence of antibiotics in glucose-free DMEM supplemented with 15% dialysed fetal bovine serum, 4 mmol L-glutamine/L, and 5 mmol galactose/L. HT-29 MTX enterocytes were seeded at 2 $x10^4$ cells per well (2 cm^2) in a 24-well plastic tissue culture dishes and incubated at 37 °C in 9.5% CO_2. These HT-29 cultures become confluent after 5-6 d and were used after 21-24 d when these enterocytes were considered polarized and differentiated. Mature HT-29 MTX cultures containing ~10^6 enterocytes per well, were >95% viable as determined microscopically using the vital dyes trypan blue (0.36%) and propidium iodide (20 mg/L).

Enterococci were grown 18 hours in Dulbecco's MOD Eagles Medium (Life Technologies) containing 2% foetal calf serum (FCS) and after growth, cell numbers of enterococci were adjusted to log 8.0 cells/ml by using a flow cytometer. One ml of this suspension was added to the tissue culture plate well. Plates were centrifuged and incubated at

37 ºC. After 1 h incubation, the monolayers were washed 3 x with sterile phosphate buffered saline solution (pH 7.4). Detached bacteria were enumerated by plate counting. HT-29 MTX cells were lysed by adding 1% Triton X-100 solution and incubated for 10 min at 37 ºC and the cell count (CFU/ml) of adhering bacteria was determined in triplicate.

2.2.2.4 Hydrophobicity

For hydrophobicity testing, strains were grown in MRS broth at 37 ºC for 18 h. Cells from 5 ml of culture were collected by centrifugation at 7500 rpm at 4ºC for 6 min. Cells were washed twice with quarter-strength Ringers solution, using the same centrifugation conditions as described above. The quarter-strength Ringer's solution containing enterococci cells provided the watery suspension. One ml of this suspension was used to determine the OD_{580}. In duplicate assessments, a further 1.5 ml of this suspension was added to an equal volume of n-hexadecane (organic phase)(Merck) and thoroughly mixed for two minutes using a vortex. The phases were allowed to separate at room temperature for 30 min, after which one ml of the watery phase was removed and the OD_{580} was determined. The OD_{580} of duplicate assessments was meaned and used to calculate hydrophobicity. Percentage hydrophobicity was calculated as follows:

(OD_{580} reading 1 - OD_{580} reading 2 / OD_{580} reading 1) x 100 = % hydrophobicity.

Hydrophobicity determinations were done in triplicate in independent experiments.

2.2.3 Investigations into virulence traits of potentially probiotic enterococci

2.2.3.1 DNAse production

A loopful of an overnight *Enterococcus* culture was streaked out onto DNAse test agar containing methyl green and plates were incubated at 37 ºC for 24 hours. A clear, pinkish zone around the colonies after incubation was considered positive for DNAse production.

2.2.3.2 Production of gelatinase

Production of gelatinase was determined on Todd-Hewitt agar containing 30 g/l gelatine. Five microliter of overnight culture grown in MRS broth was spotted onto the gelatine agar. Plates were incubated at 37 ºC for 24 h and cooled for 5 h at 4 ºC. Appearance of a turbid halo around the colonies was considered positive for hydrolysis of gelatine.

2.2.3.3 Clumping assay for presence of aggregation substance

The ability of bacteria to clump in presence of pheromone was investigated. The pheromone producer *Enterococcus faecalis* JH2-2 and a plasmid-free, negative control strain *Enterococcus faecalis* OG1X were used. For the assay, 2 µl of an *Enterococcus* test strain were grown overnight at 37 ºC in Todd-Hewitt broth (THB) were used to inoculate 198 µl of THB in a microtiter plate (negative control). Supernatant from an overnight culture of the pheromone producer *E. faecalis* JH2-2 was prepared, autoclaved and diluted 1:5 in sterile THB. Two microliter of culture were inoculated into198 µl of this pheromone containing broth in a microtiter plate. Microtiter plates were incubated at 37 ºC and inspected for clumping on an hourly basis up to eight hours.

2.2.3.4 Adhesion to extracellular matrix proteins

The ability of enterococci isolates to adhere to rat collagen I and human fibrinogen and fibronectin extracellular matrix protein was studied using microtiter plates which were commercially coated with either of these proteins. Enterococci were grown 18 h in Dulbecco's MOD Eagles Medium (Life Technologies) containing 2% foetal calf serum (FCS). After growth, cell numbers of enterococci were adjusted to log 8.0 cells/ml by using a flow cytometer. Microtiter plates were saturated with Dulbecco's MOD Eagles Medium before addition of cells. Enterococci were incubated in coated microtiter plates at 37 ºC for 1 h to allow binding of cells, after which the number of the adherent bacteria was established By removing the suspension and again counting with a flow cytometer and expressed as a percentage of the initial inoculum. Results shown are means of duplicate experiments.

2.2.3.5 Haemolysin production

Human blood (all 4 blood types) was collected, defribinated and added (5%) to Columbia agar base. Plates were pre-incubated 3 days to check for sterility. *Enterococcus* isolates were streaked out onto the blood agar, and the plates were incubated at 37 ºC for 48 h after which they were checked for zones of α- or β-hemolysis surrounding the colonies.

2.2.3.6 Mucin degradation assay

Partially purified hog gastric mucin (HGM) Type III (Sigma, Germany) was further purified according to Zhou et al., 2001. For this, 10 g of HGM were stirred for 24 h at room temperature in 500 ml of 0.1 M NaCl containing 0.02 M phosphate buffer (pH 7.8), and a few drops of toluene. The pH of this suspension was readjusted to 7.2 with 2 M NaOH after 1

hour. The mucin preparation was centrifuged (10 000 x *g*, 4 °C) and the supernatant was cooled to 0 ± 2 °C and pre-chilled ethanol (pre-chilled at –20 °C) was added to a final concentration of 60% (v/v). The resulting precipitate was dissolved in 0.1 M NaCl and precipitated with ethanol two more times. The final mucin precipitate pellet was washed once with ethanol and then dissolved in and dialysed against distilled water (4 L) for 24 h at room temperature with four changes. Dialysed mucin solution was then lyophilized and used as a source of purified mucin in all experimentation. The mucin substrate incorporated in culture medium was autoclaved before use.

Purified HGM was incorporated with medium B (see section 2.1.3.13) at concentrations of 0.5% (w/v) with and without 3% glucose. The presence of glucose in medium B as carbohydrate source affect the mucinolytic activity. Ten microlitres of 24h viable bacterial cultures were inoculated onto the surface of the medium contained in a Petri dish. The plates were incubated at 37 ºC anaerobically for 72 h and subsequently stained with 0.1% amido black in 3.5 M acetic acid for 30 min. They were then washed with 1.2 M acetic acid until the mucin lysis zone (discoloured halo) around the colony of positive control cultures (faecal flora obtained from male Fischer rat faeces) appeared. The mucin degradation activity was defined as the size of the mucin lysis zone.

2.2.3.7 Antibiotic resistance

Antibiotic susceptibility was tested using the E-test (Viva Diagnostika, Cologne, Germany). Commercial Mueller–Hinton agar (see 2.1.2.10) plates (Becton Dickinson, Heidelberg, Germany) For preparation of the inoculum opf the test strain, the entrococci were streaked out for single colonies on MRS agar plates and plates were incubated at 37°C for 48 hours. Individual colonies were resuspended in 5 ml quarter-strength Ringers solution to a density of 0.5 on the McFarland scale. The Mueller-Hinton II agar plates were seeded with enterococci using a cotton swab. Plates were incubated at 37ºC and results were read after 48 h incubation according to the E-test manufacturer's intructions. The antibiotics used included: ampicillin (0.016 to 256 μg/ml); benzylpenicillin (0.002 to 32 μg/ml); chloramphenicol (0.016 to 256 μg/ml); tetracycline (0.016 to 256 μg/ml); erythromycin (0.016 to 256 μg/ml); ciprofloxacin (0.002 to 32 μg/ml); streptomycin (high range, 0.064 to 1024 μg/ml); and gentamicin (high range, 0.064 to 1024 μg/ml).

The minimum inhibitory concentration (MIC) value determination was based on the reference agar dilution method described in 'performance standards for antimicrobial susceptibility testing', ninth informal supplement, National Committee for Clinical

Laboratory Standards (NCCLS), Vol. 19 (10), 1999. Resistance was intrepreted according to the breakpoint values supplied in this document for enterococci, i.e., resistance to ampicillin, penicillin and tetracycline at a minimum inhibitory concentration (MIC) of ≥ 16 μg/ml; chloramphenicol and vancomycin, MIC of ≥ 32 μg/ml; erythromycin, MIC ≥ 8 μg/ml; ciprofloxacin, MIC ≥ 4 μg/ml; high level gentamicin MIC > 500 μg/ml; and high level streptomycin, MIC> 1000 μg/ml.

2.2.3.8 PCR amplification of virulence factors

The genetic determinants for the enterococcal virulence factors AS (aggregation substance), Esp (enterococcal surface protein), Ace (adhesin to collagen from *E. faecalis*), gel (gelatinase) and Cyl (cytolysin) were amplified from the DNA of the potentially probiotic enterococci strains by PCR using primers specific for these genes (Table 6). The PCR reactions all contained 10 ng template genomic DNA, 200 μM desoxyribonucleotide triphosphates (dNTPs), 25 pMol of each primer (Table 6), 1 x *Taq* polymerase buffer (Pharmacia, Freiburg, Germany) and 1.5 U of *Taq* polymerase (Pharmacia). PCR conditions and primers for amplification of the virulence genes are shown in Table 6. As a positive control, the DNA of an *Enterococcus* strain known to be positive for the virulence trait was used. The PCR products were run on 1.5% agarose gels and photographed under UV light.

Table 5: Primers and amplification conditions used for PCR amplification of enterococcal virulence genes.

Virulence factor	Primer sequences	PCR amplification conditions (for all 35 cycles)	Size of amplification product
Esp	5'-TTG CTA ATG CTA GTC CAC GAC C-3' (forw) 5'-GCG TCA ACA CTT GCA TTG CCG AA-3' (rev[a])	94 °C, 1 min; 61 °C, 1 min; 72 °C, 1.5 min	900 bp
Ace	5'-GAA TTG AGC AAA AGT TCA ATC G-3' (forw) 5'-GTC TGT CTT TTC ACT TGT TTC-3' (rev)	94 °C, 1 min; 54 °C, 1 min; 72 °C, 1 min	960

[a] forw = forward (sense) primer, rev = reverse (antisense) primer

2.2.4 Cloning of a bile salt hydrolase gene from *Enterococus faecium* FAIR E-345

Genomic DNA from *Enterococus faecium* FAIR E-345 was isolated according to the methods of Pitcher et al. (1989). For this, 5 ml of an overnight culture of *E. faecium* FAIR-E 345 grown in MRS broth at 37°C was centrifuged at 13 000 x *g* (6 min, 4 °C). The cell pellet was resuspended in 1.5 ml of TE solution which contained 0.5% NaCl and the suspension was centrifuged again (13,000 x *g*, 6 min, 4 °C). The cell pellet was then resuspended in 100 µl of TERLMS solution and incubated at 37 °C for 1 h for lyser to occur. For removal of protein, 500 µl GES solution was added. The tube was inverted until lysis was observed. The sample was cooled on ice for 5 min and after that 250 µl of cold 7.5 ammonium acetate was added. The sample was mixed and cooled on ice for 10 min, after which a 0.5 ml volume of chloroform-2-pentanol (ratio 24:1) was added. The phases were mixed thoroughly by shaking vigorously and the mix was centrifuged (13 000 x *g*, 10 min, 4 °C). The upper aqueous phase was transferred to a new 1.5-ml Eppendorf tube using a Pasteur pipette. Cold (-20 °C) 2-propanol was added (0.54 volume) and the tube was inverted until the DNA precipitated. The precipitate was centrifuged (13 000 x*g*, 6 min, 4 °C) and the aqueous solution was aspirated. The DNA pellet was washed to remove salt with 500 µl of 70% ethanol. The washing step (centrifugation at 13 000 x *g*, 6 min, 4 °C) was repeated 2 times. The DNA pellet was dried in a vacuum concentrator for 5 to 10 min and the dried DNA pellet was then dissolved in 150 µl of TE solution overnight at room temperature and then stored at 4 °C.

Genomic DNA was partially digested with *Sau*3AI (New England Biolabs, Frankfurt am Main, Germany) and subjected to electrophoresis on a 1.5% agarose gel. Following electrophoresis, fragments of various sizes (1.5-2.5 kb, 1.5-3.0 kb, 2.5-4.0 kb, 2.5-4.5 kb and 4.5-6.0 kb) were extracted directly from the agarose gel using the QIAEX II gel extraction kit (Qiagen, Hilden, Germany). Vector pSKII+/- was digested with *Bam*HI and dephosphorylated using calf intestinal phosphatase (New England Biolabs). The vector was ligated to the DNA fragments and ligated constructs (1.2 µl) were used for electrotransformation into electrocompetent *E. coli* TOP10 cells (Invitrogen, Groningen, the Netherlands) at 2.5 kV, 200Ω reistance and 25 µF capacitance. Transformed cells were incubated for 1 h in SOC solution (Sambrook et al., 1989) and spread-plated onto Luria Bertani (LB) agar containing X-Gal and IPTG at standard concentrations (Sambrook et al., 1989) and 150 µg/ml ampicillin. Plates were incubated at 37ºC overnight. Colonies containing an insert were selected by α-complementation and replica-plated onto LB agar and LB-based differential medium as described by Christians et al. (1992), to detect BSH-positive clones. The plates were incubated anaerobically at 37 ºC for two days and the presence of a white zone of

precipitation around around the colony on differential medium was indicative of BSH activity (Christians et al., 1992).

Plasmid pAW01 isolated from a BSH-positive clone was isolated using the QIAGEN plasmid midi kit (Qiagen, Hilden, Germany) and used for DNA sequencing. It contained a 3-kbp insert that was sequenced bi-directionally using M13 universal and custom made primers in a primer walking strategy. Sequencing was done at MWG Biotech (Ebersfeld, Germany) and GATC (Konstanz, Germany). The DNA sequence was analyzed using the DNASTAR programme. Homology searches of the amino acid sequence to known protein sequences in the databanks was done using the BlastX search of the National Center of Biotechnology Information (NCBI). BSH homology determinations were done by cluster of amino acid sequences contained in the GenBank databank using the DNASTAR program.

2.2.5 Preparation of a BSH gene probe and determination of the genomic location of the *bsh* gene in BSH-positive enterococci

2.2.5.1 Preparation of the gene probe and isolation of plasmid and total genomic DNA of BSH-positive enterococci

The *bsh* gene was PCR amplified using primers Aw-01 (5‘-TAT ATC TAG AGG AGT AAT ATG AAC GTG GA-3‘) and primer Aw-02 (5‘- TAT AGC ATG CAA ATA ATC AAT CAC AAC ACA C-3‘) complementary to the 5‘ and 3‘ ends of this gene in pAW01, respectively, and contained *Xba*I and *Sph*I restriction enzyme sites (underlined). DNA was amplified in a 50 µl volume and the PCR mixture contained 100 ng of template DNA, 200 µM concentration of dNTP’s, 25 pMol of each of the respective primers, 1 U *Taq* DNApolymerase (Amersham Pharmacia, Freiburg, Germany), and 1X reaction buffer (Amersham Pharmacia). DNA amplification was done in 32 cycles (denaturation, 1 min at 94 °C; annealing, 1 min at 52°C, extension, 1 min at 72°C). The resulting PCR fragment was cloned into the *Xba*I and *Sph*I sites of plasmid pUC19, resulting in plasmid pAW02. Plasmid pAW02 was digested with the restriction enzyme *Xba*I to linearize the plasmid and DIG labeled using the DIG dUTP labeling and detection kit (Roche, Mannheim, Germany) according to the manufacturer‘s instructions.

Plasmid DNA was isolated from BSH-positive enterococci isolated from food (Franz et al., 2001) for determination of the genomic location of BSH genes among these strains. These food enterococcal strains investigated by Franz et al. (2001) were used, because the

potentially probiotic strains subject of this study did not contain plasmid DNA. Plasmid DNA was isolated according to methods of van Belkum and Stiles (1995). Briefly, 5.0 ml of overnight culture was centrifuged at 7 500 rpm for 6 min at 4 °C to harvest the cells. The cell pellet was washed with 1.5 ml of TE solution containing 0.5% NaCl and this suspension was transfered to an Eppendorf tube and centrifuged (7,500 rpm, 5 min, 4 °C). Supernatant was removed and the cell pellet was resuspended with 100 µl of TELS solution. This suspension was incubated at 37 °C for 1 h. A 200 µl volume of fresh lysis solution was added and mixed until the suspension was clear. Then 150 µl volume of 3 M potassium acetate, pH 5.0 was added and mixed. After incubated on ice for 12 min, the suspension was centrifuged (14 000 rpm, 10 min, 4 °C) and the supernatant was transfered to a new sterile Eppendorf tube. For the phenol/chloforom purification, 200 µl phenol was added to supernatant. This suspension was mixed and 200 µl chloroform was added. The suspension was then mixed and centrifuged (15 000 rpm, 10 min, 4 °C). The supernatant was removed to a new Eppendorf tube, after which 200 µl chloroform:isoamyl alcohol (24:1) was added. The suspension was centrifuged (15,000 rpm, 6 min, 4 °C) and the supernatant was removed. A 1/10 volume of 3 M sodium acetate and 2x volume of 95% ethanol were added to the supernatant to precipitate the DNA at –20 °C overnight. The sample was then centrifuged (15 000 rpm, 8 min, 4 °C) and the DNA pellet was washed with 70% ethanol and dried for 5 to 10 min in a vacuum drier and resuspended in 10 µl TE containing RNAse (20 µg/ml). The suspension was gently mixed and finally incubated at 37 °C for 20 to 30 min.

Total genomic DNA was isolated using the methods of Pitcher et al. (1989) as described above. Total genomic DNA was digested with *Xba*I and *Hind*III and subjected to electrophoresis using a 1.4% agarose gel. Plasmid DNA was subjected to electrophoresis on these agarose gels without restriction enzyme cutting. DNA was transferred to Hybond N^+ membrane by Southern blotting according to standard techniques (Sambrook et al. 1989) and the DIG-dUTP-labeled probe was used to detect the location of the *bsh* gene according to the DIG dUTP manufacturer‘s (Roche) instructions. Prehybridization was done at 65°C for 4 hours, while hybridization was done at 60-70 °C overnight (methods see below).

2.2.5.2 Southern hybridization

The isolated plasmid and total genomic DNA was separated on a 1% agarose gel in TBE buffer (Sambrook et al. 81989) at 100 V for 3 h. The position of the gel pockets and the plasmid bands on the gel was marked by placing cellophane paper over the gel, inspecting the gel under UV light, and marking these positions with a marker before the gel was soaked in

0.25 M HCl for 5 min. The gel was then washed with bidest water and soaked in denaturing solution for 30 min. After this, the gel was soaked in transferring solution for 30 min. The dimension of the gel was measured and a piece of nylon blotting paper and two pieces of Whatman paper were cut according to it's size. DNA was transferred using a transfer box containing transferring solution overnight at room temperatureas as described in Sambrook et al. (1989). After transferring and removal of the gel, the gel pocket and the corners of gels were marked on the nylon membrane. The membrane was rinsed in 2x SSC and dried at room temperature and baked in oven at 80 °C for 2 h.

For prehybridization, the membrane was placed in plastic bag and prehybridization solution was added and then sealed. Prehybridization was conducted at 65 °C for 4 h in hybridization chamber (Amersham Pharmacia Biotech, Freiburg, Germany). For hybridization, the DNA probe was boiled for 5 to 10 min and immediately cooled in ice slush. Prehybridization solution from the bag was poured off and the membrane in the bag was put on ice. The probe was added and the bag was quickly sealed. Hybridization was done in hybridization chamber at 60-70 °C overnight. After prehybridization, a corner of the bag was cut and probe was removed for reuse with Pasteur pipette. The membrane was washed 2 times (each time 5 min at room temperature) with washing buffer 1 (see 2.1.7.1) and 2 times (each time 15 min at 68 °C) with washing buffer 2 (see 2.1.7.2). The membrane was then dried before being equilibrated in buffer 1 for 1min. Subsequently, blocking was done for 30 min in buffer 2 at room temperature and the membrane was slowly shaken on shaking platform. The membrane was then washed for 1 min in buffer 1 and anti-DIG antibody-conjugate was added. Incubation was at room temperature for 30 min. The membrane was then washed 2 times in buffer 1, each time for 15 min before being equilibrated 2 min with buffer 3 (see 2.1.7.3). The colour solution (200 µl NTB/BCIP in 10 ml volume of buffer 3) was added and the bag was sealed before being put in the dark to allow to develop. After the signals were detected, the reaction was stopped by washing the membrane with distilled water.

2.2.6 Animal experiment

2.2.6.1 Experimental rats

Thirty six male Sprague-Dawley rats were weighed and acclimatized for 1 week. Two rats were housed per cage with wooden granulate bedding and received tap water *ad libitum*, with light and dark periods for 12 hours, and fed once a day with Basal Diet Altromin C 1000. After this period, rats were housed individually and divided into 4 groups:

- Group A (Control 1, n = 6, Diet Altromin C1000)
- Group B (Control 2, n = 6, Diet Altromin C1000 + heat inactivated 1% *Enterococcus faecalis* BFE 1071 Biomass)
- Group C (n = 12, Diet Altromin C1000 + 1% *Enterococcus faecalis* BFE 1071 Biomass)
- Group D (n = 12, Diet Altromin C1000 + 1% *Enterococcus faecalis* BFE 1071/79 (-) Biomass).

Diet treatments were given to rats once a day at 20 g at 9:00 a.m. Faecal samples were collected 4 hours after feeding time and pre-diluted 10 times with reduced Brain Heart Infusion Broth (see 2.1.2.11), homogenised with stomacher for 1 min, and stored at -20 °C. The experiment was conducted for 8 days and after that all the rat groups were fed with basal diet.

In the first week of the animal experiment (Day1 to 7) no animals were sacrificed. At certain intervals, the rats were sacrificed after day 7 and their organs (caecum, small and large intestines) were removed and stored at –80°C for future use (see Table 4). Analysis of the microflora of the organs was, however, not subject of this study. The intervals in which rats from which groups were sacrificed is given in Table 4 below. The number of rats in the different groups were minimized by the animal welfare expert to minimize the number of animals which had to be put to death. Permission for the animal experiments was obtained from the local ethics committee and the animal experiments were done under the guidance, supervision and participation of an experienced animal expert and animal caretaker.

Table 6: Time plan for sacrifice of animals in probiotic feeding experiment.

No. of animals/ sacrificed animals	Experimental animal group (see above)			
	group A	group B	group C	group D
total no. of live animals at start of experiment (day 1) to day 7 of experiment	6	6	12	12
No. of animals sacrificed on day 8	2	6	0	0
No. of animals sacrificed on day 9	0	0	6	8
No. of animals sacrificed on day 10	0	0	6	4
No. of sacrificed animals on day 11 (end of experiment)	0*	0	0	0

*Animals were kept for other experimental purposes which were not part of this study.

2.2.6.2 Preparation of feed

Biomass from *Enterococcus faecalis* BFE 1071 and *Enterococcus faecalis* BFE 1071/79(-) were prepared as follows: The culture was prepared by inoculating 1% overnight culture in 1L MRS broth (pH 6.5) and incubated overnight in shaking incubator (37 °C, 100 rpm). Cells were harvested by centrifugation (7 500 rpm, 6 min, 4 °C) and resuspended with 10 % skim milk solution (20 ml/L culture). This suspension was then frozen at -80 °C and freeze-dried for 3 days. This freeze-dried biomass was stored at –20 °C. Average cell counts for *Enterococcus faecalis* BFE 1071 and *Enterococcus faecalis* BFE 1071/79 (-) were 3.6×10^{11} and 1.9×10^{11} CFU/g, respectively with end cell counts after being mixed with diet Altromin C100 (each 1%) were 3.6×10^{9} and 1.9×10^{9} CFU/g, respectively.

2.2.6.3 Isolation of bacteria isolated from rat faecal samples

Faecal samples were diluted 10 times with reduced brain heart broth and homogenized with a stomacher for 1 min before being stored at -20°C. Samples were subjected to a serial ten-fold dilution series (10^{-1} to 10^{-8}) in quarter-strength Ringer's solution and plated onto selective media using the drop plate method using a μl volume.

Eight bacterial groups were isolated from faecal samples with selective media. Gram-positive anaerobic cocci were isolated on Bacto Azide agar base supplemented with 5% sheep blood clostridia on DRCM agar, Bacteroides and Fusobacteria on Schaedler agar supplemented with 5% horse blood, lactobacilli on Rogosa Agar, enterococci on Kanamycin Aesculin Azide Agar (see section 2.1.3.7.), *Enterobacteriaceae* on VRBD agar, bifidobacteria with Bifidobacteria agar (see section 2.1.3.9.), and a total count was assessed on Columbia agar supplemented with 5% sheep blood. All plates were incubated anaerobically at 37 °C for 3 to 5 days. All counts were done in duplicate.

2.2.6.4 Presumptive identification of lactobacilli and enterococci from rat faeces by phenotypic tests

Two dominant colonies from the Rogosa and Kanamycin Aesculin Azide Agar plates of the highest dilution factor were picked and streaked out onto MRS agar plates (pH 6.5) several times until the single, pure colonies were obtained. This was done from plates for using cell counts from faecal samples of all the animals in each of the four experimental groups. These isolates were kept in cryo vials containing 15% glycerol at –80 °C for further identification. Phenotypic tests for the presumptive identification of strains belonging to the genus *Enterococcus,* included determinations of cell morphology by phase contrast microscopy,

catalase test, gas production from glucose, growth in 6.5% NaCl, growth at pH 9.6, and growth at 45 °C. In addition fermentation of the sugars raffinose, mannitol, arabinose and sorbitol was tested to presumptively identify the enterococci to species level. Phenotypic tests for the presumptive identification of strains belonging to the genus *Lactobacillus*, isolates included determination of cell morphology by phase contrast microscopy, catalase test, gas production from glucose, growth at 15 and 45 °C, determination of *meso*-diaminopimelic acid in the cell wall and determination of the enantiomer of lactic acid produced.

2.2.6.5 Genotypic identification

2.2.6.5.1 Random Amplified Polymorphic DNA (RAPD)-PCR fingerprinting

Total genomic DNA from enterococci and lactobacilli were isolated using method from Pitcher et al. (1993). The concentration of this genomic DNA was adjusted to 100 ng/µl in bidest water for use as template in amplification with RAPD-PCR. DNA was amplified in 50 µl volumes containing 100 ng template DNA, 200 µM dNTPs, 50 pM of primer M13, 2.5 U *Taq* DNA polymerase (Amersham Pharmacia, Freiburg, Germany) and 1 x *Taq* polymerase buffer. The concentration of $MgCl_2$ in this buffer was adjusted to 3 mM. The primer sequence and PCR conditions used were: M13 (5'-GAG GGT GGC GGT TCT-3') (Huey and Hall, 1989); 35 cycles of: 94°C for 1 min, 40°C for 20 sec, ramp to 72°C at 0.6°C s^{-1}, 72°C for 2 min.

PCR products were separated by electrophoresis on a 1.8% (w/v) agarose gel at 48 V for 16 h using 1 x TBE buffer (Sambrook et al., 1989). The gels were stained in ethidium bromide and photographed on an UV transilluminator. Photo-positives were digitalized by scanning and scanned images were normalised and subsequently analysed using the Bionumerics (version 2.5) software (Applied Maths, Kortrijk, Belgium). Groupings of the RAPD-PCR fingerprints was performed by means of the Pearson product moment correlation coefficient and the unweighted pair group with mathematical average (UPGMA) clustering algorithm.

2.2.6.5.2 Species-specific PCR

Lactobacillus isolates which were presumptively identified as belonging to the *L. acidophilus* group (catalase-negative, homofermentative, growing at 45 but not at 15°C producing DL-lactate) were subjected to group-specific PCR according to the methods of Roy

et al. (2001) to confirm this presumptive identification. The group specific PCR utilises primers that target variable regions of the 16SrDNA which are specific for the *L. acidophilus* group. The *L. acidophilus* group consists of the closely related species *L. acidophilus, L. crispatus, L. johnsonii, L. gasseri, L. gallinarum* and *L. amylovorus*. The primers used in this study targeted the whole group and individual species can not be discerned. Thus, using primer combination LBL R1 and Lho the species *L. acidophilus, L. gallinarum, L. crispatus* and *L. amylovorus* were amplified. these primers do not amplify the 16S rDNA region of *L. gasseri* and *L. johnsonii*. For specific amplification of the *L. gasseri/johnsonii* species, the primers Lgj/LBL R1 were used. the DNA sequence of these primers is shown below:

Primer LBL R1: 5’ - CCA TGC ACC ACC TGT C-3’
Primer Lgj: 5’- ACA CTA GAC GCA TGT CTA-3’
Primer Lho: 5’- CTG GGA TAC CAC TT-3’

The PCR reaction mixture consisted of 100 ng of total genomic DNA of the test strain as template, 200 µM dNTPs, 25 pMol of each primer, 1 x *Taq* polymerase buffer and 1 U of *Taq* polymerase (Amersham Pharmacia). PCR amplification was done for 35 cycles at the following conditions: 94 °C for1 min, 50 °C for 1 min and 72 °C for 1.5 min for both reactions, i.e with primer LBL R1/Lgj or LBL R1/Lho. PCR products were subjected to electrophoresis in 1.0% agarose gels for 2 hours at 100V in TBE buffer (Sambrook et al., 1989). Thus all strains that were presumptively identified as belonging to the *L. acidophilus* group were subjected to group-specific PCR twice, i.e., once with each different primer combination.

3.0 RESULTS AND DISCUSSION

3.1 Technological and functional properties of potentially probiotic lactic acid bacteria

3.1.1 Technological properties

3.1.1.1 Acid and bile tolerance

Studies on acid resistance and bile tolerance were done *in vitro* to assess the ability of potential probiotic lactic acid bacteria to survive transit the stomach and conditions encountered in the small intestine. These studies were done using fifty-five enterococci strains isolated from food as well as the bacteriocin-producing strains *Enterococcus faecalis* BFE 1071 (produces enterocin 1071), it's mutant *Enterococcus faecalis* BFE 1071/79(-), the enterocin A and B producer *Enterococcus faecium* BFE 900 and the sakacin A producer *L. sakei* Lb706 in the investigation. Thus a large amount of bacteria, mostly enterococci, were tested, in order to compare their acid resistance and bile tolerances relative to the bacteriocin-producing strains, and thus obtain a measure for the latter's ability to survive acid and bile stress. The capability of bacteria to survive acid conditions in the *in vitro* gastrointestinal model of this study were arbitrarily defined as not resistant (survival of <50% of cells), weakly resistant (survival of 50-75% of cells), moderately resistant (survival of 75-90% of cells), and strongly resistant (>90% survival of cells).

With the exception of *E. faecalis* FAIR-E 115 and *E. malodoratus* FAIR-E 169, all strains survived at a level of more than 50% of the initial inoculum in MRS broth at pH 3.0 and may thus be considered acid resistant (Table 7). For *E. faecium* 48,8% (21 of 43 strains), while for *E. faecalis* 46,5% (20 of 43 strains) were strongly resistant to this low pH, respectively. Thus, for just under half of the strains of these two species, more than 90% of cells survived more than one hour's exposure to this low pH. This exemplifies that enterococci are actually quite acid resistant. This feature, together with the heat resistance and salt tolerance of enterococci, is well known for these bacteria and is one of the reasons often cited for the robust nature of these bacteria (Franz et al., 1999; Giraffa et al., 2003). This resistance of low pH was also expected for enterococci, as these bacteria naturally occur in the gastrointestinal tract, or can be transmitted from environmental sources or food sources to the human gastrointestinal tract (Van den Bogaard et al., 1997; Berchieri, 1999; Stobberingh et al., 1999; Gelsomino et al., 2003).

The non-bacteriocin producing, probiotic enterococci candidates from food (see Materials and Methods Table 4) all exhibited either strong (FAIR-E 6, 9, 243, 345, 349, 365) or moderate (FAIR-E 15, 210) acid resistance (Table 7). As future potential probiotic

candidates, these acid resistance point towards the fact that these strains would probably survive stomach passage. In addition, a probiotic control strain *E. faecium* SF68 (deposited in the FAIR-E culture collection as FAIR-E 24) also showed strong acid resistance. Interestingly, the bacteriocin-producers *E. faecalis* BFE 1071 and *L. sakei* Lb706 showed a weak resistance to low pH, while *E. faecium* BFE 900 and the *E. faecalis* BFE 1071/79(-) showed a strong resistance to low pH (Table 7). Nevertheless, this means that these bacteriocin-producing strains could all be expected to survive the low pH conditions of the stomach to some extent in an animal feeding experiment. It is unclear why the *E. faecalis* mutant was more acid resistance than the wild-type strain. Speculatively, loss of the plasmid in the mutant may be energetically favourable for the mutant, in that it does not need to spend energy in plasmid replication functions and bacteriocin production which require ATP. This may mean, that the strain is more capable to withstand adverse conditions and can spend energy in protection of the cell by increased production of acid shock proteins involved in the stress response (Abee and Wouters, 1999).

Table 7: Survival of enterococci strains in presence of hydrochloric acid at pH 3.0.

Strains	Acid tolerance			
	not resistant	weak resistance	Moderate resistance	strong resistance
E. durans	-	FAIR-E140, 357	FAIR-E1, 231, 251, 364	FAIR-E2, 326, 332, 373
E. faecium	-	FAIR-E41, 118, 119, 121, 128, 131, 132, 133, 401, 402, 403	FAIR-E13, 14, 15, 80, 129, 130, 189, 210, 396, E398, 399	FAIR-E3, 6, 9, 24, 38, 40, 83, 120, 134, 135, 137, 171, 243, 345, 349, 365, 394, 395, 397, 400, BFE 900
E. faecalis	FAIR-E115	FAIR-E82, 109, 111, 112, 116, 183, 186, 188, 193, 224, 235, 236, BFE 1071	FAIR-E39, 69, 124, 125, 185, 191, 302, 307, 313	FAIR-E71, 88, 108, 113, 114, 117, 122, 123, 184, 190, 192, 237, 279, 292, 315, 339, 363, 385, 404, BFE 1071/79(-)
Others	*E. malodoratus* FAIR-E169	*L. sakei* Lb 706	*E. casseliflavus* FAIR-E230	-

The bile tolerance and tolerance to duodenum secrete of enterococci is shown in Table 8. This tolerance was assessed by viable counts after 2 hours of anaerobic incubation following bile and duodenum secrete addition. A strong bile and duodenum secrete tolerance in this study was arbitrarily defined as survival of more than 5% of cells of the initial inoculum, while a moderate tolerance was defined as 2.5 to 5 % survival of cells. A weak tolerance was defined as survival of 1-2.5% of cells of the initial inoculum and a negative result implied failure to grow after bile and duodenum juice treatment. In contrast to acid resistance (Table 7), only 1 *E. durans* strain, 28 *E. faecium* strains, and 2 *E. faecalis* strains, including the bacteriocin producing *E. faecalis* BFE 1071, were weakly, moderately or strongly tolerant against physiological bile salt and duodenum juice concentrations as used in this study (Table 8). Therefore, *E. faecium* strains seem to be more tolerant to bile and duodenum juice than *E. faecalis* strains.

Table 8: Survival of enterococci strains in presence of physiological concentrations of bile and artificial duodenum secrete in an intestinal tract model.

Strains	Bile and duodenum secrete tolerance			
	negative	weak	Moderate	strong
E. durans	FAIR-E 1, 2, 140, 231, 251, 326, 332, 357, 373	FAIR-E 364	-	-
E. faecium	FAIR-E 3, 24, 40, 41, 83, 121, 130, 131, 132, 134, 135, 137, 189, 400, BFE 900	FAIR-E 38, 129, 133, 396, 401, 402, 403,	FAIR-E 9, 13, 80, 118, 119, 128, 210, 243, 349, 395, 399,	FAIR-E 6, 14, 15, 120, 171, 345, 365, 394, 397, 398,
E. faecalis	FAIR-E 39, 69, 71, 82, 88, 108, 109, 111, 112, 113, 114, 115, 116, 117, 122, 123, 124, 125, 183, 184, 186, 188, 190, 191, 192, 193, 224, 235, 236, 237, 279, 292, 302, 307, 313, 315, 339, 363, 385, 404, BFE 1071/79(-)	FAIR-E185, BFE 1071	-	-
Others	*E. malodoratus* FAIR-E169, *E. casseliflavus* FAIR-E230, *Lactobacillus sakei* Lb 706	-	-	-

Bile and small intestine secrete tolerance:
> 5% survival : +++ (strong)
2.5-5% survival : ++ (moderate
1-2.5% survival : + (weak)

Thus 23.3% of *E. faecium* strains in this study showed a strong tolerance to bile and duodenum secrete at the conditions used in this study. These strains, including the two weakly tolerant *E. faecalis* strains BFE 1071 and FAIR-E 185 thus have desirable properties for probiotics from a technical point of view. The other potential future *E. faecium* probiotic candidates, which do not produce bacteriocin (see Table 4), all showed either a moderate (FAIR-E 9, 210, 243, 349) or strong (FAIR-E 6, 15, 345, 365) tolerance to bile and duodenum secrete (Table 8). This implies, that these strains are probably well suited to survive conditions in the small intestinal tract.

Once bacteria reach the small intestinal tract, their ability to survive depends on their resistance to bile (Gilliland, 1987). Bile entering the duodenal section of the small intestine was found to reduce survival of bacteria. This is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids which are very susceptible to destruction by bile salts which have detergent characteristics. Hence, the success of a probiotic also depends on the selected strain possessing bile-resistance qualities (Jin et al., 1998).

However, the bile concentration used in this study was very high, i.e. 10%. Although this was a physiological concentration, bile in the gastrointestinal tract is usually secreted following a meal and then concentrations decrease, as the bile is re-absorbed from the small intestine. In other words, the bile concentration in the small intestine does not remain at the constant high concentration as used in this experimental model. Consequently, moderately and highly tolerant enterococci, including the bacteriocin-producer *E. faecalis* BFE 1071 in this study, can be considered to be very tolerant to bile and duodenum secrete and probably would be well equipped to survive actual bile and duodenal secrete stress in the gastrointestinal tract. The failure of other enterococci and bacteriocin-producing strains to survive the bile and duodenum secrete in this study, however, does not imply that these organisms could not survive actual bile and duodenum secrete stress *in vivo*, as the conditions used in the *in vitro* study probably underestimated *in vivo* tolerance. To demonstrate this point, the successful probiotic for human and animal use, *E. faecium* SF 68 (FAIR-E 24), was not resistant to conditions of bile and duodenal secrete used in this study (Table 8). Nevertheless, it is well known that this strain survives gastrointestinal passage and is metabolically active in the gastrointestinal tract of the pig (Havenaar and Huis in ‘t Veld, 1993).

Enterococci are well known to be commensals of the gastrointestinal tract of human and animals, and in this ecological niche, these bacteria come into contact and interact with bile salts. Thus, it is not surprising when enterococci are resistant to bile acid (Franz et al.,

2001). However, probiotic bacteria vary considerably in their levels of bile tolerance. The mechanism of tolerance is not understood and the minimum acceptable level of bile tolerance for a candidate probiotic remains unknown (Klaenhammer and Kullen, 1999). The fact that relatively many enterococci strains were bile tolerant in this study, showed that these strains probably have a high chance to survive in gastrointestinal tract.

3.1.2 Functional properties of potentially probiotic lactic acid bacteria

3.1.2.1 Bacteriocin production

Bacteriocin production of probiotic strains was suggested to be one of the functional traits of probiotics. As this investigation concerned the influence of a bacteriocin-producing strain on the microflora of the rat gastrointestinal tract, we investigated the activity spectra of three potentially probiotic bacteriocin-producing strains against indicator bacteria which typically are associated with this environment.

Activity spectra of bacteriocins produced by *E. faecalis* BFE 1071, it's bacteriocin-negative *E. faecalis* BFE 1071/79(-) (used as negative control), *L. sakei* Lb706 and *E. faecium* BFE 900 are presented in Table 9. *E. faecalis* BFE 1071 showed bacteriocin activity against six *Lactobacillus acidophilus* strains, two strains of *L. crispatus* (20584 and NCFB CHN-1-4) and *L. gallinarum* DSM 10532 and NCFB 2235, as well as *L. gasseri* P79 and *L. fermentum* DSM 20852 (Table 9). As expected, the bacteriocin-negative mutant *Enterococcus faecalis* BFE 1071/79(-) showed no bacteriocin towards any of the indicators. *Enterococcus faecium* BFE 900 showed antimicrobial activity only against *L. gallinarum* NCFB 2235, while *L. sakei* Lb706 inhibited *L. acidophilus* NCFB 1417 and NCFB 104 (Table 9).

Enterococcus faecalis BFE 1071 produces 2 antimicrobial peptides called enterocin 1071A and enterocin 1071B. These peptides are heat resistant (100 °C, 60 min), remain active after 30 min of incubation at pH 3 to 12, and are sensitive to treatment with proteolytic enzymes, namely to α-chymotrypsin, papain, pepsin, pronase, proteinase K, and trypsin (Balla et al., 2000). In this study *E. faecalis* BFE 1071 inhibited lactobacilli that are typically associated with the gastrointesinal tract, i.e. *L. acidophilus, L. crispatus, L. gasseri, L. gallinarum,* and *Lactobacillus fermentum* strains (Reuter, 2001). In addition, Balla et al. (2000) showed that *E. faecalis* BFE 1071 inhibited the growth of selected *Enterococcus* spp., *Lactobacillus salivarius* subsp. *salivarius*, and a few other Gram-positive bacteria such as, ,*Peptostreptococcus aerogenes, Streptococcus agalactiae, Listeria innocua* and *Clostridium tyrobutyricum.*

Table 9: Activity spectra of bacteriocins from *E. faecalis* BFE 1071, *L. sakei* Lb 706, and *E. faecium* BFE 900. The bac$^-$ negative mutant *E. faecalis* BFE 1071/79(-) served as negative control, and showed no bacteriocin antagonism against any of the indicator strains.

Indicator bacteria	Diameter of inhibition zone (mm) produced by bacteriocin-producing strain		
	*E. faecalis*BFE 1071	*E. faecium* BFE 900	*L. sakei* Lb706
L. acidophilus DSM 20242	-	-	-
L. acidophilus NCFB 1360	5	-	-
L. acidophilus NCFB 1417	5	-	3
L. acidophilus NCFB 2261	-	-	-
L. acidophilus NCFB 2658	-	-	-
Lb. acidophilus NCFB 2660	4	-	-
Lb. acidophilus NCFB 2662	-	-	-
Lb. acidophilus NCFB 2663	-	-	-
Lb. acidophilus NCFB 2473	4	-	-
Lb. acidophilus NCFB 2745	-	-	-
Lb. acidophilus NCFB 2471	3	-	-
Lb. acidophilus NCFB 104	7	-	7
Lb. rhamnosus DSM 20245	-	-	-
Lb. rhamnosus DSM 20711	-	-	-
Lb. rhamnosus DSM 8745	-	-	-
Lb. rhamnosus DSM 20247	-	-	-
Lb. reuteri DSM 20053	-	-	-
Lb. reuteri DSM 20016	-	-	-
Lb. reuteri DSM 20015	-	-	-
Lb. crispatus NCFB 2752	-	-	-
Lb. crispatus 20584	-	-	-
Lb. crispatus NCFB CHN-1-4	6	-	-
Lb. gasseri DSM 28077	-	-	-
Lb. gasseri P79	3	-	-
Lb. gallinarum T50	-	-	-
Lb. gallinarum DSM 10532	7	-	-
Lb. gallinarum NCFB 2235	7	6	-
Lb. amylovorus NRRLB 4540	-	-	-
Lb. fermentum DSM 20852	6	-	-
Lb. salivarius NCFB 15555	-	-	-

Enterococcus faecium BFE 900, which produces enterocins A and B, suppressed only the growth of *Lactobacillus gallinarum* (Table 9). However, in the study of Franz et al. (1999) *E. faecium* BFE 900 was antagonistic towards *L. sakei, Clostridium butyricum,* enterococci and *Listeria* spp. including *Listeria monocytogenes.* The sakacin A producer *L. sakei* Lb706 in this study inhibited the growth of only two *L. acidophilus* strains. Thus the antimicrobial activity spectra of *L. sakei* Lb706 and *E. faecium* BFE 900 were very narrow towards the selected indicator strains that can be regarded as typical of the intestinal flora. In contrast, the enterocin 1071 producer *E. faecalis* BFE 1071 showed antimicrobial activity against a wider

range of indicator bacteria. This may be a reflection of the fact that *E. faecalis* BFE 1071 was isolated from an intestinal environment, while *E. faecium* BFE 900 and *L. sakei* Lb706 were isolated from food sources (Axelsson and Holck, 1995; Franz et al., 1999; Balla et al., 2000). Because *E. faecalis* BFE 1071 showed a relatively wide antimicrobial activity spectrum against lactobacilli strains typical of the gastrointestinal tract this bacteriocin-producer would probably be more suitable for studies regarding the influence of bacteriocin-activity on the gastrointestinal microflora in an animal model.

3.1.2.2 Bile salt hydrolase activity

3.1.2.2.1 Detection of BSH activity among probiotic candidates as well as food and clinical enterococci strains

When the potentially probiotic strains and other enterococci strains from food and clinical sources were spotted onto MRS agar plates supplemented with the sodium salt of taurodeoxycholic acid and $CaCl_2$, a white zone of precipitation occurred when these strains were BSH-positive (Figure 4).

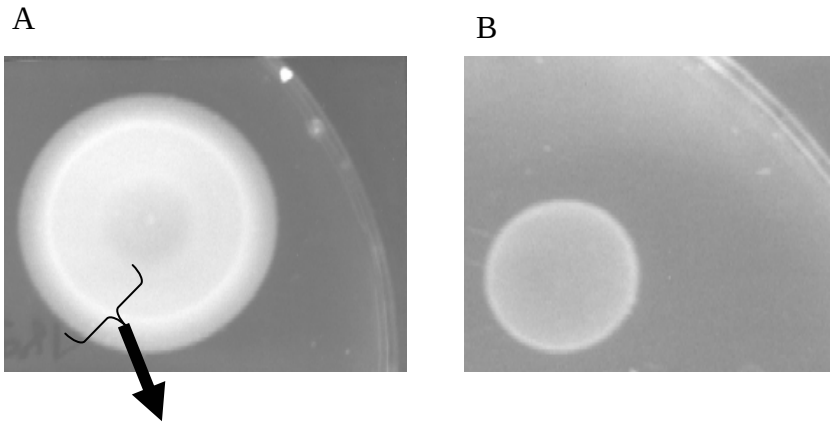


Figure 4: Example of bile salt hydrolase activity showed by BSH-positive *E. faecium* FAIR-E 189 (A) and negative control *E. faecium* E 179 (B).

Using this plate screening assay, 43 *E. faecalis*, 43 *E. faecium* and 10 *E. durans* strains and one each strain of *E. malodoratus* and *E. casseliflavus* from food, human faeces and clinical sources were screened for BSH activity, in addition to the potentially probiotic and bacteriocin-producing strains *E. faecalis* BFE 1071, *E. faecium* BFE 900 and *L. sakei* Lb706

(Table 10). This wide selection of strains was chosen to determine the incidence of BSH activity among enterococci.

Table 10: Detection of bile salt hydrolase (BSH) activity among enterococci strains.

Strain	Bile salt hydrolase activity[a]	
	positive	negative
Enterococcus durans	FAIR-E1, 140, 231, 326, 332,	FAIR-E2, 251, 357, 364, 373
Enterococcus faecalis	FAIR-E69, 71, 82, 88, ***111***, ***115***, ***117***, **123**, **124**, **125**, 186, ***190***, ***191***, ***192***, ***193***, 224, 235, 236, 237, 279, 292, 302, 307, 313, 315, 339, 363, 385, 404	FAIR-E***39***, ***108***, ***109***, ***112***, ***113***, ***114***, ***116***, **122**, ***183***, ***184***, ***185***, ***188***, BFE 1071, BFE 1071/79(-),
Enterococcus faecium	FAIR-E3, 6, 14, 15, 24, 38, **120**, ***128***, ***129***, **134**, **137**, 171, 189, 345, **394**, **395**, **396**, **397**, **398**, **399**, **400**, **401**, **402**, **403**, BFE 900	FAIR-E9, 13, ***40***, ***41***, 80, 83, **118**, **119**, **121**, ***130***, ***131***, 132, **133**, ***135***, 210, 243, 349, 365
Others	-	*E. malodoratus* FAIR-E169, *E. casseliflavus* FAIR-E230, *L. sakei* Lb 706

[a] strains in bold typeface are human faecal isolates, strains in bold typeface and cursive are clinical strains, other strains of enterococci were food isolates

Strains of the enterococcal species (*E. faecalis*, *E. faecium* and *E. durans*) in this study showed BSH activity, which was not surprising, since enterococci are well known to be commensals of the gastrointestinal tract of humans and animals. In this ecological niche, enterococci come into contact and interact with bile salts (Franz et al., 2001). Among the 43 *E. faecalis* strains, 29 (67.4%) were BSH positive, whereas of the 43 *E. faecium* strains, 25 (58.1%) exhibited BSH activity (Table 10). Five of the 10 *E. durans* strains (50%) were BSH positive. The only *E. malodoratus*, *E. casseliflavus*, and *Lactobacillus sakei* (strains Lb 706) strains tested were BSH negative. From the non-bacteriocin-producing, potential future probiotic candidate enterococci, some were BSH-positive (FAIR-E 6, 15, 345) while others were negative (FAIR-E 9, 210, 243, 349, 365). The control probiotic strain *E. faecium* SF68 (FAIR-E 24) was BSH-positive. Interestingly, of the bacteriocin-producing, potentially probiotic strains investigated in this study, only the *E. faecium* BFE 900 strain isolated from black olives was BSH-positive (Table 10). This may be explained by the fact that this strain may have originally stemmed from a gastrointestinal source and occurred in the olives as a

contaminant. The food enterococcal strains in this study are mainly isolated from cheese. The fact that many of these strains exhibited BSH activity (see Table 10) may be explained by the fact that cheese enterococci isolates are known to originate as faecal contaminants in the dairy environment as result of poor hygienic practices (Litopoulou-Tzanetaki, 1990; López-Diaz et al., 1995).

In contrast, the *E. faecalis* BFE 1071 strain isolated from the faeces of Göttingen mini-pigs was BSH-negative. However, our extended investigation on incidence of BSH activity among human faecal and clinical isolates (see Table 10) showed that BSH-negative enterococci can be isolated from intestinal sources and thus BSH activity probably is not a prerequisite for intestinal survival. The ecological significance of bacterial BSH activity has also been subject to debate. De Smet et al. (1995), suggested that it serves as a detoxification reaction for lactobacilli. However, Moser and Savage (2001) tested a wide spectrum of BSH^+ lactobacilli for their resistance towards bile salt toxicity, and could not support this hypothesis. This led Moser and Savage [13] to suggest that instead of protecting lactobacilli from toxicity of conjugated bile salts, BSH activity may rather be important for the bacteria to grow in and colonise the intestine.

3.1.2.2.2 Hydrophobicity

Testing for the hydrophobicity of bacterial cell surfaces was done to assess their potential for adhesion to intestinal epithelial cells. Based on results from acid and bile salt tolerance, some selected *E. faecium* (28 strains) and *E. faecalis* (25 strains) from food and human faecal sources as well as the bacteriocin-producing probiotic candidates of this study were subjected to hydrophobicity testing using the partitioning test in a watery suspension and n-hexadecane. Tests were done in triplicate and standard deviation of these tests generally was less than 10%, usually around 5%. Hydrophobicity of selected enterococcal strains was arbitrarily defined as weak (0-30% hydrophobicity), moderate (31-60% hydrophobicity), and strong (61-100% hydrophobicity). Most of *E. faecium* strains, including the bacteriocin-producer *E. faecium* BFE 900, showed weak hydrophobicities (26 strains), while one strain each showed a moderate (FAIR-E 41) and strong hydrophobicity (E 118) (Table 11). Among the *E. faecalis* strains, five strains exhibited moderate hydrophobicity, including the bacteriocin producing *E. faecalis* BFE 1071, *E. faecalis* BFE 1071/79(-), FAIR-E113, 184, 185, and 191. The other strains exhibited only weak hydrophobicities. The non-bacteriocin producing, potential probiotic enterococci candidates mostly were weakly hydrophobic (FAIR-E 6, 9, 15, 349) while some strains possessed moderate (FAIR-E 243, 345, 365) or strong (FAIR-E 210)

hydrophobicity (Table 11). The bacteriocin-producer *L. sakei* Lb706 strain was also shown to have moderate hydrophobicity value (Table 11).

Table 11: Hydrophobicity of selected enterococcal strains.

Strains	Hydrophobicity		
	weak (0-30%)	moderate (31-60%)	strong (61-100%)
E. faecium	FAIR-E 6, 9, 15, 24, 38, 40, **119**, **120**, **121**, 128, 129, 130, 131, 132, **133**, **134**, 135, **137**, 189, 349, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, BFE 900	FAIR-E 41, 243, 345, 365	FAIR-E 118, 210
E. faecalis	FAIR-E 39, 108, 109, 111, 114, 115, 116, 117, **122**, **123**, **124**, **125**, 183, 185, 186, 188, 190, 192, 193	FAIR-E 113, 184, 191, BFE 1071, BFE 1071/79(-)	-
Others	-	*L. sakei* Lb 706	-

[a] strains in bold typeface are human faecal isolates, strains in bold typeface and cursive are clinical strains, other strains of enterococci were food isolates

Bacterial adhesion has been interpreted in terms of hydrophobicity. Some authors have also indicated an influence of the electrical charges of bacteria and solid surface on adhesion (van Loosdrecht et al., 1987). Hydrophobicity is directly related to concentration of carbon in hydrocarbon form and inversely related to oxygen concentration or to the nitrogen/phosphate ratio (Mozes et al., 1988). Greater hydrophobicity of cells is postulated in greater attractive forces and higher levels of adhesion (Marin et al., 1997).

The hydrophobicity of bacteria can be determined with contact angle method (van Loosdrecht et al., 1987), partitioning bacteria between two aqueous phases (between polyethylene glycol and a dextran phase), or by partitioning bacteria into either the aqueous or organic phase. For example using a aqueous and n-hexadecane solution in hydrophobicity testing bacteria would adhere to droplets of organic solvents (n-hexadecane) if their surface is hydrophobic (Rosenberg, 1984). Similar to our study, a hexadecane adherence assay was applied by Greene at al. (1992) to measure hydrophobicity of *Staphylococcus intermedius* and *S. hyicus* isolates, Ding and Lammler (1992) to study hydrophobicity of *Actinomyces pyogenes* strains and Del Re et al. (2000) who studied adhesion, autoaggregation and hydrophobicity of 13 probiotic strains of *Bifidobacterium longum.*

Twenty six of the twenty eight *Enterococcus faecium* strains in this study (92.9%) exhibit only weak hydrophobicity, as well as 19 of 24 strains of *Enterococcus faecalis*

(79.2%). This indicated, that enterococci in general do not have a high hydrophobicity and may indicate , that based on this characteristic, they may not have high adhesion capacity. Nevertheless, 5 of 24 strains of *E. faecalis* (20.8%) exhibited moderate hydrophobicity (Table 11). In contrast Del Re et al. (2000) found a high hydrophobicity potential among 13 strains of *Bifidobacterium longum* and thus this genus may have a higher capacity to adhere to gastrointestinal cells when compared to enterococci.. Similarly, among 42 *Actinomyces pyogenes* strains, Ding and Lämmler (1992) found that 60% of cells were strongly hydrophobic, while 40% showed a weak or moderate hydrophobicity as defined using their criteria.

The bacteriocin-producing probiotic candidates *E. faecalis* BFE 1071 and *L. sakei* Lb706 showed a moderate hydrophobicity value in this study which may indicate that these bacteria would be better suited for adhesion to intestinal cells in vivo than the bacteriocin-producer *E. faecium* BFE 900. A high cell surface hydrophobicity is considered an advantage in the colonisation of mucosal surfaces Ljungh and Wadstöm (1982). However, specific surface structures called adhesins may also play an important role in adhesion of bacteria to epithelial cells. Such adhesins for enterococci, for instance, include surface proteins such as aggregation substance, enterococcal surface protein, endocarditis antigen and adhesin to collagen from *E. faecalis*, which are also known virulence traits (Franz et al., 1999). In addition enterococci were shown to contain ligands involved in binding that contain D-glucose and D-mannose (Guzmàn et al., 1991). For this reason, hydrophobicity testing was only considered as an indication of binding capacity, and the probiotic candidates were also investigated for their binding capability in tissue culture, using the polarised, mucus-secreting and enterocyte-like HT-29 MTX cell line. This cell line is the closest mimick of gastrointestinal enterocytes currently employed in tissue culture experimentation.

3.1.2.2.3 Adhesion to HT29 MTX-cell line

Adherence of probiotic bacteria to enterocytes could confer a competitive advantage in the gut ecosystem (Holzapfel et al. 1998). This may also be an important technological property of these bacteria, as it may increase their residence time in the gastrointestinal tract. In this study, we used the human intestinal epithelial, mucus-secreting cell line HT29-MTX. The use of this cell line is thought to represent the *in vivo* situation closely (Holzapfel et al., 1998).

Adherance to HT29-MTX cells was studied only for selected enterococci probiotic candidates, including the bacteriocin-producers *E. faecium* BFE 900 and *E. faecalis* BFE

1071. The bacteriocin-producer *L. sakei* Lb706 was not included as at this stage of the study, as it was unlikely that this strain would be chosen as a probiotic candidate for animal experiments or future development, because the strain was from non-intestinal origin, is not usually associated with the gastrointestinal tract, was not bile tolerant, was BSH-negative and did not show a broad range of bacteriocin-activity against lactobacilli typical of the gastrointestinal tract. The other enterococci tested included non-bacteriocin producing food isolates (see Table 4) which in a previous EU study showed non-virulent characteristics like absence of plasmid DNA, absence of vancomycin resistance and gelatinase activity in a plate screening assay,a s well as a probiotic control strain *E. faecium* SF68 (FAIR-E 24). The enterococci were capable of adhering to HT29-MTX cells, however at a low level of generally less than 30% (Fig. 5). Bacteriocin-producing *Enterococcus faecium* BFE 900 had the lowest level of adherence (3%) whilst *E. faecium* FAIR-E 15 the highest (31%). *E. faecium* FAIR-E6, E 210 and E 243 exhibited moderate adherence values (27%, 26%, and 28%, respectively. The Enterocin 1071-producing strain *E. faecalis* BFE 1071 also exhibited a moderate to high adherence of 17% when compared to the other enterococci cells. The probiotic control strain *E. faecium* SF68 did not show high (<15%) adherence to HT29 MTX cells.

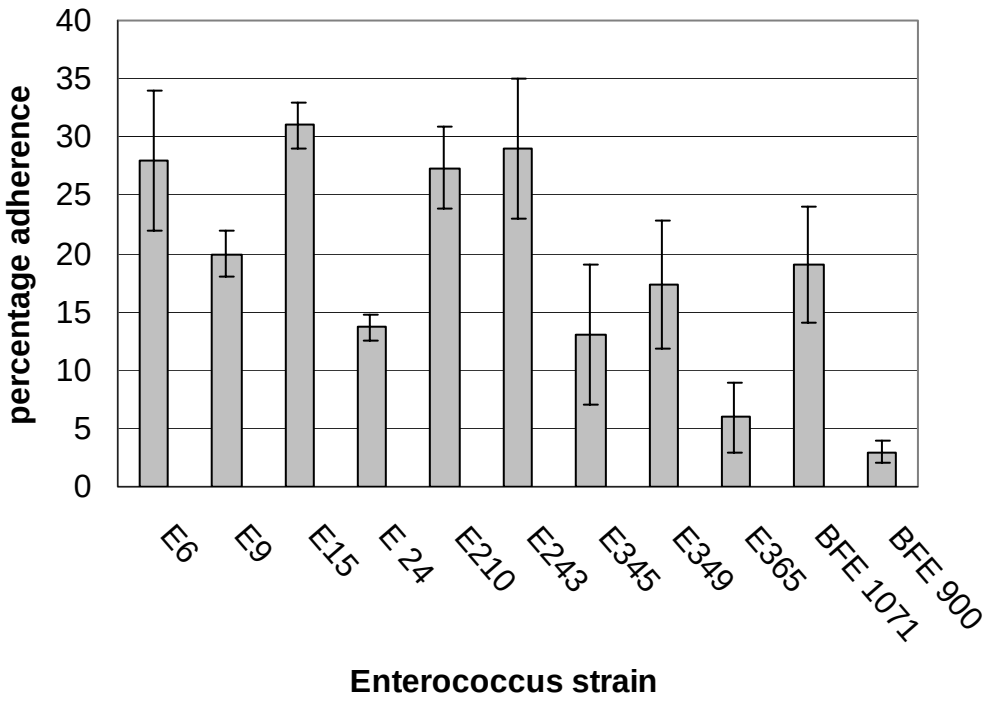


Fig. 5: Percentage adherence of enterococci probiotic candidates to HT29-MTX-cells in tissue culture

It is generally believed that adherence to mucosal surfaces contributes to the efficacy of a probiotic strain, since adherent strains could confer a competitive advantage, which is important for bacterial maintenance in the gastrointestinal tract. By blocking the atachment sites, probiotics might contribute to the prevention of infection by pathogenic microorganisms (Holzapfel et al., 1998).

Adhesion of probiotic candidate enterococci to HT29 MTX-cell line was shown to be at low level (between 3 and 31% adherence values). By contrast, it was shown that *L. acidophilus* and *L. rhamnosus* strains adhere in relatively high numbers and are able to prevent attachment pathogenic microorganisms such as *Salmonella typhimurium, Yersinia enterocolitica,* and enteropathogenic *E. coli* (Coconnier et al., 1993; Bernet et al., 1994; Hudault et al., 1997). However, other probiotic strains such as *E. faecium* SF68 are known not to adhere strongly in the gastrointestinal tract, and were shown in animal experiments and with human volunteers to be washed out after 1 to 2 weeks after cessation of probiotic treatment (Havenaar and Huis in't Veld, 1993; Vael and Goossens, 2002). This, however, does not imply that the cells are not metabolically active and it is thought that the probiotic activity is still sustained even though the strain cannot adhere effectively (Havenaar and Huis in 't Veld, 1993). Although adherence to such cell line may indicate the possibility of a strain to adhere *in vivo*, it does not always mean that these enterococci could not have long residence time in the gastrointestinal tract even when they do not adhere effectively. Havenaar and Huis in 'Ät Veld (1993) and Vael and Goossens (2002) showed that *E. faecium* SF68 could still be detected in the faeces at least one week after stopping probiotic treatment. It is important to note that results from such studies may not be predictive of the actual *in vivo* situation.

The studies on technological and functional probiotic candidates are summarised in Table 12. All of the non-bacteriocin producing enterofcocci probiotic candidates appear to be suited for development of probiotics, as they have good technological characteristics (mostly resistant to acid and tolerant to bile and duodenum secrete). The adhesion capability as judged by their hydrpophobicity and adhesion to HT-29 cells differs somewhat (see Table 12) but strong adherance to gastrointestinal cells may not necessarily be considered as a absolutely required characteristic for a probiotic strain. Some strains were bile salt hydrolase positive, although this characteristic is considered to be a probiotic trait the potential risks associated with BSH activity, i.e. conversion of primary to secondary bile salt in the intestine, which are procarcinogenic (Marteau et al., 1995), should be kept in mind. Among the bacteriocin-

producers, *E. faecalis* BFE 1071 showed the greatest promise for use as a probiotic candidate, also for studying the effects of this strain on the autochthonous flora of an experimental animal. This is because the strain is probably well adapted to survive gastrointestinal conditions, as it was isolated from faeces. Furthermore, the findings of this study show that this strain indeed was relatively acid resistant and tolerant to bile and duodenum secrete. Furthermore, this strain showed bacteriocin-activity against a broad range of typical gastrointestinal lactobacilli. As for the enterococci isolates the strain did, however, not show a high adherence capability. Nevertheless this strain appeared to be most suited for studying the effect of a bacteriocin-producer on the microflora of rats in a model study and was chosen for this purpose (see below).

Table 12: Summary of technological and probiotic properties of candidate probiotic strains investigated in this study.

Strain	**Acid resistance**	**Tolerance to bile and duodenum secrete**	**Bile salt hydrolase activity**	**hydro-phobicity**	**Adherence (%) to HT29 MTX cells (average of 3 trials)**	**Bacteriocin activity spectrum against typical intestinal lactobacilli**
Non-bacteriocin producing probiotic enterococci candidates						
FAIR-E 6	strong	strong	+	weak	28	-
FAIR-E 9	strong	moderate	-	weak	20	-
FAIR-E 15	moderate	strong	+	weak	31	-
FAIR-E 210	moderate	moderate	-	strong	27	-
FAIR-E 243	strong	moderate	-	medium	29	-
FAIR-E 345	strong	strong	+	medium	13	-
FAIR-E 349	strong	moderate	-	weak	17	-
FAIR E 365	strong	strong	-	medium	6	-
Bacteriocin-producing probiotic candidates						
E. faecalis BFE 1071	weak	weak	-	moderate	19	broad
E. faecium BFE 900	strong	negative	+	weak	3	narrow
L. sakei Lb706	weak	negative	-	moderate	n.d.	narrow

n.d. = not determined

Because of the asociation of enterococci with human disease, it was considered important to investigate the safety of the non-bacteriocin producing enterococci candidates and strain *E. faecalis* BFE 1071. In addition, it was considered necessary to investigate the virulence potential of *E. faecalis* BFE 1071 before use in an animal experiments.

3.2 Investigation into the safety of potentially probiotic enterococci

3.2.1 Antibiotic resistance

The antibiotic resistance of non-bacteriocin producing, potentially probiotic *E. faecium* strains, as well as the bacteriocin-producing *E. faecalis* BFE 1071 was investigated using the E-test (Viva Diagnostika, Cologne, Germany). The results of antibiotic resistance tests are shown in Table 13.

Table 13: Minimum inhibitory concentrations for inhibition of probiotic enterococci candidates with antibiotics ampicillin (Amp), benzylpenicillin (Bpen), Chloramphenicol (Chloram), Tetracycline (Tet), Erythromycin (Eryth), Ciprofloxacin (Cipro), Streptomycin (Strep) and Gentamicin (Gent). Resistances[a] are indicated by shaded boxes.

Strain	Amp	Bpen	Chloram	Tet	Eryth	Cipro	Strep	Gent
FAIR-E 6	0.125	0.5	8.0	0.75	4.0	6.0	48.0	24.0
FAIR-E 9	0.5	>32.0	6.0	0.25	3.0	0.25	192.0	32.0
FAIR-E 15	1.0	16.0	16.0	1.5	6.0	2.0	64.0	8.0
FAIR-E 210	1.5	16.0	24.0	1.0	32.0	0.5	48.0	24.0
FAIR-E 243	0.125	4.0	6.0	0.75	1.0	6.0	64.0	32.0
FAIR-E 345	0.190	0.38	8.0	0.5	>256.0	6.0	32.0	24.0
FAIR-E 349	0.5	0.75	12.0	0.5	24.0	1.5	32.0	8.0
FAIR-E 365	2.0	>32.0	8.0	0.75	4.0	0.38	128.0	8.0
E. faecalis BFE 1071	0.5	2.0	6.0	0.75	0.75	0.5	192	12.0

[a] Resistance interpreted on the basis of minimum inhibitory concentration values for enterococci supplied in 'Performance standards for antimicrobial susceptibility testing', ninth informal supplement, National Committee for Clinical laboratory standards (NCCLS) Vol. 19 (1) from 1999. Minimum inhibitory concentration values for ampicillin, ≥16 μg/ml; penicillin, ≥16 μg/ml; chloramphenicol ≥32 μg/ml; tetracycline, ≥16 μg/ml; erythromycin ≥8 μg/ml; ciprofloxacin ≥4 μg/ml; highlevel streptomycin >1000 μg/ml and high level gentamycin >500 μg/ml.

All of the strains, except the bacteriocin-producer *E. faecalis* BFE 1071 exhibited resistance to at least one of the antibiotics tested (Table 13). *E. faecium* strains E 210 and E 345 even contained resistance towards two antibiotics, i.e. penicillin and erythromycin, and erythromycin and ciprofloxacin, respectively. However, multiple antibiotic resistances among enterococci are quite common and can be explained by the fact that enterococci are naturally (intrinsically) resistant towards a variety of antibiotics (Murray, 1990, Franz et al., 1999). The

EU Scientific Committee on Animal Nutrition (SCAN) has been called by the EU to provide guidelines to regulate bacteria used in feeds (i.e. probiotic bacteria, starter bacteria) and according to the SCAN guidelines, these should not contain any acquired antibiotic resistances (SCAN, 2002). Such strains can, however, possess intrinsic resistances, when it can be shown that these are not transferable. For the enterococci, resistance to ampicillin, streptomycin, gentamicin, chloramphenicol, tetracycline, and erythromycin are considered to be acquired resistances. Thus the strains above (FAIR-E 9, 15, 210, 345, 349 and 365) containing resistances to some of these antibiotics (Table 13) would be difficult to develop as an animal probiotic strain. Although the EU has not yet established similar guidelines for human probiotics, this is expected to follow soon. Thus realistically, the only strains with a potential for future probiotic development that are considered safe from an antibiotic resistance point of view, would be the bacteriocin-producer *E. faecalis* BFE 1071 and the non-bacteriocin-producing *E. faecium* strains FAIR-E 6 and FAIR-E 243 (Table 13). Although the latter strains exhibit ciprofloxacin resistance, this trait is considered to be intrinsic to some enterococci strains.

3.2.2 Physiological investigation of virulence potential

The non-bacteriocin producing *E. faecium* probiotic candidates and bacteriocin-producing strains *E. faecalis* BFE 1071 were investigated using physiological tests for their virulence potential. This included investigations for production of DNAse, haemolysin, gelatinase and mucin degradation activity, as determined on agar plates, as well as testing for production of aggregation substance, as determined using a clumping assay. In addition, the strain were assessed for their ability to bind extracellular matrix proteins. The results for testing of these virulence determinants are shown in Table 14 and 15.

Table 14: Physiological testing for virulence of probiotic enterococci candidates.

Enterococci strains	DNAse	Gelatinase	Aggregation substance	Hemolysin	Mucin Degradation
FAIR-E6	-	-	-	-	-
FAIR-E-9	-	-	-	-	-
FAIR-E-15	-	-	-	-	-
FAIR-E-210	-	-	-	-	+
FAIR-E-243	-	-	-	-	-
FAIR-E-345	-	-	-	-	-
FAIR-E-349	-	-	-	-	-
FAIR-E-365	-	-	-	-	-
E. faecalis BFE 1071	-	-	-	-	-

None of the nine strains produced DNAse, gelatinase, aggregation substance and hemolysin, which are important virulence traits of enterococci associated with human infection. Only one strain, *E. faecium* FAIR-E 210 hydrolysed mucin, which can be considered as a potential safety concern. In addition, this strain also was resistant to penicillin and erythromycin (Table 13) and would thus probably not be suitable for further development as probiotic strain.

Table 15: Binding of potential probiotic enterococci to extracellular matrix proteins.

strains	Rat collagen I (%)	Human fibrinogen (%)	Human fibronectin (%)
FAIR-E 6	25	0	32
FAIR E 9	21	27	31
FAIR E 15	25	16	0
FAIR E 210	18	48	14
FAIR E 243	65	59	57
FAIR E 345	84	28	35
FAIR E 349	88	46	35
FAIR E 365	84	4	48

All eight non-bacteriocin producing enterococci strains did not bind well to extracellular matrix (ECM) proteins, as binding to these was generally lower than 50% (Table 15). However, some strains bound well to specific ECM proteins (especially rat collagen I), indicating that this may be a potential virulence trait, should enterococci be able to reach the ECM. The extracellular matrix (ECM) is a stable macromolecular structure underlying epithelial and endothelial cells and surrounding connective tissue cells (Westerlund and Korhonen, 1993). The ECM underlying the cells comprises several collagens, fibronectin, laminin, vitronectin, thrombospondin and a number of glycosaminoglycans. Bacteria may come into contact with the ECM if this is exposed, as occurs in wounds, or if the bacteria have tissue-invasive capability or produce toxins or proteases (Ljungh and Wadström, 1996). Specific binding to an ECM component may lead to colonisation and possibly the development of an infection. Thus the relative importance of binding of enterococci probiotic candidates to ECM proteins should be seen in connection of presence of their other virulence factors such as invasins, proteases or toxins, as it would not be expected that binding of ECM proteins would play a role if such factors are not present. For this reason, the presence of virulence genes for production of the adhesins enterococcal surface protein (Esp) and the

adhesin to collagen from *E. faecalis* (Ace) and invasins (aggregation substancewas also investigated on the molecular biological level.

3.2.3 Molecular biological investigation into potentially probiotic enterococci virulence factors

The presence of genes for the virulence factor enterococcal surface protein (Esp) and adhesin to collagen from *E. faecalis* (Ace) was investigated by PCR amplification of these genes. The results for the PCR amplification for Ace and Esp are shown in Fig. 6.

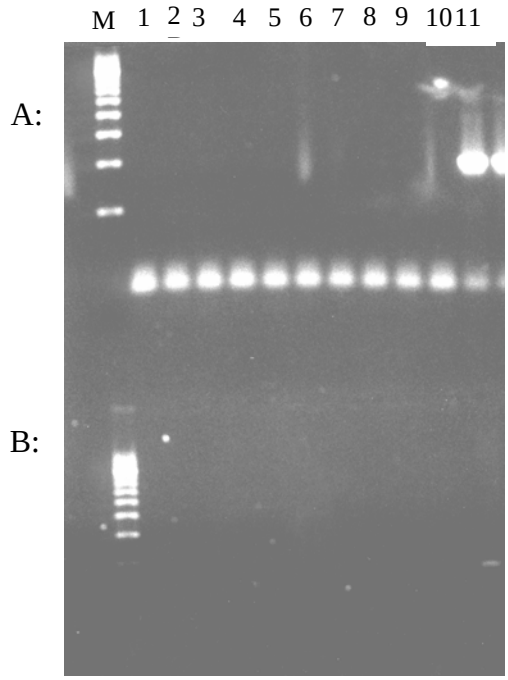


Fig. 6: PCR amplification of Ace (A) and Esp (B). Lane 1, *faecium* E 6, Lane 2, E 9, lane 3, E 15, lane 4, E 210, lane 5, E 243, lane 6, E 345, lane 7, E 349, lane 8, E 365, lane 9, BFE 1071, lane 10 BFE 1071/79(-), lane 11, positive conrol *E. faecalis* E 69 (A) or *E. faecalis* V594 (B). M: 500 bp marker (Biorad).

None of the strains produced either Ace or Esp. The gene could however, be amplified at correct size from the DNA of control strains (see Fig. 6)..

In general, the strains of potential probiotic enterococci appeared to be clear of virulence determinants. Production of virulence determinants appears to be common among enterococci from clinical and food sources, especially in *E. faeclis* strains. Franz et al., 2001 investigated 47 *E. faecalis* strains from food and showed that 78.7% of food isolates produced one or more

virulence determinants (AS, Cyl, Esp or gelatinase). Many of the *E. faecalis* strains tested produced multiple virulence determinants. In contrast, only 10.4% of the food *E. faecium* strains produced one or more of these virulence determinants. Eaton and Gasson (2001) also reported a much higher incidence of virulence determinants from food enterococcal strains for *E. faecalis* strains, when compared to *E. faecium* strains. Eaton and Gasson (2001) reported the incidence of virulence factors (aggregation substance, Esp, Ace, gelatinase) to be highest among clinical isolates, followed by food isolates and then probiotic isolates. The absence of virulence factors of enterococci in this study was comparable to that reported for other probiotic strains (Eaton and Gasson, 2001) and is considered as an advantage and prequisite for the development of these strains as future probiotics.

Based on the ability of *E. faecalis* BFE 1071 to resist acid and tolerate bile salt and duodenum secrete, it's gastrointestinal origin, it's ability to produce bacteriocin with activity against lactobacilli which are typically associated with the gastrointestinal tract, the strain was chosen as the probiotic candidate for use in animal experiments. *E. faecalis* BFE 1071 did not possess BSH activity, which may be a positive characteristic when considering that there is some debate about the value of bile salt hydrolase activity of a probiotic strain. This controversy is based on the fact that the primary bile salt which is produced in the deconjugation reaction by BSH-positive bacteria, can be further hydrolysed to secondary bile salts by other intestinal bacteria, and these secondary bile salts may be procarcinogenic.

Nevertheless, it was considered important to study the genetic basis of bile salt hydrolase activity of BSH-positive probiotic enterococci candidates. To date, the BSH gene has never been cloned from an *Enterococcus* strain and the production has not been investigated on a molecular biological level. For this reason, the genetic basis for BSH activity of one of the potentially probiotic enterococci strains, i.e. *E. faecium* FAIR-E 345, was elucidated. One aim of this study was also to determine, whether the gene for BSH activity is plasmid located among BSH-positive enterococci, and can thus be transferrred to other enterococci strains.

3.3 Bile Salt Hydrolase

3.3.1 Cloning of *bsh* gene, nucleotide sequence and amino acid homology

One of approximately 4000 colonies screened after transformation of *E. coli* TOP10 with chromosomal DNA fragments of 2.5-4.0 kbp length expressed the BSH gene, as was demonstrated by the presence of a large zone of precipitation around the colony growing on LB-based differential agar (Fig. 7).

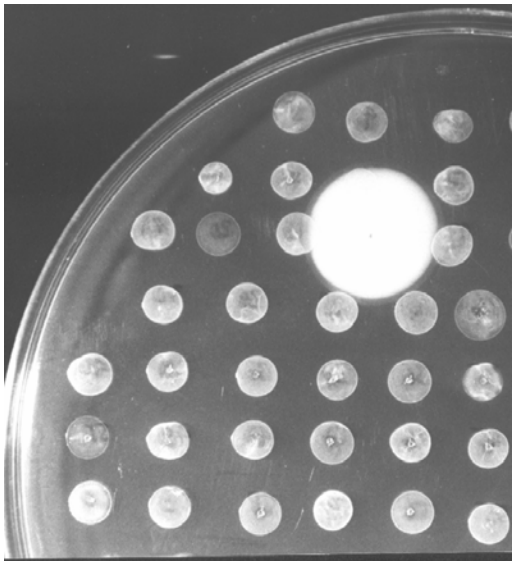


Fig. 7: Photograph showing the appearance of BSH-positive *E. coli* TOP 10 colony on the LB-based differential agar plate. A large zone of precipitation around the colony was evident of BSH activity, while BSH-negative colonies did not show a zone of precipitation.

The insert in the plasmid pAW01 contained by this clone was completely sequenced in both directions. Analysis of the DNA sequence revealed the presence of one open reading frame (ORF) encoding a protein of 324 amino acids with an isoelectric point of 4.877 (Fig. 8). A presumptive ribosome binding site (GGAGGAA) was located 8 bases upstream of the ATG start codon for this ORF. Presumptive –10 (TATAGT) and –35 (TTGATA) promoter sequences were located upstream of this RBS (Fig. 8). A possible terminator with dyad

symmetry occurred 164 bp downstream of the TGA stop codon of this ORF. A databank search indicated that the amino acid sequence deduced from this ORF showed highest homology to that of the BSH protein from *Listeria monocytogenes, Lactobacillus* (*L.*) *plantarum* (68.5 % identity), while lower homologies to BSH proteins reported previously for *L. johnsonii* (52.8 % identity), *L. gasseri* (49.2 % identity), *Clostridium perfringens* (42.2 % identity), *Bifidobacterium longum* (38.2 % identity) and *L. acidophilus* (34.8 % identity) were found (Christiaens et al., 1992; Coleman and Hudson, 1995; Elkins and Savage, 1998; Tanaka et al., 2000; Russell and Klaenhammer, 2001). In addition, The BSH protein from *E. faecium* FAIR-E 345 also showed homology to the penicillin V acylase (PVA) of *Bacillus sphaericus* (32.7 % identity) (Suresh et al., 1999).

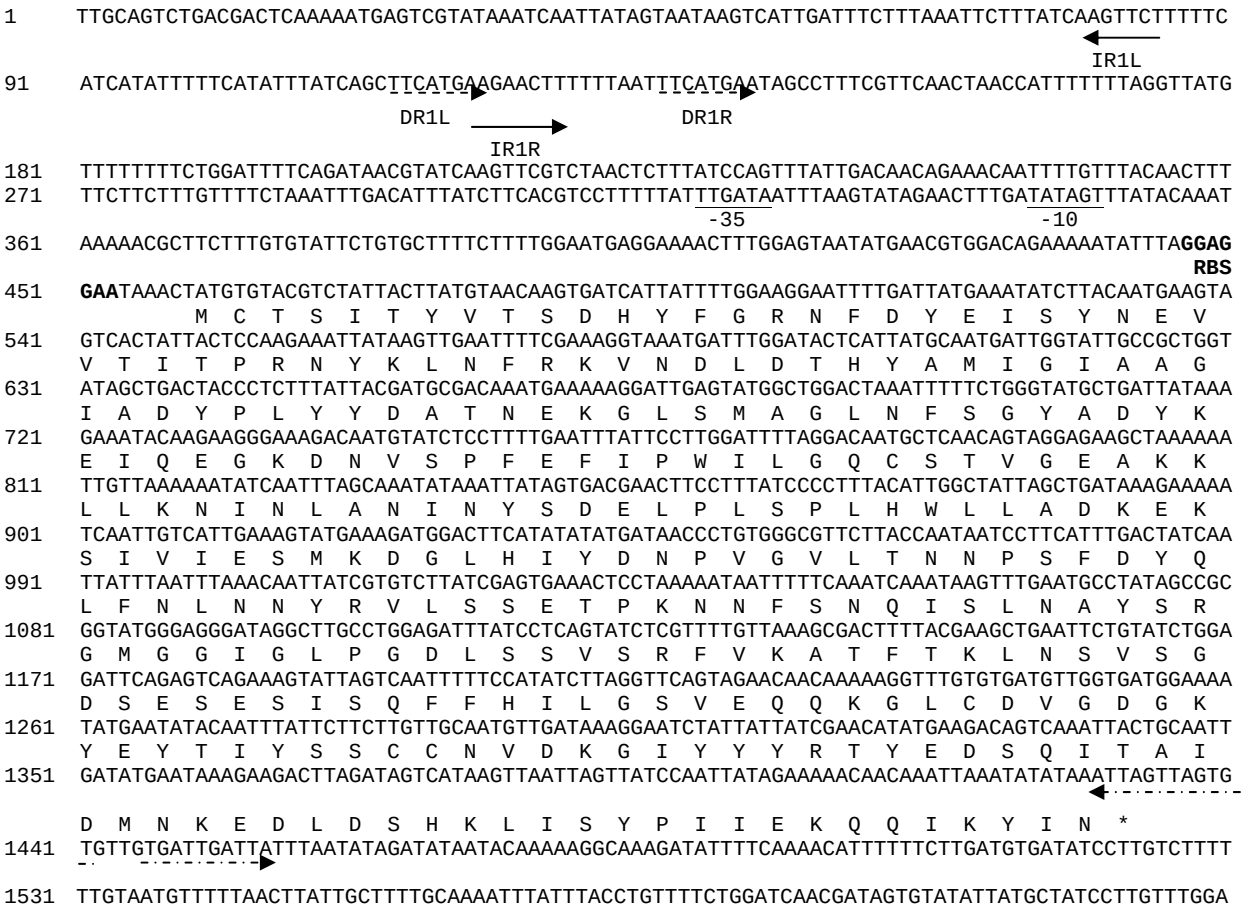


Fig. 8: DNA sequence of *bsh* gene isolated from *E. faecium* FAIR-E 345. The ribosome binding site (RBS) is indicated in bold print, inverted (IR) or direct (DR) repeats are indicated by arrows. L=left, R= right.

The amino acids of the active site of PVA were determined by crystal structure analysis (Suresh et al., 1999) to be Cys-1, Asp-20, Tyr-82, Asn-175 and Arg 228. As pointed out by Tanaka et al. (2000), all of the BSH proteins characterised so far also have these conserved amino acids at these positions, with the exception of Tyr-82, which is replaced by Asn-81 in the BSH proteins. This was also the case for the amino acid sequence for the BSH protein of *E. faecium* FAIR-E 345 as deduced from the nucleotide sequence of the *bsh* gene in this study (Fig. 9). Tanaka et al. (2000) purified the BSH protein and determined that the N-terminal amino acid sequence for purified BSH did not include a Met residue. The authors suggested that the formylmethionine was processed and that the Cys was the first amino acid of the mature protein. This finding was also obtained with PVA, in which Cys-1 plays a central role in the active site (Suresh et al., 1999; Tanaka et al., 2000).

Using site-directed mutagenesis, Tanaka et al. (2000) furthermore showed that replacing Cys-1 with Ala completely abolished BSH activity. The highly conserved Cys residue in all BSH proteins (Fig. 9) confirms the importance of the cysteine in the active site and may imply, that for all the BSH proteins shown aligned in Fig. 9, the formylmethionine is processed and Cys is the first amino acid of the mature BSH protein. If this is the case, the BSH protein from *E. faecium* FAIR-E 345 would consist of 324 amino acids and the protein would have a pI of 4.877.

Multiple alignment of the BSH amino acid sequences (Fig. 9) also showed that there was a considerable degree of heterogeneity among BSH proteins and that long stretches of conserved regions, which may have aided development of PCR primers for detection of BSH genes, did not occur. For this reason, the strategy to use the cloned BSH gene itself as a gene probe to detect the genomic location of BSH genes among enterococci was adopted.

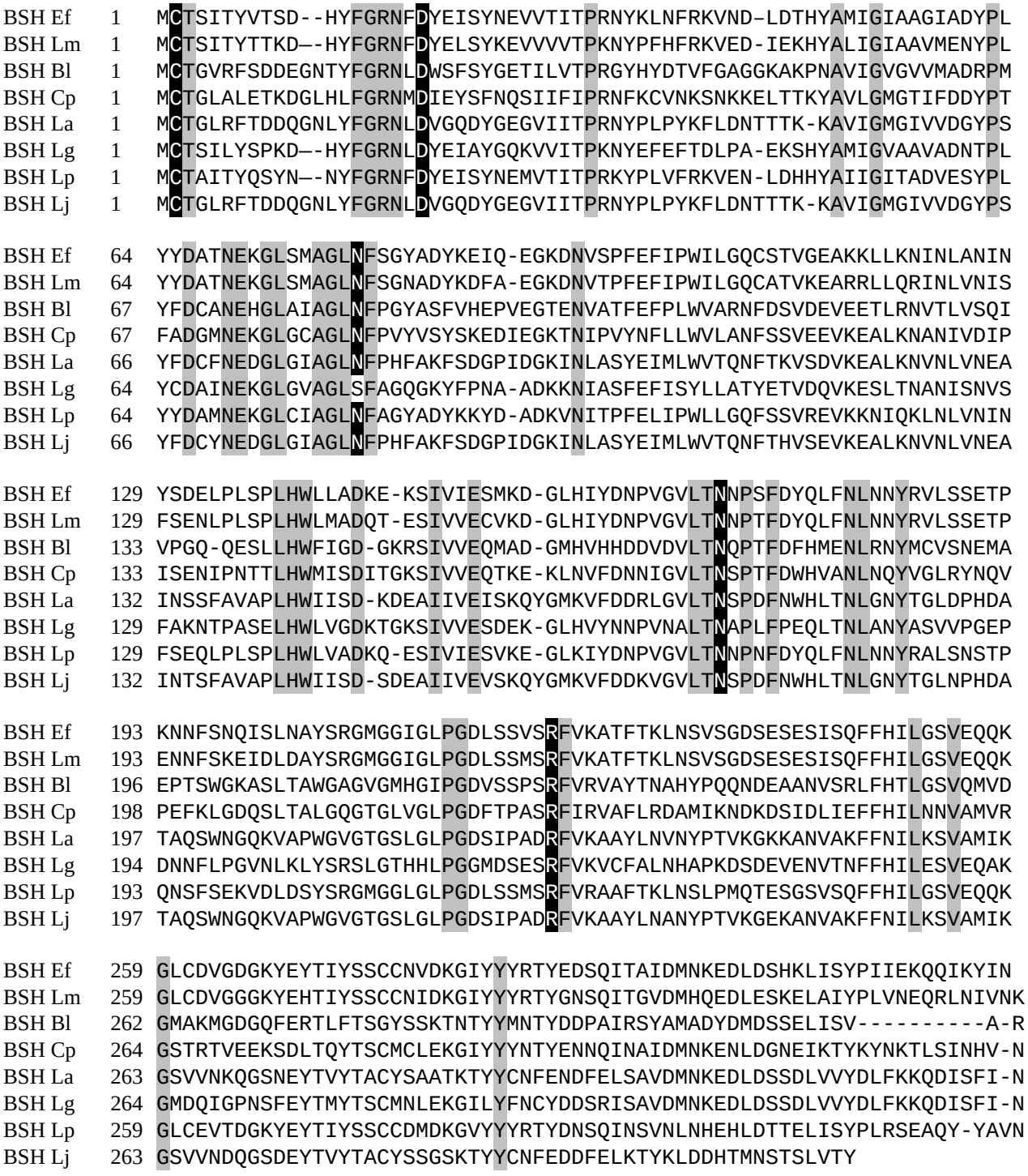


Fig. 9: Alignment of the BSH protein sequences from *Enterococcus faecium* FAIR-E 345 (BSH Ef), *Listeria monocytogenes* (BSH Lm), *Bifidobacterium longum* (BSH Bl), *Clostridium perfringens* (BSH Cp), *Lactobacillus acidophilus* (BSH La), *L. gasseri* (BSH Lg), *L. plantarum* (BSH Lp), and *L. johnsonii* (BSH Lj) by Lipman-Pearson alignment. Identical amino acids are indicated in black and grey boxes, amino acids hypothesised to be part of the active site are indicated in black boxes.

3.3.2 Genomic localisation of the *bsh* gene among *Enterococcus* strains

Use of the BSH probe gave good hybridisation signals when probing the genomic DNA of some of the strains (Fig 10).

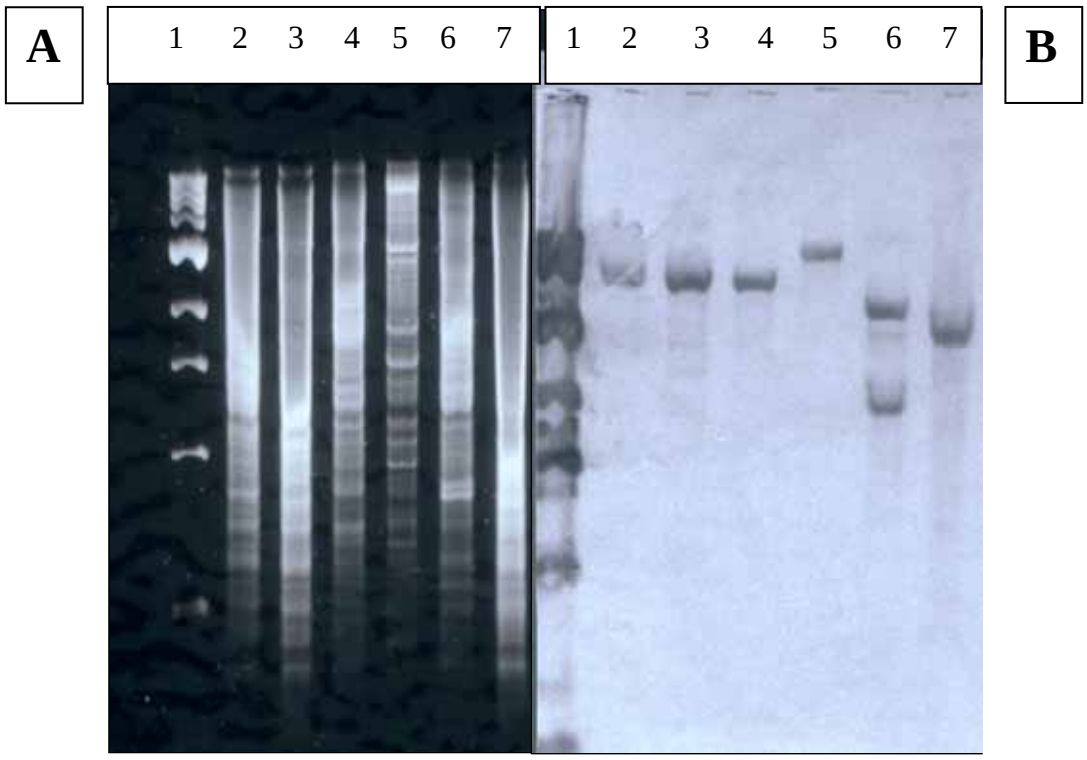


Fig. 10: Photograph of agarose gel with total genomic DNA from enterococci strains that was cut with the restriction enzymes *Xba*I and *Hin*dIII (a) and nylon membrane after Southern blotting of the agarose gels and probing with the BSH probe (b). Lane 1, 1 kb DNA ladder; lane 2, *Enterococcus* (*E.*) *faecium* FAIR-E 6; lane 3, *E. faecium* FAIR-E 14; lane 4, *E. faecium* FAIR-E 15; lane 5, *E. faecium* FAIR-E 20; lane 6, *E. faecium* FAIR-E 25; lane 7, *E. faecium* FAIR-E 34

When the BSH gene probe was hybridised to the genomic DNA of the negative control strains *E. faecalis* FAIR-E 29 and *E. faecium* FAIR-E 80, only a very weak signal could be observed (result not shown). This signal was noticeably weaker and could be well distinguished from positive signals obtained with most of the BSH positive strains (Fig. 10). The probe hybridised well to and positive signals were obtained with the genomic DNA of 22 enterococci strains (Table 16). Although ten of these strains harboured plasmid DNA, the

probe was found to hybridise only to the total genomic DNA and not to the plasmid DNA isolation of these strains (Table 16).

Table 16: Occurence of *bsh* gene among *Enterococcus* strains based on hybridisation using a BSH gene probe and the DIG-dUTP labeling and detection kit

Strains	Plasmid		Encoded *bsh* gene		
	detected	not detected	plasmid	chromosome	not both
Enterococcus durans	FAIR-E1, 231, 261, 389	FAIR-E326, 332	-	FAIR-E1, 326, 332,	FAIR-E 231, 261, 389
Enteroococcus faecium	FAIR-E3, 6, 20, 24, 25, 84, 243	FAIR-E14, 15, 34, 154, 170, 171, 217, 225, 227, 254, 263, 345, 349	-	FAIR-E3, 6, 14, 15, 20, 24, 25, 34, 84, 154, 170, 171, 217, 225, 227, 243, 254, 263, 345, 349	-
Enterococcus faecalis	FAIR-E63, 69, 71, 85, 88, 229, 237, 255, 259, 279, 313, 339, 342, 351, 363, 404	FAIR-E82, 176, 224, 226, 235	-	FAIR-E69, 71, 82, 85, 176, 224, 229, 237, 313	FAIR-E63, 88, 226, 235, 255, 259, 279, 339, 342, 351, 363, 404

For twelve other enterococci strains that contained plasmids, weak signals were obtained only when probing the total genomic DNA. These weak signals could represent hybridisation signals to the BSH genes, but which could not be unequivocally differentiated from the negative control. This may indicate that there is a certain degree of heterogeneity among the nucleotide sequences of BSH genes of enterococci, which did not allow the probe to bind equally well in all cases and result in signals of the same intensities. Nevertheless, in this study hybridisation signals could not be detected from plasmid DNA preparations of any of the strains tested. Therefore, our results indicated that the probe could in many cases specifically detect the *bsh* gene, and that this gene is probably located on the chromosome in enterococci strains. Such a chromosomal location of the BSH gene would be consistent with all previous reports on the cloning of these genes from *Listeria monocytogenes*, lactobacilli, *Bifidobacterium longum* and *Clostridium perfringens.*

Should BSH activity be regarded as an important functional property of probiotic enterococci strains, the apparent chromosomal location of BSH genes would imply, that this

trait is likely to be stable in such BSH^+ probiotic enterococci and most likely not subject to plasmid loss. Furthermore, this would imply that if BSH activity is considered as a colonisation factor which favours intestinal growth, as suggested by Moser and Savage (2001), it may be viewed as a possible virulence factor for enterococci strains which carry additional virulence determinants. Thus, it is considered as unlikely that this trait is transferable by conjugation from BSH^+ food strains or probiotic strains which carry no virulence determinants, to such food strains which do harbour virulence determinants and which could have, as a consequence of BSH activity, been better equipped for intestinal survival.

3.4 Animal Experiment

Bacteriocin production has been described as a functional trait of probiotic bacteria, and probiotic strains that produce bacteriocins have found application for human consumption. To date, however, data on the influence of bacteriocin production by probiotic strains on the autochthonous flora of the host are scarce. In this study, the bacteriocin-producing *E. faecalis* BFE 1071, which has probiotic potential, was used in an animal study to determine its influence on the gut flora of healthy male Sprague-Dawley rats. The animal experiment consisted of four groups, i.e. (1) group A fed control diet; (2) group B fed control diet and heat inactivated, non-viable biomass of the bacterioicin producer *E. faecalis* BFE 1071; (3) group C fed control diet and viable bacteriocin-producer *E. faecalis* BFE 1071 and (4) group D fed control diet and bacteriocin-negative mutant *E. faecalis* BFE 1071/79(-). Bacterial counts done on faeces of animals of group A were done in quadruplicate before the begin of experimentation, at the end of the animal acclimatisation stage. Counts were done in quadruplicate and after this, during the duration of the experiment, faeces from animals of group A were not further analysed, as it was not expected that the faecal counts and composition of the microflora of the faeces of the control group of animals would change during the experiment.

3.4.1 Bacterial counts of faeces of rats in the animal experiment

3.4.1.1 Total anaerobic count

Total anaerobic cell counts from faeces of animals belonging to experiment group B, C and D were compared (Fig. 11). The mean anaerobic total count on day 1 from faeces of animals belonging to groups B, C, and D were 8.2, 8.6, and 8.6 log CFU/g, respectively. After day 1, there were a general increase in total anaerobic cell count of the faeces of animals belonging to group B, C and D. Total anaerobic cell counts of group C tended to increase until the last day of treatment (day 7). The difference in total anaerobic cell counts between group C and control group B at the different sampling times ranged from about 0.5 to 1.5 log CFU/g, which is explained by the influence of feeding of the bacteriocin-producing strain *E. faecalis* BFE 1071 on the total count of bacteria.

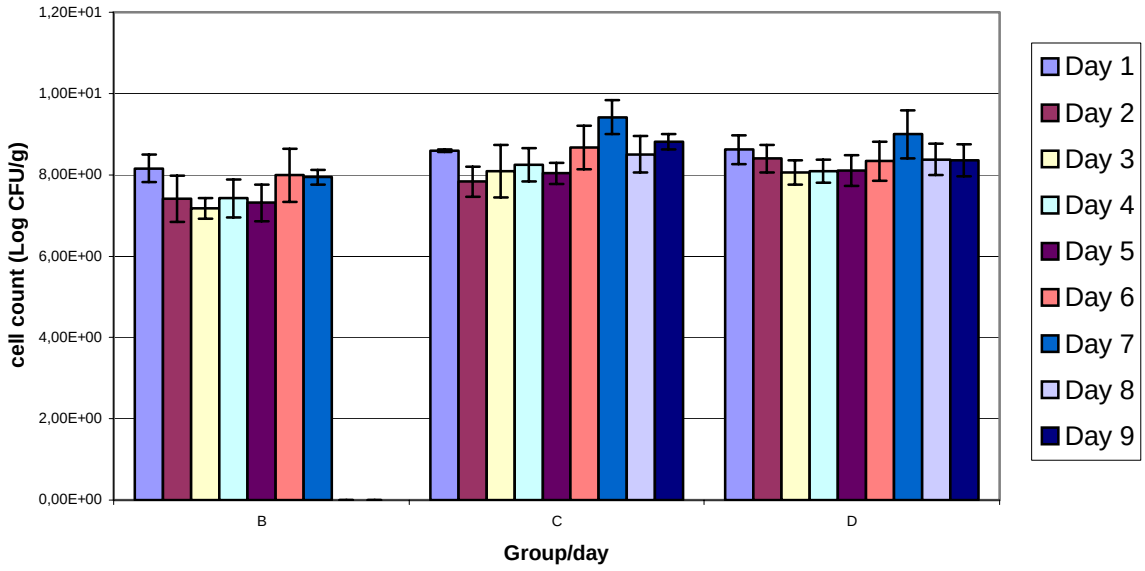


Fig. 11: Comparison of total anaerobic cell count from faeces of animals belonging to groups B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-).

3.4.1.2 *Enterococcus* counts

Counts of enterococci from experiment groups B, C and D were compared (Fig. 12). On day 1, faeces of animals from groups B, C, and D had enterococcal counts of 8.15, 8.11, and 8.5 log CFU/g, respectively. At day 1, there were a slight increase in cell count in group

B, C and D. The enterococci counts, however, decreased on day 2 from 0.5 to 1.0 log CFU/g. Cell count of group C tended to increase until the last day of treatment (day 7). The difference in cell count between group C and control group B was up to 1.5 log CFU/g.

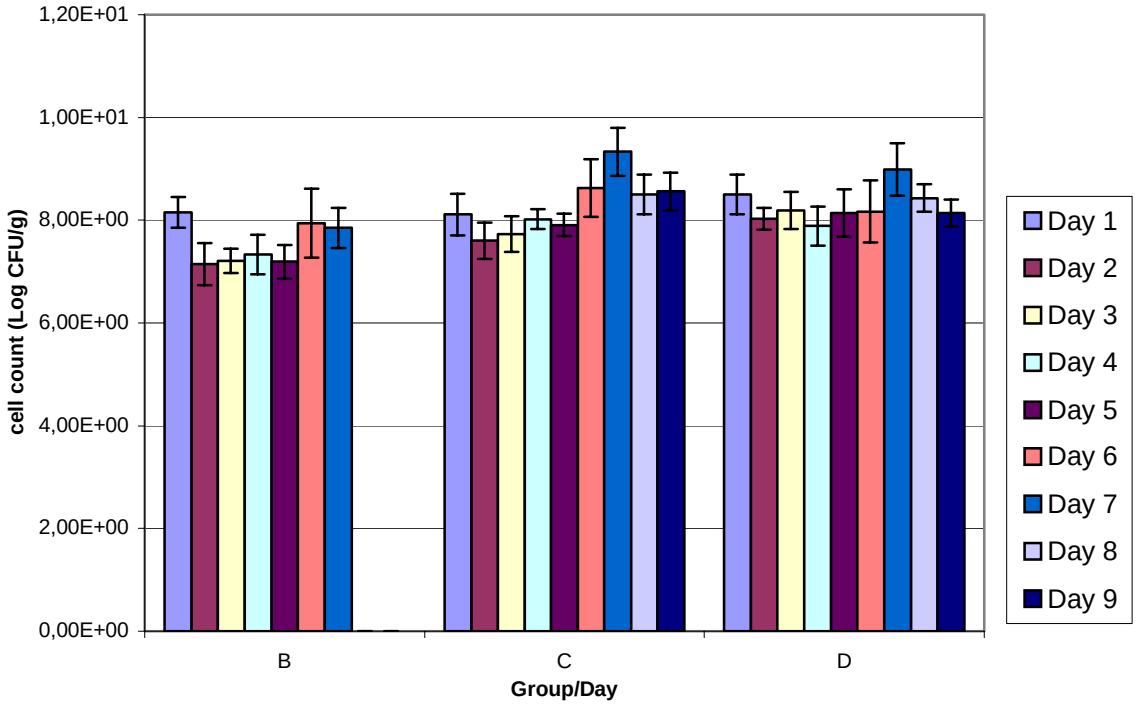


Fig. 12: Comparison of enterococci cell counts in faeces of animals from treatment groups B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-).

3.4.1.3 *Lactobacillus* counts

Counts of lactobacilli from rat faeces belonging to experiment groups B, C and D were compared (Fig. 13). On day 1, groups B, C, and D had cell counts of 8.2, 8.6, and 8.6 log CFU/g, respectively. Cell count on the next days increased until the last day of treatment (day 7). The difference in cell counts between group C and control group B was at a maximum of about 1.25 log CFU/g. At day 8 and 9 (group C and D) at which the treatment was no longer applied, there were decrease in cell count from ca. 0.3 log CFU/g.

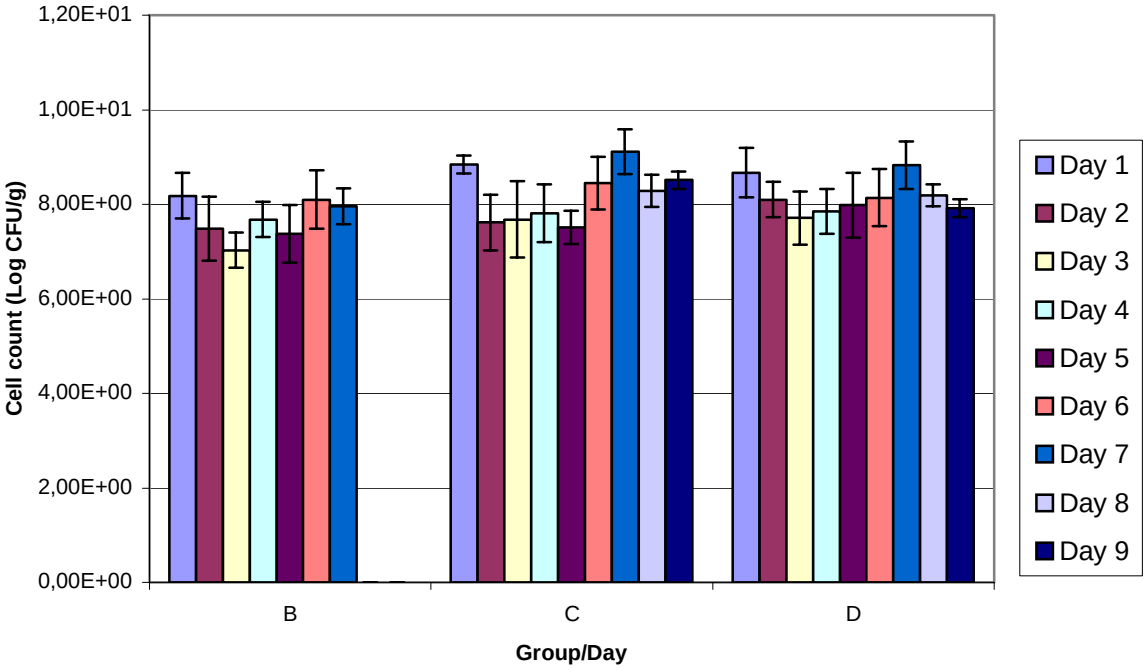


Fig. 13: Comparison of lactobacilli cell count in rat faeces of animals groups B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-).

3.4.1.4 *Bifidobacterium* counts

Counts of bifidobacteria from rat faeces of experiment groups B, C and D are shown in Fig. 14. On day 1, the day treatment was applied, groups B, C, and D had cell counts of 8.2, 8.9, and 8.7 log CFU/g, respectively. Bifidobacteria counts of the next days increased until the last day of treatment (day 7), especially in group C, showing the effect of bacteriocin-producing *E. faecalis* BFE 1071 which supported the growth of bifidobacteria. The difference in cell count between group C and control group B ranged from 0.8 to 1.8 log CFU/g. At day 8 and 9 (group C and D) at which the treatment was no longer applied, there were decreases in bifidobacterial counts from ca. 0.3 to 1.0 log CFU/g.

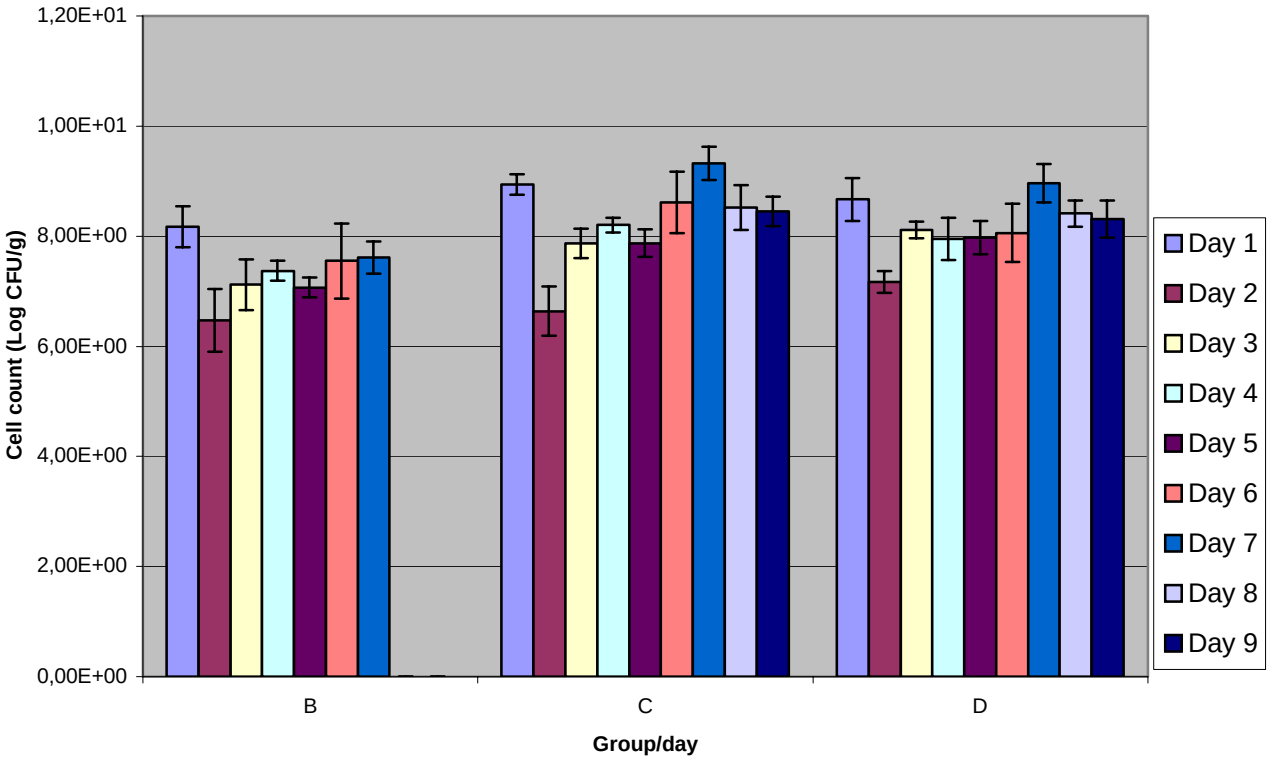


Fig. 14: Comparison of bifidobacteria cell counts in rat faeces of groups B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-).

3.4.1.5 *Clostridium* counts

Cell counts of clostridia from rat faeces of experiment groups B, C and D were compared as shown in Fig. 15. On day 1, faeces from animals of groups B, C, and D had cell counts of 8.4, 9.4, and 9.0 log CFU/g, respectively. Cell counts of all treatment groups decreased during the next days until the day 5, especially in rat faeces of group C, showing the effect of bacteriocin-producing *E. faecalis* BFE 1071 which may have inhibited the growth of clostridia. At group C the decrease reached a maximum of 2.0 log CFU/g. At day 6 and 7, however, the clostridial counts were shown to increase again.

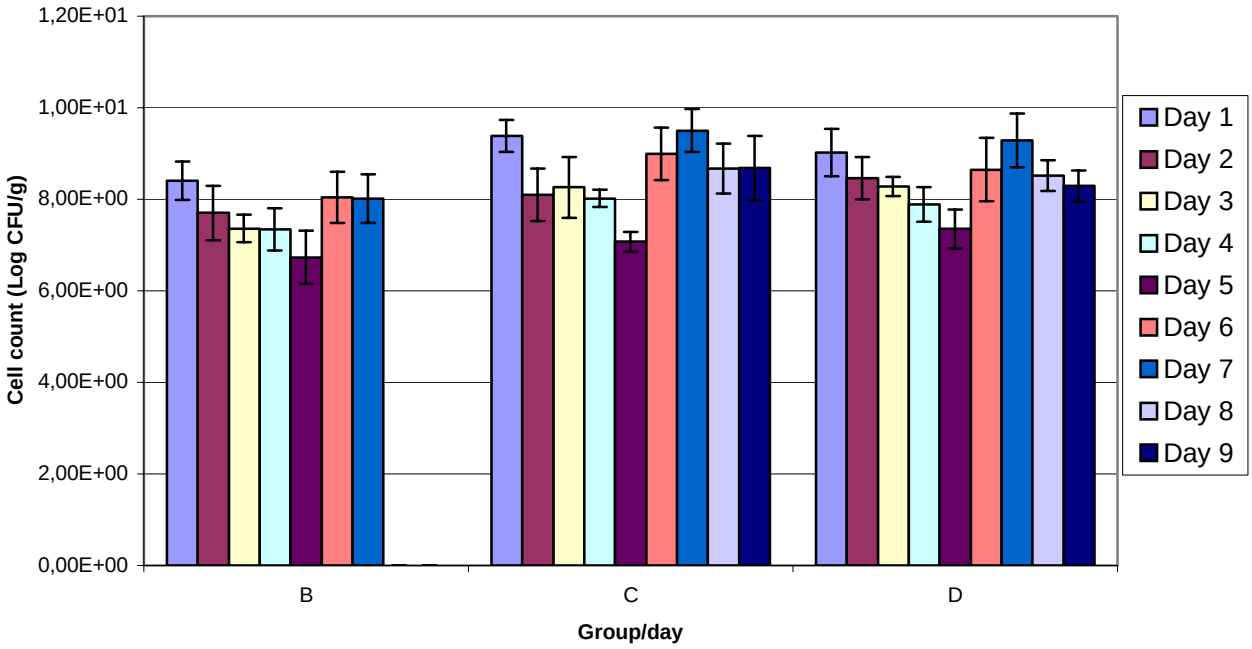


Fig. 15: Comparison of clostridia cell count in rat faeces of animals belonging to treatment groups B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-).

3.4.1.6 Eubacterium count

Cell counts of eubacteria from rat faeces of experiment groups B, C and D were compared as shown in Fig. 16. On day 1 counts of eubacteria in the rat faeces of animals of group B, C, and D were 7.7, 8.5, and 8.5 log CFU/g, respectively. Cell counts on the next days increased and decreased only slightly without noticeable effect, showing that the bacteriocin-producing *E. faecalis* BFE 1071 had no or relatively very little effect on eubacteria.

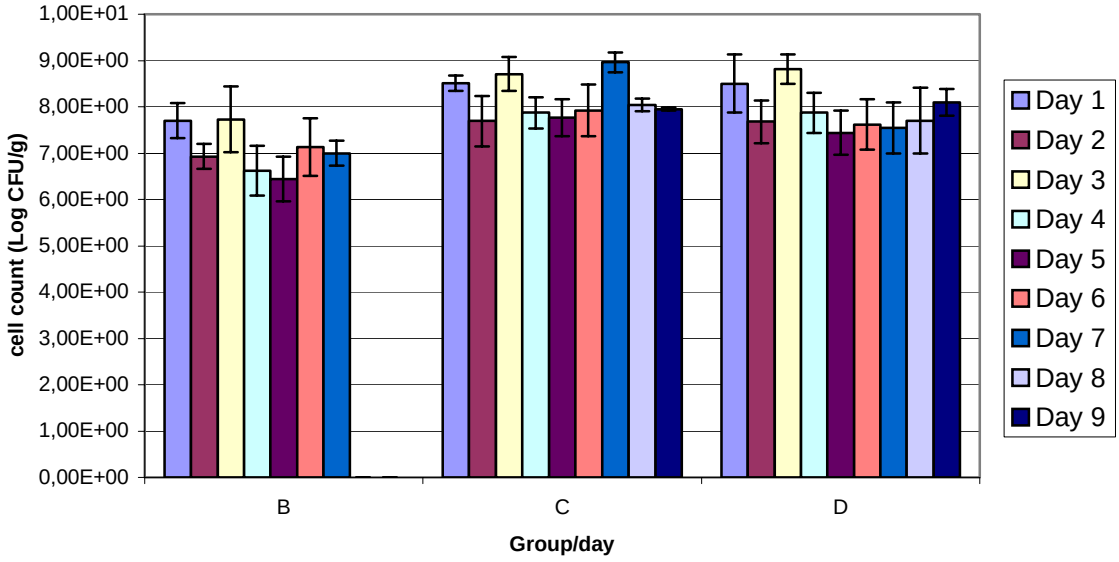


Fig. 16: Comparison of eubacteria cell count of rat faeces belonging to animals of groups B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-).

3.4.1.7 Gram-positive anaerobic cocci

Cell counts of anaerobic cocci from rat faeces of animals from experiment groups B, C and D were compared as shown in Fig. 17. On day 1, the day treatment was applied, anaerobic cocci counts rat faeces from groups B, C, and D were 8.4, 9.2, and 8.9 log CFU/g, respectively. Cell counts on the next days decreased and increased again slightly in groups B and C until day 5. The difference in cell count between group C and control group B was between 0.3 to 1.6 log CFU/g.

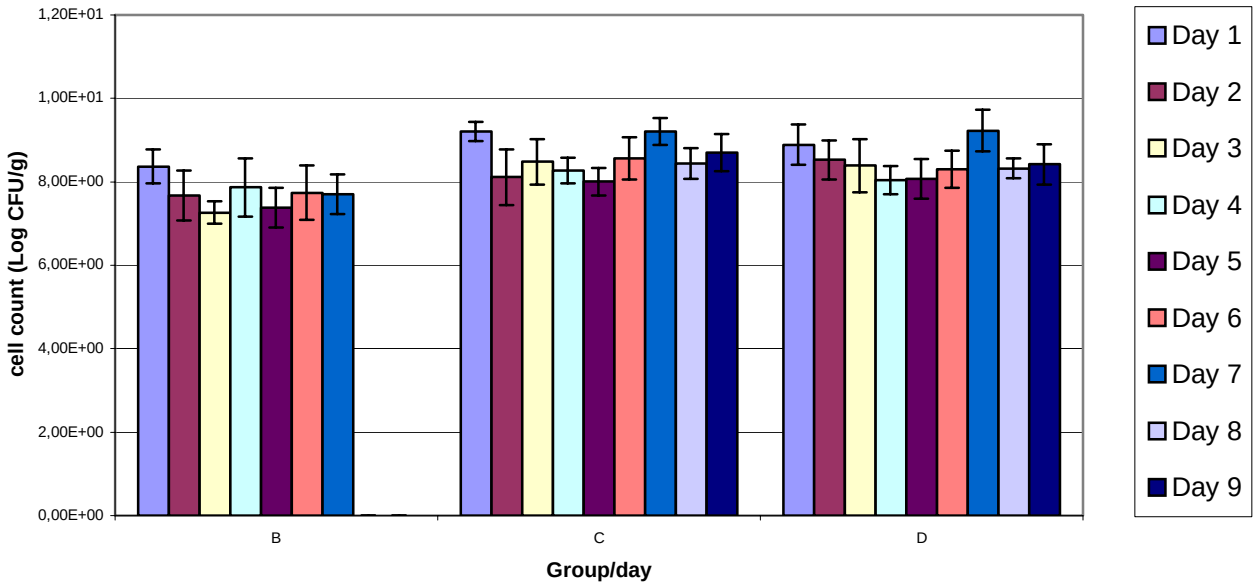


Fig. 17: Comparison of Gram-positive anaerobic cocci cell count in rat faeces from animals belonging to groups B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-).

Overall, there was no large effect of feeding the enterococci on the numbers of bacteria in the rat faeces. As expected, it appeared that feeding of enterococci increased the numbers of these bacteria in the rat faeces. This was especially obvious for the rat treatment group C in which rats were fed viable bacteriocin-producing *E. faecalis* BFE 1071 and for which there was a noticeable increased in enterococci counts from day 1 to day 7. Also, a slight increase in lactobacilli was noticed in the rat faeces of animals belonging to treatment groups C and D. Vahjen et al. (2002), who studied the effect of probiotic *E. faecium* SF 68 on the lactobacilli microflora in the small intestine of growing poultry poults, also noticed an increase in lactobacilli numbers in the intestine after administration of probiotic. They concluded that the enterococci lowered the pH of the gastrointestinal tract and thus stimulated lactobacilli growth by creating a suitable environment for lactobacilli proliferation (Vahjen et al., 2002). Similarly, the increase in lactobacilli numbers noted in this study may be as a stimulatory

effect of the bacteirocin-producing enterococci strain. However, the bacterial counts could not give any information on the composition of the enterococci and lactobacilli microflora; for this reason predominant isolates were isolated and identified using phenotypic and genotypic methods (see below). Administration of enterococci also led to a slight decrease in numbers of clostridia in faeces from animals treated with the bacteriocin-producer from day 1 of treatment till day 5, which may be explained by the antimicrobial activity of the bacteriocin produced by *E. faecalis* BFE 1071. However, after day 5 clostridial counts increased again, which may imply that clostridia became resistant to the bacteriocin. Otherwise, treatment of either bacteriocin-producing *E. faecalis* BFE 1071 or its bacteriocin-negative mutant was not deemed to have any effect on counts of the other bacterial groups, i.e. eubacteria, Gram-positive anaerobic cocci and bifidobacteria.

3.4.2 Phenotypical characterisation

3.4.2.1 *Enterococcus* isolates

One hundred and thirty-nine enterococci isolates from the animal experiment were phenotypically characterised by determining whether they possessed coccoid morphology, production of CO_2 from glucose, ability to grow at 45 °C, and ability to grow in MRS broth medium containing 6.5% NaCl and in MRS broth at a pH of 9.6. The ability to ferment key sugars, i.e. mannitol, sorbitol, arabinose and raffinose allowed the presumptive identification of enterococcal species (see Fig. 3). According to these phenotypic tests the isolates from rat faeces were dominated by *Enterococcus faecalis* (121 isolates) strains could ferment mannitol and sorbitol but not arabinose. There were also 12 isolates which could be presumptivly identified as *Enterococcus faecium* based on their ability to utilise mannitol and arabinose. Some strains of *E. faecium* can ferment sorbitol, while others do not, therefore sorbitol fermentation alone is not a useful fermentation characteristic for identification of *E. faecium.* Four presumptive *Enterococcus durans* strains could not utilise mannitol, sorbitol, arabinose and raffinose. Two presumptive *Lactococcus* spp. or unidentifiable enterococci were isolated from faecal samples and characterised by their ability to ferment mannitol but not sorbitol and arabinose (Fig. 18).

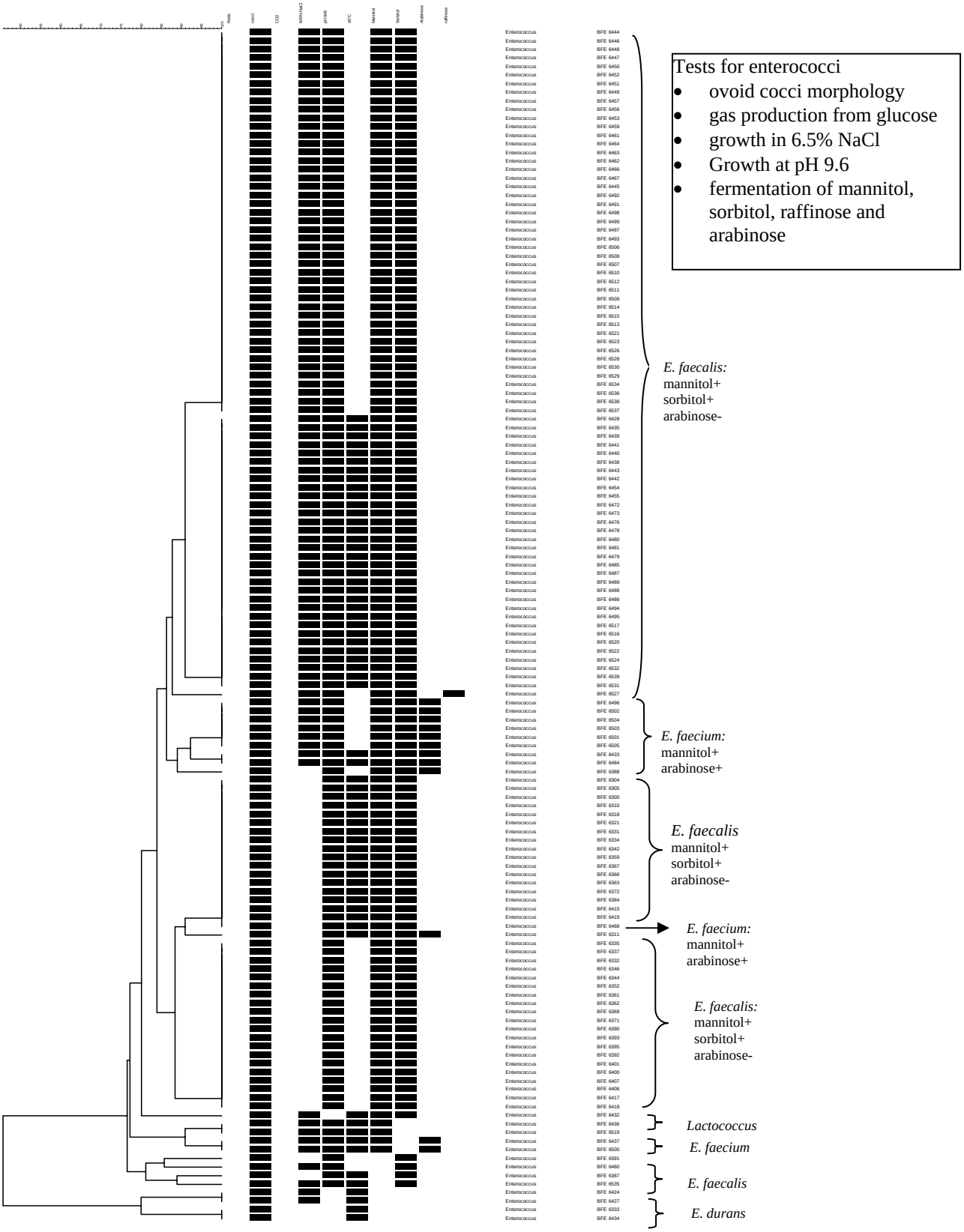


Fig. 18: A dendrogram showing phenotypical comparison of enterococci strains. The black small boxes showed positive results for the tests as given in the box at the right top of the figure.

When every single treatment group of the animal experiment was compared with one another, we could see that group A isolates consisted of *E. faecalis* (12 strains), *E. faecium* (1 strain), and *E. durans* (2 strains); group B isolates consisted of *E. faecalis* (25 isolates), *E. faecium* (4 strains), *E. durans* (1 strain) and *Lactococcus* (1 strain); group C consisted of *E. faecalis* (41 isolates), *E. faecium* (7 strains), and *E. durans* (1 strain); and group D consisted of *E. faecalis* (43 isolates) and *Lactococcus* (1 strain).

Groups C and D had relatively more *E. faecalis* isolates than group A and B. This could be explained by the fact that animals in these groups were fed with diet containing lyophilized biomass of *E. faecalis* BFE 1071 and its mutant. *E. faecalis* BFE 1071 could probably survive and support other *E. faecalis* strains. Hence, *E. faecalis* dominated the population.

E. faecium strains were isolated much less than *E. faecalis*. The possibility that growth of autochthonous *E. faecium* strains was inhibited by bacteriocin-producing *E. faecalis* BFE 1071 seemed to be not relevant, since only low numbers of isolates of *E. faecium* strains could be shown also on group A and B which acted as control groups.

3.4.2.2 Lactobacilli isolates

One hundred lactobacilli isolates were characterised phenotypically to determine whether they were rod-shaped, catalase-negative, produced or CO_2 from glucose, and their ability to grow at the temperatures of 15, 45 and 19 °C (for presumptive *L. reuteri* isolates only). In addition, it was investigated which lactic acid enantiomer was produced. This allowed preliminary identification of the *Lactobacillus* group or species. Six isolates were presumptively identified as *Lactobacillus reuteri*, fifty isolates were presumptively identified as members of the *Lactobacillus acidophilus* group and forty-four isolates were presumptively identified as to be *Lactobacillus murinus* group. The latter group consists of *L. murinus*, *L. salivarius* and *L. agilis* (Fig. 19).

Although phenotypic identification could in many cases presumptively identify the enterococci and lactobacilli to species level, in some cases (especially for the lactobacilli), the presumptive identification was only to group level, e.g., the *L. acidophilus* or *L. murinus* groups. Thus, for accurate identification of the isolates, and to follow the bacteriocin-producer isolate in the faeces of the rat, genotypic methods such as RAPD-PCR strain typing or group-specific PCR were used .

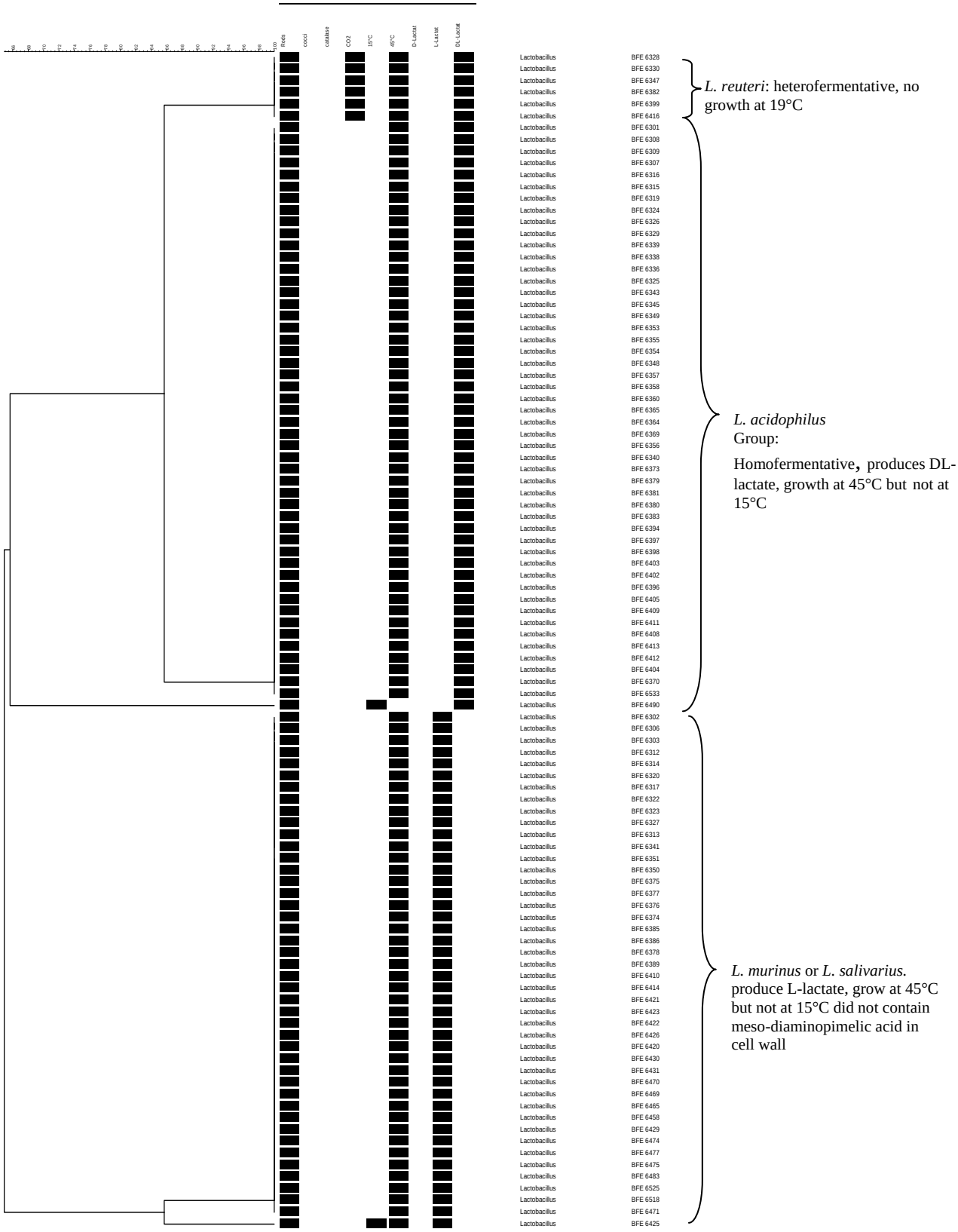


Fig. 19: Dendrogram showing the phenotypical comparison of lactobacilli. Black boxes showed positive results for the tests: morphology (cocci or rods), growth at 15°C or 45°C, production of gas from glucose, and enantiomer of lactate produced (D, L or DL).

Strains that could presumptively be identified as *L. reuteri* or *L. fermentum* by phenotypic tests produced DL-lactate and were heterofermentative. After testing their ability to grow at 19 °C, however, it was shown that these strains failed to grow, confirming that these strains could be presumptively identified as *L. reuteri*. Strains belonging to the *L. acidophilus* group also produced DL-lactate but in contrast to the *L. reuteri* strains these were homofermentative. Similar to *L. reuteri* and *L. acidophilus* strains, *L. murinus* group-strains could also grow at 45 °C. One strain could even grow at 15 °C. These strains belonging to the *L. murinus* group produced L-lactate. The peptidoglycan type of this group was Lys-DAsp. Based on this result, *L. agilis* could be excluded from the presumptive identification of these strains, as *L. agilis* posesses meso-diaminopimelic acid in the cell wall.

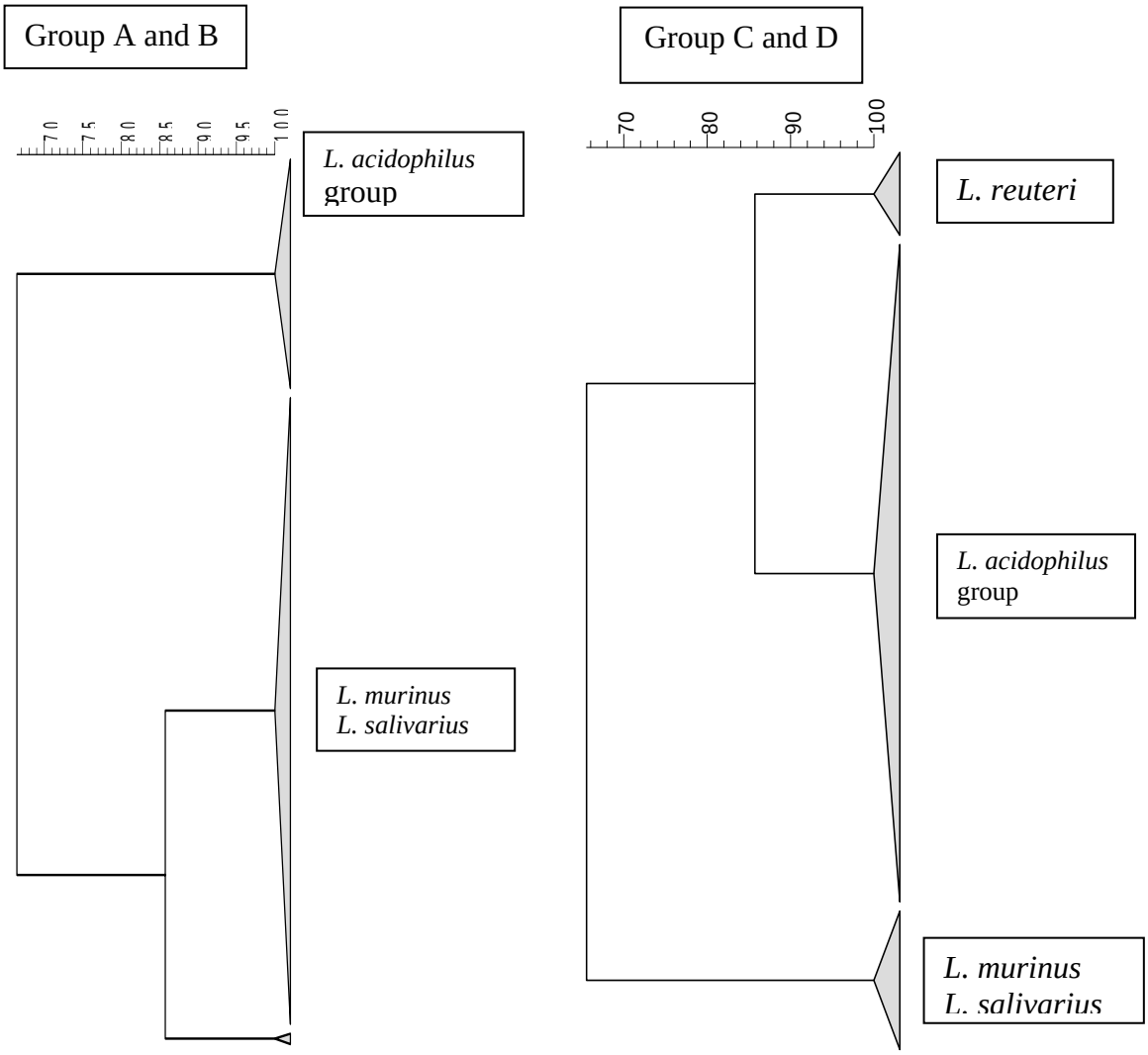


Fig. 20: Comparison of dendrograms obtained by clustering of phenotypic properties of lactobacilli species from experiment group A (control diet) B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-)

When the distribution of lactobacilli strains from the different experimental groups are compared, it was interesting to note that groups A and B (control groups) only consisted of *L. acidophilus* and *L. murinus* group strains, where strains from the *L. murinus* group dominated the population. In contrast, for group C and D treatment groups, the population were dominated by *L. acidophilus* group strains and strains belonging to the *L. murinus* group made up only a minor proportion of the population . In addition, in the groups and D treatment groups all of a sudden also *L. reuteri* strains were isolated, albeit at a low incidence and these made up the smallest proportion of the strains of these treatment groups (Fig. 20). When every single group of experiment was compared with one-another, it was evident that both groups A and B had relatively the same composition of lactobacilli consisting of *L. acidophilus* group and *L. murinus* group strains (Fig. 21). Group C had a higher proportion of presumptive *L. reuteri* strains(5 strains) than group D (1 strain). The strains belonging to the *L. murinus* group were more numerous in faeces from animals treated in group D (8 strains) than in group C (2 strains), while both groups C and D had almost the same number of strains belonging to the *L. acidophilus* group (5 and 8 isolates, respectively) (Fig 21).

A bacteriocin test with nine indicator strains (*Enterococcus faecium* FAIR E 29, *Lactobacillus sakei* DSM 20017, *Lactobacillus acidophilus* AC2, *Lactobacillus* BFE 6339, *Lactobacillus* BFE 6383, *Lactobacillus* BFE 6379, *Lactobacillus* BFE 6364, *Lactobacillus* BFE 6402, and *Lactobacillus* BFE 6370 showed, that all lactobacilli strains isolated from rat faeces did not produce bacteriocin.

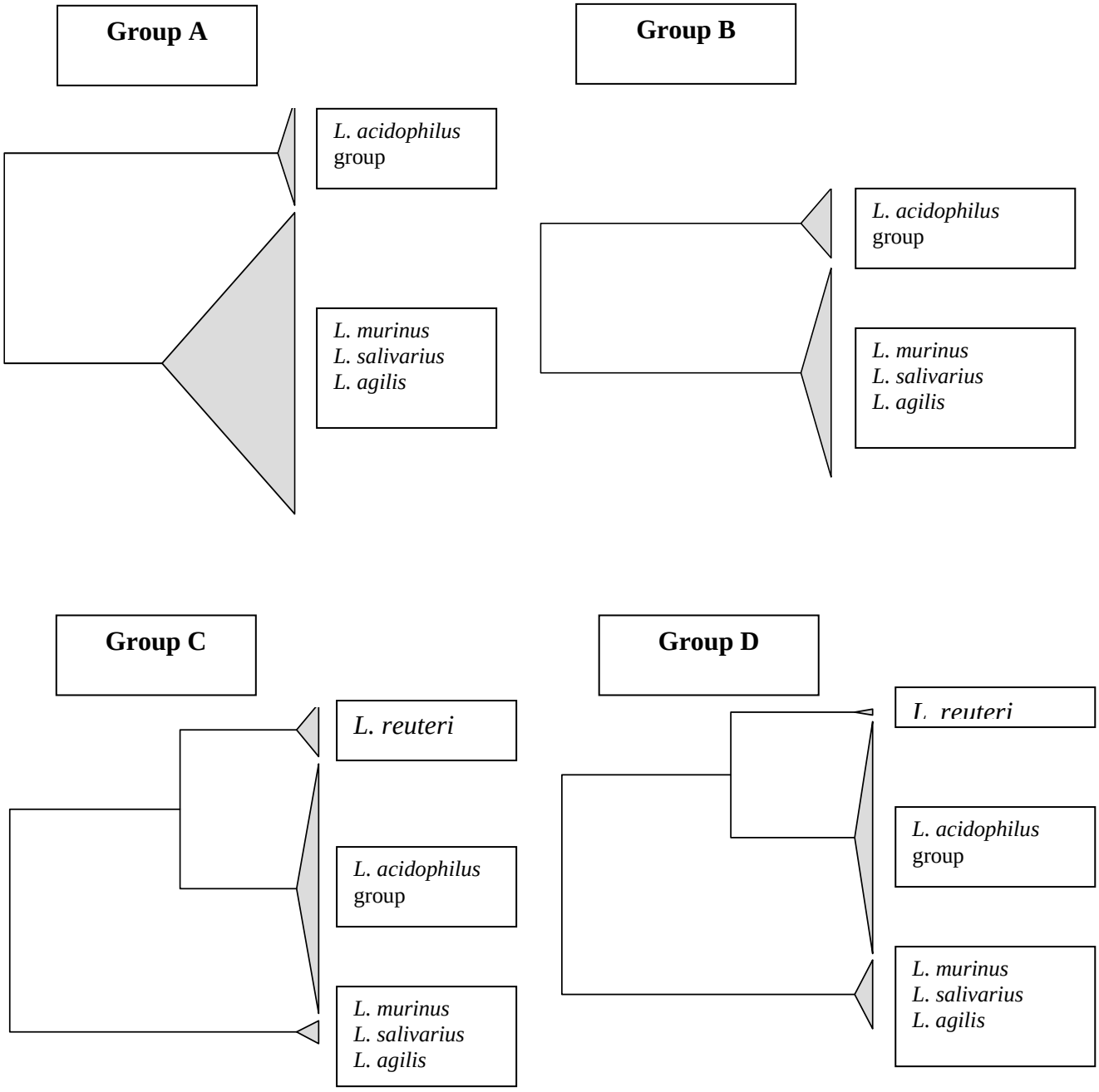


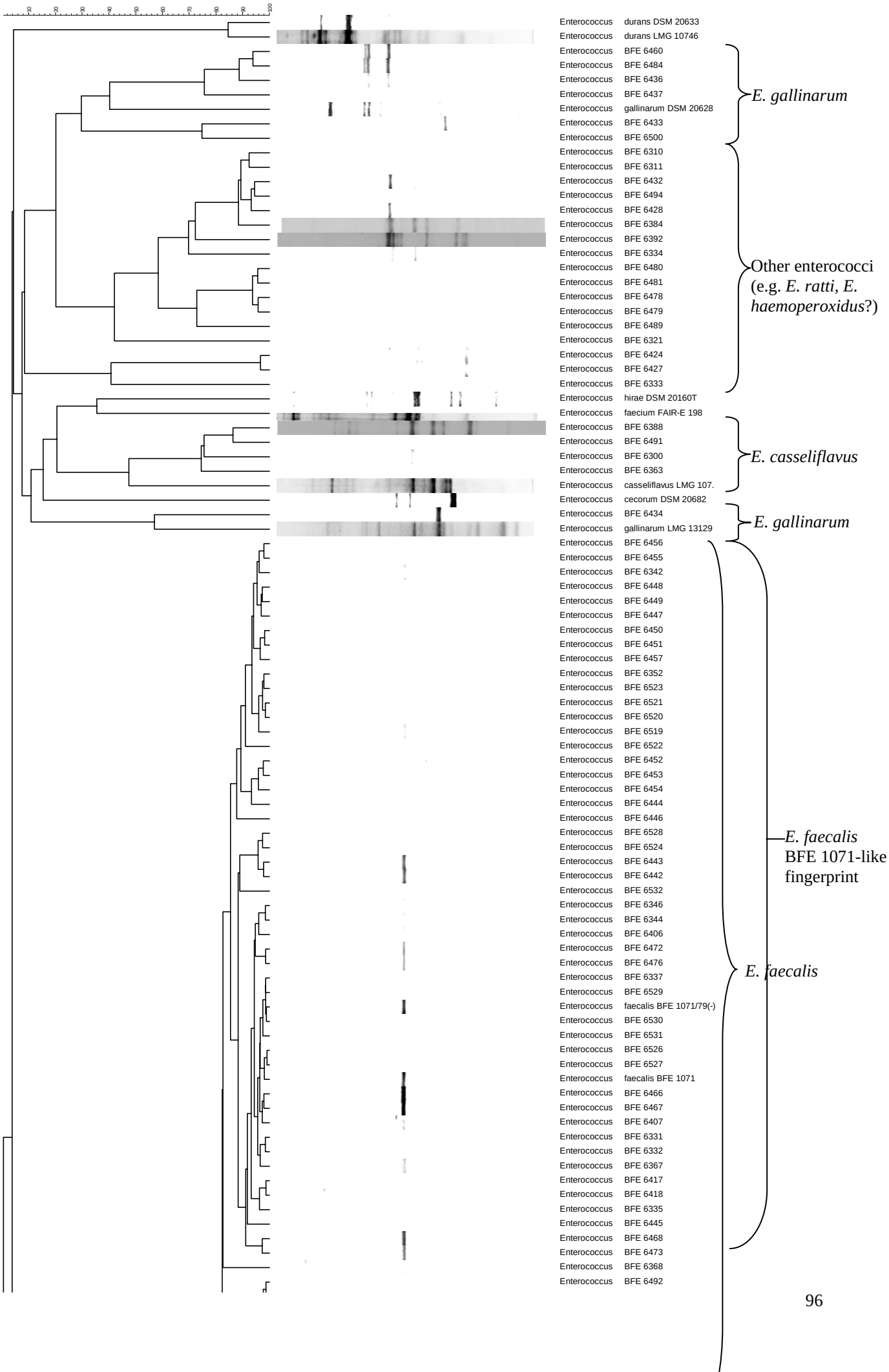
Fig. 21: Comparison of dendrograms obtained by clustering of phenotypic properties of lactobacilli species from experiment group A (control diet) B (control diet and heat inactivated *E. faecalis* BFE 1071), C (control diet containing viable bacteriocin producer *E. faecalis* BFE 1071), and D (control diet containing non-bacteriocin producer *E. faecalis* BFE 1071/79(-).

3.4.3 Genotypical characterisation

3.4.3.1 Enterococci group

By analysis of the RAPD-PCR fingerprints of isolates together with enterococci type strains, it was found that enterococci strains of rat samples consisted of *Enterococcus casseliflavus* (4 strains), *Enterococcus gallinarum* (7 strains), and mostly *Enterococcus faecalis* (111 strains). In addition, one cluster revealed enterococci that did not group together with one of the reference strains used in this study. However, not all the reference strains were used, and new enterococci have been described such as *E. ratti*, *E. haemoperoxidus* and *E. moraviensis* (Teixeira et al., 2001; Svec et al., 2001) which were not used in this study. Thus these isolates may belong to these species, or may consist of new *Enterococcus* species which have not been described so far. Although phenotypic characterisation previously indicated 12 *E. faecium* species, this could not be confirmed by genotypic identification and this serves to illustrate that phenotypic characterisation by comparisons of sugar fermentation patterns is not very reliable.

The bacteriocin-producing *E. faecalis* BFE 1071 could survive in the rat gastrointestinal tract and dominate among enterococi from the faecal flora. This was, because the fingerprint of the *E. faecalis* bacteriocin-producer and its mutants were very characteristic, and this fingerprint was almost identical from many of the *E. faecalis* isolates that were recovered from groups C and D and clustered together in cluster A (see Fig. 22). The *E. faecalis* BFE 1071 bacteriocin producer and its non-bacteirocin producing mutant fingerprints were virtually identical. All isolates from rat faeces that had a fingerprint close to the bacteirocin-producer or its mutant are shown in Fig. 22 and they clustered very closely at r = 85.47 %, indicating that these are the same strain. Thus the enterococci used as probiotic in this study could be selectively detected among enterococci from rat faeces.


10
20
30
40
50
60
70
80
90
100
Enterococcus durans DSM 20633
Enterococcus durans LMG 10746
Enterococcus BFE 6460
Enterococcus BFE 6484
Enterococcus BFE 6436
Enterococcus BFE 6437
Enterococcus gallinarum DSM 20628
Enterococcus BFE 6433
Enterococcus BFE 6500
E. gallinarum
Enterococcus BFE 6310
Enterococcus BFE 6311
Enterococcus BFE 6432
Enterococcus BFE 6494
Enterococcus BFE 6428
Enterococcus BFE 6384
Enterococcus BFE 6392
Enterococcus BFE 6334
Enterococcus BFE 6480
Enterococcus BFE 6481
Enterococcus BFE 6478
Enterococcus BFE 6479
Enterococcus BFE 6489
Enterococcus BFE 6321
Enterococcus BFE 6424
Enterococcus BFE 6427
Enterococcus BFE 6333
Other enterococci (e.g. E. ratti, E. haemoperoxidus?)
Enterococcus hirae DSM 20160T
Enterococcus faecium FAIR-E 198
Enterococcus BFE 6388
Enterococcus BFE 6491
Enterococcus BFE 6300
Enterococcus BFE 6363
Enterococcus casseliflavus LMG 107.
E. casseliflavus
Enterococcus cecorum DSM 20682
Enterococcus BFE 6434
Enterococcus gallinarum LMG 13129
E. gallinarum
Enterococcus BFE 6456
Enterococcus BFE 6455
Enterococcus BFE 6342
Enterococcus BFE 6448
Enterococcus BFE 6449
Enterococcus BFE 6447
Enterococcus BFE 6450
Enterococcus BFE 6451
Enterococcus BFE 6457
Enterococcus BFE 6352
Enterococcus BFE 6523
Enterococcus BFE 6521
Enterococcus BFE 6520
Enterococcus BFE 6519
Enterococcus BFE 6522
Enterococcus BFE 6452
Enterococcus BFE 6453
Enterococcus BFE 6454
Enterococcus BFE 6444
Enterococcus BFE 6446
Enterococcus BFE 6528
Enterococcus BFE 6524
Enterococcus BFE 6443
Enterococcus BFE 6442
Enterococcus BFE 6532
Enterococcus BFE 6346
Enterococcus BFE 6344
Enterococcus BFE 6406
Enterococcus BFE 6472
Enterococcus BFE 6476
Enterococcus BFE 6337
Enterococcus BFE 6529
Enterococcus faecalis BFE 1071/79(-)
Enterococcus BFE 6530
Enterococcus BFE 6531
Enterococcus BFE 6526
Enterococcus BFE 6527
Enterococcus faecalis BFE 1071
Enterococcus BFE 6466
Enterococcus BFE 6467
Enterococcus BFE 6407
Enterococcus BFE 6331
Enterococcus BFE 6332
Enterococcus BFE 6367
Enterococcus BFE 6417
Enterococcus BFE 6418
Enterococcus BFE 6335
Enterococcus BFE 6445
Enterococcus BFE 6468
Enterococcus BFE 6473
Enterococcus BFE 6368
Enterococcus BFE 6492
E. faecalis BFE 1071-like fingerprint
E. faecalis

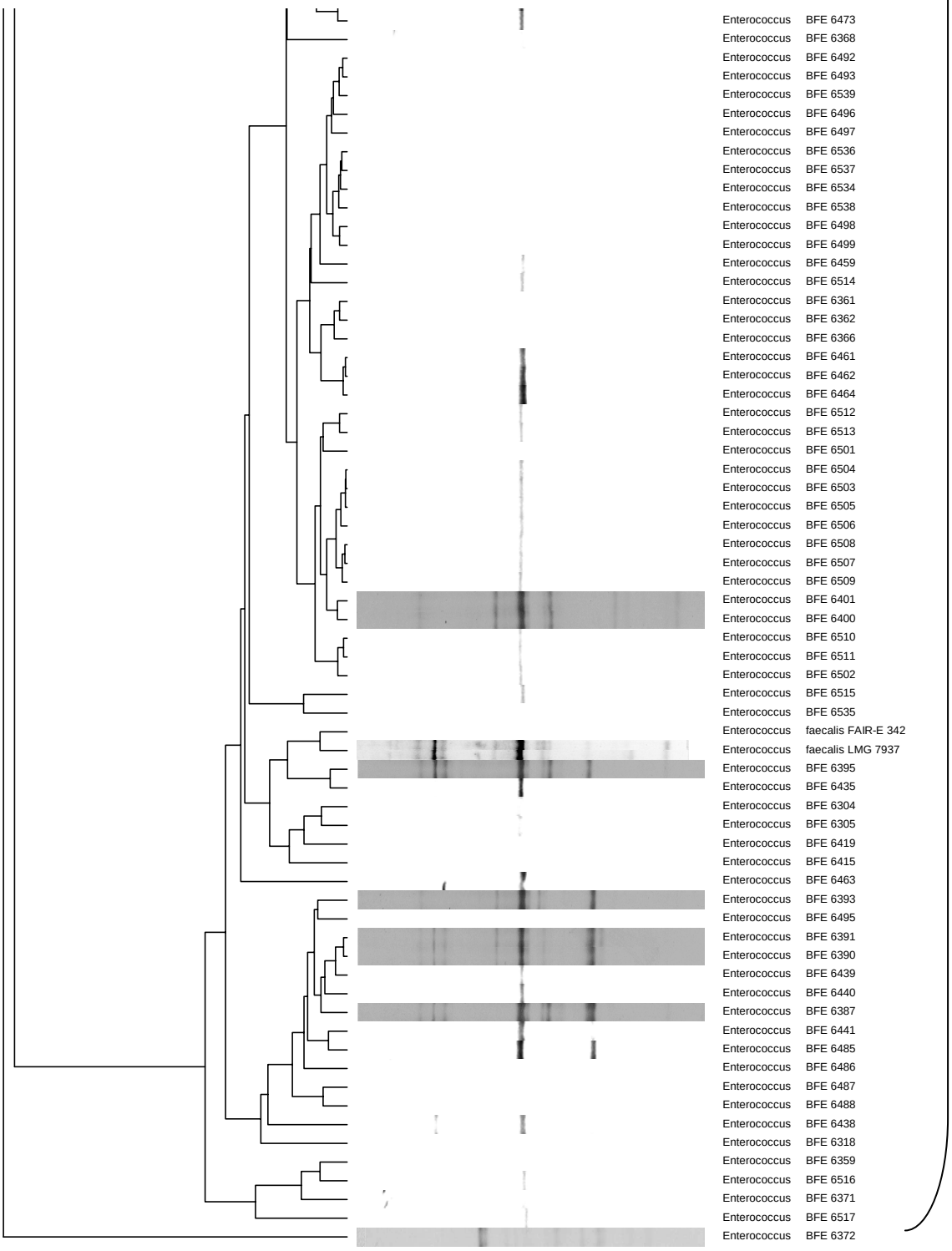


Fig. 22: Dendrogram of RAPD-PCR fingerprints of enterococci from rat faeces obtained from animals group A (control diet), B (control diet with heat inactivated, *E. faecalis* BFE 1071), group C (control diet with viable bacteriocin-producing *E. faecalis* BFE 1071) and D (control diet with viable non-bacteriocin-producing *E. faecalis* BFE 1071/79(-).

3.4.3.2 Lactobacilli group

Comparing the RAPD PCR along with suitable type strains, it was found that lactobacilli strains of rat faeces consisted of *Lactobacillus reuteri* (5 strains), *Lactobacillus salivarius* (45 strains), while strains from the *Lactobacillus acidophilus* group consisted of 2 species, i.e. *Lactobacillus johnsonii* (32 strains), and *Lactobacillus gasseri* (16 strains) (Fig. 23). The strains were identified to species level using group-specific PCR (result not shown). Thus, all strains that reacted with the *L. gasseri/L. johnsonii* group primers and not with the *L. acidophilus* group primers (reacting with *L. acidophilus*, *L. crispatus*; *L. gallinarum*, *L. amylovorus*) could be identified as either *L. gasseri* or *L. johnsonii*. Grouping of the isolates with a reference strain in RAPD PCR fingerprinting enabled identification of the isolate to species level. One isolate each had RAPD-PCR fingerprints which were similar to *L. cripatus* and *L. murinus*, respectively, and thus could be tentatively identified as such.

Our results thus confirmed the phenotypic identification of lactobacilli isolated from rat faeces. This study gives new insight on the autochthonous lactobacilli flora of the rat gastrointestinal tract. It appears that *L. salivarius* strains predominate in the normal rat and this is followed by *L. johnsonii* and *L. gasseri* strains (Fig. 24 groups A, B). Clearly, administration witht he probiotic could shift the microflora contained in the rat faeces from one that predominantly contains *L. salivarius*, to one in which members of the *L. acidophilus* groups predominate (Fig. 24 groups C, D). This '*Enterococcus* effect' particularly appeared to stimulate *L. johnsonii*, a strain which is often used as am probiotic and thus considered to be valuable in gut health (Holzapfel et al., 1998).

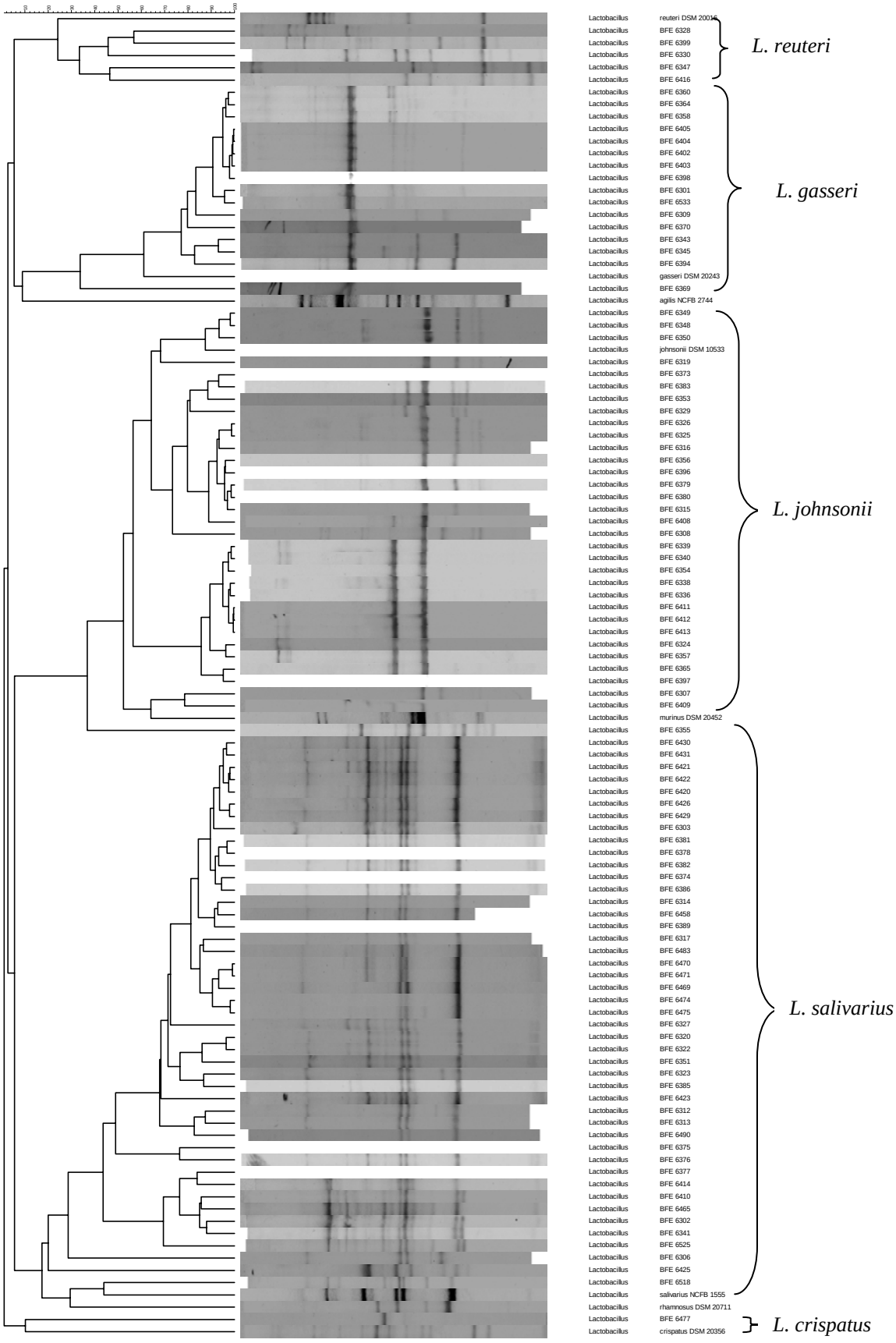


Fig. 23: Dendrogram of RAPD-PCR fingerprints of enterococci from rat faeces obtained from animals group A (control diet), B (control diet with heat inactivated, *E. faecalis* BFE 1071), group C (control diet with viable bacteriocin-producing *E. faecalis* BFE 1071) and D (control diet with viable non-bacteriocin-producing *E. faecalis* BFE 1071/79(-).

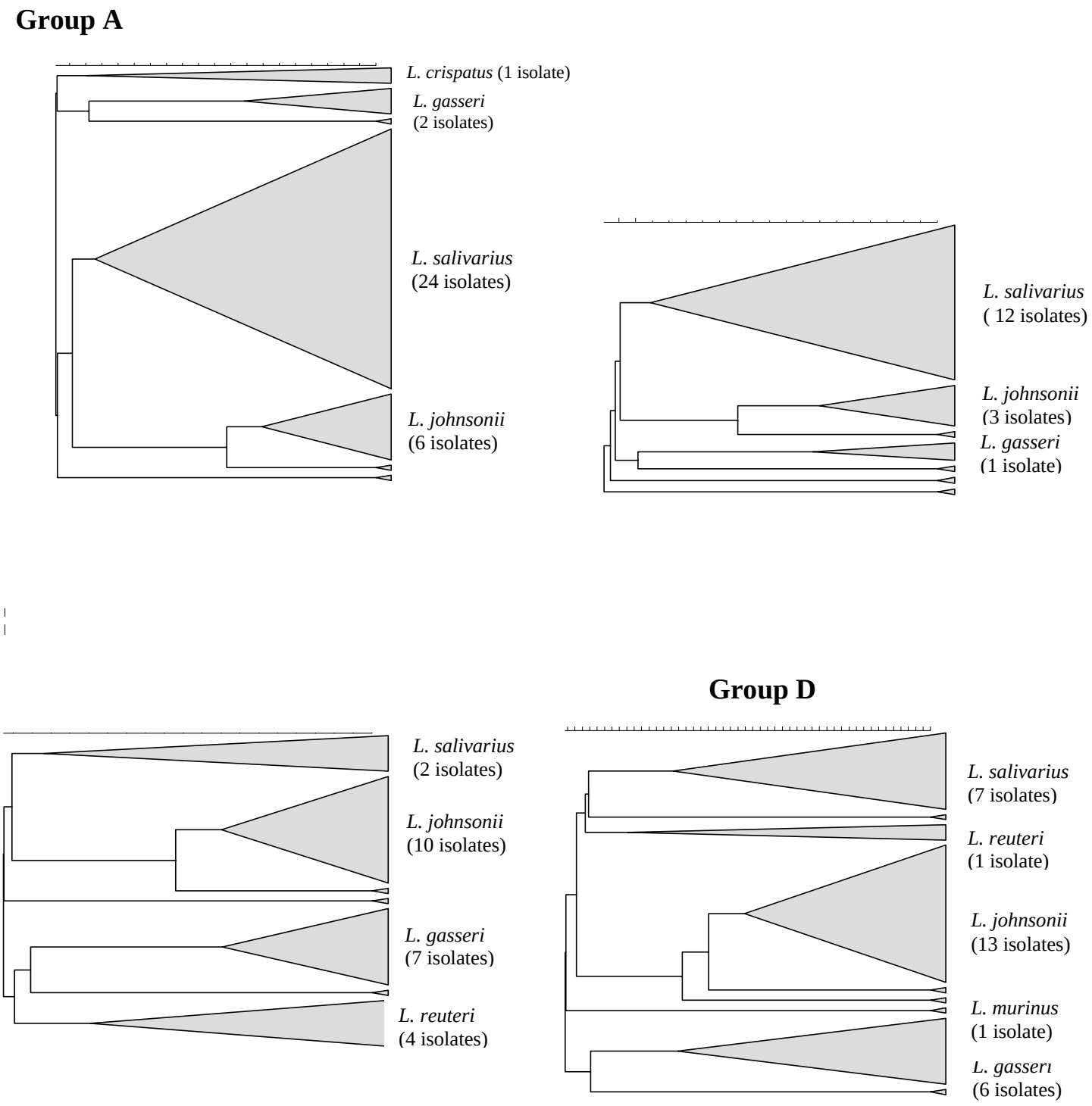


Fig. 24: Dendrograms of RAPD-PCR fingerprints of lactobacilli from animal treatment groups A (diet only), B (diet containing heat inactivated *E. faecalis* BFE 1071), C (diet containing viable bacteriocin-producing*E. faecalis* BFE 1071) and D (diet containing viable non-bacteriocin-producing*E. faecalis* BFE 1071/79(-).

RAPD-PCR is one of the most popular genotypic techniques. It was developed to reveal intra- and interspecific differences in bacterial genomes and can differentiate at the strain level (Welsh and McClelland, 1990; Williams et al., 1990). This technique has been used for the classification of a variety of food-borne microorganisms, including *Saccharomyces* spp. (Molnál et al., 1995), *Bacillus* spp. (Stephan et al., 1994), *Lactococcus* spp. (Tailliez et al., 1998), *Lactobacillus* spp. (Berthier and Ehrlich, 1999; Du Plessis and Dicks, 1995), *Penicillium* spp. (Dupont et al., 1999), and *Streptococcus* and *Enterococcus* (Moschetti et al., 1998). Spano et al. (2001) showed that use of species-specific PCR test for preliminary screening and then RAPD-PCR may be considered the most reliable method in order to perform a rapid and correct typing of *Lactobacillus plantarum* from wine must.

Lactobacillus acidophilus is a natural inhabitant of mammalian gastrointestinal systems. This species is of considerable industrial and medical interest, because *L. acidophilus* is believed to play an important role in human health and nutrition by its influence on the intestinal flora (Roy et al., 2000). The *L. acidophilus* complex strains were divided into six species including *L. acidophilus, L. crispatus, L. amylovorus, L. gallinarum, L. gasseri,* and *L. johnsonii* and strains of L. johnsonii, L. gasseri and L. acidophilus are successfully being used as probiotics (Holzapfel et al., 1998).

Our results show that feeding bacteriocin-producing enterococci shift the *Lactobacillus* flora towards the *L. acidophilus* group in the rat gastrointestinal tract, i.e. towards *L. johnsonii* and *L. gasseri* strains. However, because the same trend is observed between the animal treatment groups C and D, i.e., the groups that received the bacteriocin-producing strain and the bacteriocin-negative mutant, it appears that this effect is not due to bacteriocin production, but rather a result of the enterococci present, whether they produce bacteriocin or not. Thus overall, a net ‘enterococci effect’ was observed, which shifts the lactobacilli flora of the rat gastrointestinal tract towards the *L. acidophilus* group, while no ’bacteriocin effect’ could be noticed, apart from the fact that in the rat group D treated with the bacteriocin producer, the incidence of the heterofermentative *L. reuter*i strains increased slightly. Whether this ‘*Enterococcu*s effect’ is a result of better competition of enterococci and suppression of the *L. salivarius* group, or whether it is a result of stimulation of the growth of the *L. acidophilus* group with consequent replacement of the *L. salivarius* group, is unknown.

4.0 GENERAL DISCUSSION AND CONCLUSION

The lactic acid bacteria (LAB) are a group of bacteria united by a constellation of morphological, metabolic and physiological characteristics. Generally, LAB are described as Gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid as a major end-product during fermentation of carbohydrates (Axelsson, 1998). LAB are generally considered as 'food grade' organisms. It is assumed that most representatives of this group do not pose any health risks to healthy humans. Most LAB are designated as 'GRAS' (**g**enerally **r**ecognised **a**s **s**afe) microorganisms in the USA based on a long history of safe use in foods. However, some species may act as opportunistic pathogens in rare cases (Aguire and Collins, 1993; Gasser, 1994; Holzapfel et al., 1995).

LAB have long been known to be involved in the production of fermented foods. Presently, these products constitute one-quarter of our diet and are characterised by a safe history, certain beneficial health effects, and an extended shelf life when compared to non-fermented, fresh foods (Hammes and Tichaczek, 1994).

In recent years, the consumer has been increasingly confronted with functional food products which are claimed to promote his /her health and well-being. At the centre of these food products are the so-called pro- and prebiotics (Simmering and Blaut, 2001). Traditionally, LAB are the basic components of probiotics as they best fit the functional and safety requirements by their definitions (Reuter, 1997). Today, a wide number of microbial species and genera also are considered to meet the requirement for probiotics. Claimed health benefits exerted by probiotic include prevention or alleviation of diarrhoea, antimicrobial activity by bacteriocin production, antimutagenic and anticarcinogenic properties, reduction of serum cholesterol levels, and improvement of lactose tolerance (Shah, 2000).

Enterococci are LAB that have also been used to advantage in the food industry. Enterococcal strains are used for the production of certain types of cheeses in Southern European countries. Specific strains of enterococci are being used as probiotics for humans and animals. One of the best studied enterococci used as a human probiotic, especially in the treatment of diarrhoea, is *Enterococcus faecium* SF 68. The strain was originally isolated in Sweden and was patented in Switzerland and other countries (Lewenstein et al., 1979).

Intestinal LAB are known to produce bacteriocins which can inhibit pathogenic bacteria, for example the intestinal isolates *Lactobacillus acidophilus* 30SC (Oh et al., 2000), *Lactobacillus delbrueckii* subsp. *lactis* UO004 (Boris et al., 2001), *Lactobacillus salivarius* subsp. *salivarius* UCC118 (Flynn et al., 2002), as well as a vaginal isolate of *Lactobacillus*

salivarius CRL 1328 (Ocana et al., 1999). *L. acidophilus* LF221, isolated from infant faeces was shown to produce at least two bacteriocins (designed as acidocin LF221 A and acidocin LF221 B) which exhibited activity against different bacteria including some pathogenic species such as *Bacillus cereus, Clostridium difficile*, *Listeria innocua*, *Staphylococcus aureus* and group D streptococci. Du Toit et al. (1998. 2000) showed that enterococci isolated from the faeces of Göttingen mini-pigs had bacteriocin activity against many enterococci species, *Lactobacillus salivarius* which is often associated with the human gastrointestinal tract, as well as *Clostridium* and *Listeria* spp. One of the enterococcal bacteriocin, produced by *E. faecalis* BFE 1071, was characterised at the chemical and genetic level in detail in a follow-up study (Balla et al., 2000). This bacteriocin, termed enterocin 1071A&B was shown to be a two-peptide bacteriocin and thus can be classified as a class IIb bacteriocin according to the bacteriocin classification scheme of Nes et al. (1996).

Although a great deal of research has been dedicated to elucidate aspects of bacteriocin activity such as genetics of production, immunity and transport, as well as toxicological aspects and application of bacteriocins in foods, there are almost no data available on the effect of bacteriocins or bacteriocin-producing probiotic lactic acid bacteria on the hosts autochthonous flora. Because bacteriocin activity has been viewed as a functional characteristic of probiotic lactic acid bacteria, some research efforts have been dedicated to evaluate the effect of purified bacteriocins or bacteriocin producers on foodborne pathogens *in vitro* or in seldom cases also *in vivo*. However, no studies have yet addressed the question about the effect on the host autochthonous flora, which is surprising, considering that bacteriocins are active usually against closely related species and may thus interfere with the hosts established microflora. The hosts' own microflora is considered to have an important function itself in protecting the gastrointestinal tract from invading pathogens. Such a potential interference with the protective activity of the hosts autochthonous flora has raised concerns on whether bacteriocin-production by probiotic bacteria is actually a desirable functional characteristic. This study was motivated to determine the influence of a potentially probiotic, bacteriocin-producing *Enterococcus faecalis* strain BFE 1071 and its bacteriocin-negative mutant on the composition of the intestinal microflora of male Sprague-Dawley rats in an *in vivo* experimental model. To reach this objective the study aimed to: (1) investigate the suitability of various enterococci strains isolated from food or gastrointestinal sources for use as a probiotic on the basis of their technological and functional properties. To study bile salt hydrolase activity of enterococci, for which no information is available at the genetic level, using a molecular biological approach which included cloning and genetic

characterisation of the bile salt hydrolase gene of one of the enterococci strains. (2) to evaluate and compare the probiotic potential of the selected *Enterococcus* strains using a commercial *Enterococcus faecium* SF68 probiotic culture, and to select a bacteriocin-producing strain and its bacteriocin-negative mutant for use in an *in vivo* experiment to determine their effects on the hosts autochthonous intestinal flora, to investigate the potentially probiotic enterococci strains for virulence determinants and antibiotic resistances in a safety evaluation, (3) to determine the effect of the bacteriocin and the effect of the bacteriocin producer on the intestinal microflora of the rat by recovering predominant gastrointestinal bacteria from the faeces on selective media, and specifically by phenotypic and genotypic identification of the lactobacilli and enterococci isolates

For technological properties of potentially probiotic LAB investigated in this study, *E. faecium* strains (48.8%) and *E. faecalis* (46.5%) were considered to be acid resistant, including the bacteriocin producer *E. faecalis* BFE 1071 and *E. faecium* BFE 900. However, *E. faecium* strains were considered more resistant against bile and duodenum secrete (65%) than *E. faecalis* (5%), including the bacteriocin producer *E. faecalis* BFE 1071.

The bacteriocin producer *E. faecalis* BFE 1071 showed a relatively wide antimicrobial activity spectrum against lactobacilli strains typical of the gastrointestinal tract, compared to other bacteriocin producers *E. faecium* BFE 900 and *L. sakei* Lb706.

Twenty-nine *E. faecalis* strains (67.4%) and 25 *E. faecium* strains (58.1%) were found to posses bile salt hydrolase (BSH) activity. However, the bacteriocin producer *E. faecalis* BFE 1071 as well as the sakacin A producer *L. sakei* Lb 706 showed no BSH activity. In contrast, the other bacteriocin producer used in this study, *E. faecium* BFE 900, showed BSH activity.

Most of *E.faecium* strains, including the acteriocin producer *E. faecium* BFE 900, showed weak hydrophobicity (60.5%). Among the *E. faecalis* strains, five strains exhibited moderate hydrophobicity, including the bacteriocin-producing *E. faecalis* BFE 1071. The other *E. faecalis* strains exhibited only weak hydrophobicities.

The selected enterococci were capable of adhering to HT29-MTX cells, however at a low level of generally less than 30%. Bacteriocin-producing *E. faecium* BFE 900 had the lowest level of adherence (3%) whilst *E. faecium* FAIR-E 15 the highest (31%). The enterocin 1071-producing strain *E. faecalis* BFE 1071 also exhibited a moderate to high adherence of 17% when compared to the other enterococci cells. The probiotic control strain *E. faecium* SF68 did not show high (<15%) adherence to HT29-MTX cells.

Upon physiological testing for virulence of probiotic enterococci candidates (DNAse production, gelatinase, aggregation substance, haemolysin and mucin degradation, the

enterocin 1071- producing strain *E. faecalis* BFE 1071 showed no positive results along with other *E. faecium* probiotic candidate strains, except *E. faecium* FAIR-E 210 which is capable of degrading the mucin. These results were confirmed with PCR amplification of enterococcal surface protein (Esp) and adhesin to collagen from *E. faecalis* (Ace) of these genes, which showed that all probiotic enterococci candidates posses no such genes.

Cloning of *bsh* genes from BSH-positive *E. faecium* FAIR-E 345 and DNA sequencing of this gene showed that the *bsh* gene encodes a protein of 324 with an isoelectric point of 4.877. Amino acid sequence deducted from *bsh* revealed highest homology to that of the BSH protein from *Listeria monocytogenes, Lactobacillus* (*L.*) *plantarum (68.5% identity),* while lower homologies to BSH proteins reported previously for *L. johnsonii* (52.8% identity), *L. gasseri* (49.2% identity), *Clostridium perfringens* (42.2% identity), *Bifidobacterium longum* (38.2% identity), and *L. acidophilus* (34.8% identity) (Christiaens et al., 1992; Coleman and Hudson, 1995; Elkins and Savage, 1998; Tanaka et al., 2000; Russell and Klaenhammer, 2001). Furthermore, the BSH protein from *E. faecium* FAIR-E 345 showed homology to the penicillin V acylase (PVA) of *Bacillus sphaericus* (32.7% identity) (Suresh et al., 1999). Based on hybridisation using a BSH gene probe and the DIG-dUTP labeling and detection kit, it was found that *bsh* gene of enterococci located in the chromosome. This would imply that this trait is likely to be stable i such BSH^+ probiotic enterococci and most likely not subject to plasmid lost. It is considered as unlikely that this trait is transferable by conjugation from BSH^+ food strains or probiotic strains which carry no virulence determinants, to such food strains which do harbour virulence determinants and which could have, as a consequence of BSH activity, been better equipped for intestinal survival.

Overall, there was no large effect of feeding the enterococci on the numbers of bacteria in the rat feaces. As expected, it appeared that feeding of enterococci increased the numbers of these bacteria in the rat faeces. This was especially obvious for the rat treatment group C in which rats were fed viable bacteriocin producing *E.faecalis* BFE 1071 and for which there was a noticeable increase in enterococci counts from day 1 to day 7. A slight increase in lactobacilli was noticed belonging to treatment groups C and D, which may be as a stimulatory effect of the bacteriocin-producing *E. faecalis* BFE 1071. A slight decrease in numbers of clostridia treated with bacteriocin producing *E.faecalis* BFE 1071 strain was noticed from day 1 of treatment up to day 5, which may be explained by the antimicrobial activity of the bacteriocin produced by *E.faecalis* BFE 1071. However, after day 5 clostridial counts increased again, which may imply that clostridia became resistant to the bacteriocin. Otherwise, treatment of either bacteriocin producing *E.faecalis* BFE 1071 or its bacteriocin-

negative mutant was not deemed to have any effect on counts of the other bacterial groups, i.e. eubacteria, Gram-positive anaerobic cocci and bifidobacteria. Bcaterial counts, however, could not give any information on the composition of the enterococci and lactobacilli microflora; for this reason predominant isolates were isolated and identified using phenotypic and genotypic methods.

One hundred and thirty-nine enterococci isolates from the animal experiment were phenotypically characterised, including their ability to ferment key sugars. According to these, enterococci isolates were dominated by presumptive *E. faecalis* (121 isolates), *E. faecium* (12 strains), *E. durans* (3 isolates) and *Lactococcus* spp., or unidentifiable enterococci (2 isolates). Group C and D had relatively more *E. faecalis* isolates than group A and B. This could be explained by the fact that the animals in these groups were fed with diet containing lyophilized biomass of bacteriocin producing *E.faecalis* BFE 1071 and its mutant. *E.faecalis* BFE 1071 could probably survive and support other *E.faecalis* strains. Hence, *E. faecalis* dominated the population.

One hundered lactobacilli isolates were characterised phenotipically. It was found that lactobacilli isolates consisted of presumptive *L. reuteri* (6 isolates), strains belonging to the *L. acidophilus* group (50 isolates) and strains belonging to the *L. murinus* group (44 isolates). The latter consisted of *L. murinus, L. salivarius* and *L. agilis.* For accurate identification of the isolates from the enterococci and lactobacilli groups, genotypic methods such as RAPD-PCR strain typing or group-specific PCR were used.

By analysis of the RAPD-PCR fingerprints of isolates together with enterococci type strains, it was found that enterococci strains of rat faecal samples consisted of *E. gallinarum* (4 strains), *E. casseliflavus* (4 strains), and *E. faecalis* (111 strains). In addition, one cluster (consisted of 17 strains) revealed enterococci that did not group together with one of the reference strains used in this study. The bacteriocin-producing *E. faecalis* BFE 1071 could survive in the rat gastrointestinal tract and dominate among enterococci from the fecal flora. This was because the fingerprint of the bacteriocin producing *E. faecalis* BFE 1071 and and its mutant were characteristic, and this fingerprint was almost identical from many of the *E . faecalis* isolates that were recovered from groups C and D. Lactobacilli strains of faecal samples consisted of *L. reuteri* (5 strains), *L. salivarius* (45 strains), *L. gasseri* (16 strains) and *L. johnsonii* (32 strains). One isolate each had fingerprints which were similar to *L. crispatus* and *L. murinus*. An ‘Enterococccus effect’ was noted for bacteria isolated from faeces of rats from groups B and C and this ‘*Enterococcus* effect’ particularly appeared to stimulate *L. johnsonii*, i.e. feeding of the enterococci probiotics led to an increase in *L. johnsonii*.

The overall objective ot this thesis has been met and all of the the aims were fulfilled. Bacteriocin producing *E.faecalis* BFE 1071 has probiotic properties and showed so far no virulence traits. Bacteriocin producing *E.faecalis* BFE 1071 could survive in the gastrointestinal tract of rats and showed a possible bacteriocin activity as evidenced by decreases in numbers of clostridia as well as an '*Enterococcus* effect', that the supports the growth of particular groups of bacteria (*L. johnsonii* and *L. gasseri*) in faeces. Although an *Enterococcus* probiotic was previously described to stimulate lactobacilli growth (Vahjen et al., 2002) it was not shown whether a shift in predominance of the *Lactobacillus* species takes place as isolates were not characterised to species level. In this study, it was shown that the effect of bacteriocin production on the composition of the intestinal lactobacilli flora was probably negligible, although a slight increase in isolation frequency of *L. reuteri* strains occurred in the faeces of group C animals. However, as mentioned above, a definite *Enterococcus*-effect was noted which did influence the composition of the lactobacilli microflora. This study therefore, gives new insight on the effect of a probiotic on the composition of the gastrointestinal microflora. Furthermore, the study supplied detail on the predominant bacterial groups occurring in rat faeces, and the composition of the rat lactic acid bacterial microflora. To date, only very limited information is available on the lactic acid bacteria species that predominate in this ecosystem. This study clearly showed that in the rat faeces *L. salivarius L. reuteri*, *L. johnsonii* and *L. gasseri* strains predominate, with *L. salivarius* being most ferequently isolated. For the enterococci, *E. faecalis* strain predominated, together with enterococci which in this study could not be identified and which may either consist of presently undescribed enterococci species, or may belong to *E. ratti*, *E. moraviensis* or *E. haemoperoxidus.* Further studies would be need to identify these strains further. The effects of the bacteriocin-producing probiotic noted in this study using a rat model probably can not be directly interpolated for the human situation, as a result of differences in physiology, as well as composition of the host autochthonous microflora. However, it support the idea also in the human environment a bacteriocin-producing probiotic could potentially alter the autochthonous microflora.

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Curriculum Vitae

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Education

1975 – 1981	Primary School, SD Persit II, Palembang, Indonesian
1981 – 1984	Junior High School, SMPN 10, Palembang, Indonesian
1984 – 1987	Senior High School, SMAN 6, Palembang, Indonesian

High School Education

1987 – 1992	Under graduate study to obtain „Bachelor of Science with Honours“ Technologie of Agriculture at the University of Sriwijaya, Palembang, Indonesia
1994 – 1997	Graduate study to achieve „Master of Science“ in Biotechnology at Gadjah Mada University, Yogyakarta, Indonesia
1998 – present	Graduate study to achieve „Doctor of Philosophy“ at theUniversity of Karlsruhe, Karlsruhe, Germany. Research was conducted at the Bundesforschungsanstalt für Ernährung, Institut für Hygiene und Toxikologie, Karlsruhe, Germany, financed by DAAD (German Academic Exchange Service).

Job Experiences

1992 – 1993	Position as assistant lecturer at the University of Sriwijaya, Indonesia
1993 – 1998	Position as junior lecturer at the University of Sriwijaya, Indonesia
2001 – 2002	Position as scientific assistant at Engler-Bunte-Institut, Lehrstuhl für Wasserchemie, University of Karlsruhe, Germany

Special Knowledge

EDV (electronic data processing)-experiences in software application (Microsoft Word, Excel and Power Point, DNAStar, DNA Strider, Bionumerics)

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Karlsruhe, 29 September, 2003

Agus Wijaya

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4. Wijaya, A., Löffler, C., Hucker, S., Holzapfel, W.H., W.H., und Franz, C.M.A.P. 2000. Enterococci as potential probiotic bacteria: technological, functional and safety aspects. Poster zum „5th Karlsruhe Nutrition Congress: Functional Food, Challenges for the New Millenium. Karlsruhe, 22.-24. 10. 2000.