Characterization of bacteriocins produced by lactic acid bacteria from fermented beverages and optimization of starter cultures

by

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

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Summary

Lactobacillus plantarum JW3BZ and *Lactobacillus fermentum* JW15BZ isolated from boza, a Bulgarian cereal based fermented beverage, produce bacteriocins JW3BZ and JW15BZ active against a wide range of food spoilage and pathogenic bacteria. Strains JW3BZ and JW15BZ are resistant to low pH (pH 2.0–4.0). Both strains grow well in MRS broth with an initial pH ranging from 5.0 to 10.0. Strain JW3BZ displayed intrinsic resistance to bile salts. Strain JW15BZ, on the other hand, is sensitive to bile salts exceeding concentrations of 0.3% (w/v). Both strains are weakly hydrophobic and are resistant to a wide range of antibiotics, anti-inflammatory drugs and painkillers. Strains JW3BZ and JW15BZ adhered at 4% to Caco-2 cells and they did not compete with *Listeria monocytogenes* Scott A for adhesion. A homologue of *MapA,* a gene known to play a role in adhesion, was detected in *L. plantarum* JW3BZ. Both strains have high auto- and co-aggregation properties.

Bacteriocin JW15BZ was partially purified with ammonium sulfate, followed by separation on Sep-Pak C_{18} and reverse phase High Pressure Liquid Chromatography (HPLC). Two separate peaks with antimicrobial activity were recorded for bacteriocin JW15BZ, suggesting that it consists of at least two antimicrobial peptides. *Lactobacillus plantarum* JW3BZ contains genes homologous to *plnE*, *plnF* and *plnI* of the *plnEFI* operon that encode for two small cationic bacteriocin-like peptides with double-glycine-type leader peptides and its respective immunity proteins. The antimicrobial activity displayed by strain JW3BZ may thus be ascribed to the production of plantaricins E and F. Bacteriocin JW3BZ and JW15BZ displayed activity against herpes simplex virus (HSV-1) (EC_{50}=200 µg/ml).

Both strains were identified in boza after 7 days at storage at 4 ^{o}C and repressed the growth of *Lactobacillus sakei* DSM 20017, indicating that the bacteriocins are produced *in situ*. The sensory attributes of boza prepared with different starter cultures did not vary considerably, although statistical differences were observed for acidity and yeasty aroma.

Encapsulation of strain JW3BZ and JW15BZ in 2% sodium alginate protected the cells from low pH (1.6) and 2.0% (w/v) bile. The rate at which cells were released from the matrix varied, depending on the conditions. Better survival of strains JW3BZ and JW15BZ encapsulated in 2% (w/v) alginate was observed during 9 h in a gastro-intestinal model. Highest release of cells was observed at conditions simulating colonic pH (pH 7.4), starting

from 56-65% during the first 30 min, followed by 87%. Complete (100%) release was recorded after 2.5 h at these conditions.

Strains JW3BZ and JW15BZ could be used as starter cultures in boza. The broad spectrum of antimicrobial activity of bacteriocins JW3BZ and JW15BZ is an added advantage, rendering the cells additional probiotic properties. Encapsulation of the cells in alginate gel increased their resistance to harsh environmental conditions and may be the ideal method to deliver viable cells *in vivo*.

Opsomming

Lactobacillus plantarum JW3BZ en *Lactobacillus fermentum* JW15BZ, geïsoleer uit boza, ‘n Bulgaarse graan-gebaseerde gefermenteerde drankie, produseer bakteriosiene JW3BZ en JW15BZ met aktiwiteit teen ‘n wye verskeidenheid voedselbederf- en patogeniese bakterieë. Beide stamme weerstaan lae pH (2.0-4.0) en groei goed in vloeibare MRS medium met ‘n aanvanklike pH van 5.0–10.0. Stam JW3BZ het ‘n goeie intrinsieke weerstand teen galsoute, terwyl stam JW15BZ sensitief is vir galsoutkonsentrasies hoër as 0.3% (m/v). Beide stamme is swak hidrofobies en toon weerstand teen ‘n wye reeks antibiotika, anti-inflammatoriese middels en pynstillers. Beide stamme heg teen 4% aan Caco-2 en kompeteer nie met *L. monocytogenes* Scott A vir aanhegting nie. Stam JW3BZ besit ‘n homoloog van *MapA*, ‘n geen wat vir adhesie kodeer. Beide stamme toon hoë outo- en ko-aggregasie eienskappe.

Bakteriosien JW15BZ is gedeeltelik met behulp van ammonium sulfaat gesuiwer, gevolg deur skeiding in ‘n Sep-pak C_{18} kolom en tru-fase hoëdruk vloeistofchromatografie (“HPLC”). Twee afsonderlike pieke met antimikrobiese aktiwiteit is vir bakteriosien JW15BZ waargeneem. Bakteriosien JW15BZ bestaan dus uit ten minste twee antimikrobiese peptiede. Stam JW3BZ besit die operon *plnEFI* wat kodeer vir twee klein kationiese bakteriosienagtige peptiede met dubbel-glisien leierpeptiede en ‘n immuniteitsproteïen. Beide die bakteriosiene toon antivirale aktiwiteit (EC_{50}= 200µg/ml) teen die herpes simpleks virus (Tipe 1).

Beide stamme is oor ‘n periode van 7 dae van berging by 4 ^{o}C in boza waargeneem. Die onderdrukking van *Lactobacillus sakei* DSM20017 in boza deur beide die stamme dui op moontlike *in situ* produksie van die twee bakteriosiene. Boza wat met verskillende suurselkulture berei is, het nie noemenswaardig verskil op grond van hul sensoriese eienskappe nie, alhoewel betekenisvolle verskill waargeneem is betreffende die suurheidsgraad en gis-aroma.

Enkapsulering van *L. plantarum* JW3BZ en *L. fermentum* JW15BZ met ‘n 2% (m/v) alginaat matriks het die selle teen lae pH (1.6) en 2.0% (m/v) galsoute beskerm. Spesifieke toestande het die tempo waarteen selle uit die matriks vrygestel is bepaal. Ge-enksapsuleerde selle het strestoestande oor ‘n periode van 9 uur in ‘n gastro-intestinale model beter oorleef. Die grootste aantal selle is vrygelaat onder toestande wat die pH van die kolon simuleer (pH 7.4). ‘n Aanvanklike sel vrystelling van 56-65% is waargeneem gedurende die eerste 30 min, maar het tot 87% toegeneem. Totale sel vrystelling (100%) is na 2.5 ure waargeneem.

Stamme JW3BZ en JW15BZ kan dus as suurselkulture gebruik word in die produksie van boza. Die wye spektrum antimikrobiese aktiwiteit van bakteriosiene JW3BZ en JW15BZ is ’n bykomende voordeel en verleen addisionele probiotiese eienskappe aan die selle. Enkapsulering van die selle in alginaat jel het hul meer bestand gemaak teen stresvolle omgewingstoestande en dit mag dalk die ideale metode wees om lewende selle *in vivo* vry te stel.

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To Mom Santie and Dad Louis

My pillars of strength

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CHAPTER 1

Introduction

Introduction

The concept of probiotic foods has gained significant interest since its introduction to clinical nutrition and food science during the 1980's (Fuller, 1989; Shortt, 1999). Most probiotic foods are milk-based, although many cereals with added probiotic cultures are appearing on the market. Cereal has a high nutritional value (vitamins, proteins, dietary fiber, energy, and minerals) and is cultivated on more than 73% of agricultural soil while contributing to more than 60% of the world's food production (Charalompopoulos et al., 2002; Angelov et al., 2006).

Most lactic acid bacteria naturally present in food and strains used as starter cultures are also present in the gastrointestinal tract of humans and animals (Ahrné et al., 1998; Vogel et al., 1999; Molin, 2001). A number of papers have been published on the identification and classification of lactic acid bacteria in cereal-based food and a few reported on their probiotic and bacteriocinogenic properties (Kimura et al., 1997; Choi et al., 1999; Kabadjova et al., 2000; Todorov and Dicks, 2004, 2005; Todorov et al., 2006; Von Mollendorff et al., 2006). Research on the application of probiotic strains as starter cultures is lacking and need to be addressed.

Starter cultures have to meet certain selection criteria to be considered probiotic, i.e. they have to survive at low pH and in the presence of high bile salt concentrations, and need to adhere to the mucosa or epithelial cells (Salminen et al., 1996; Mattila-Sandholm et al., 1999; Reid and Burton, 2002). Colonization is important, as it plays a vital role in survival of the strains, stimulation of the immune system, enhanced healing of damaged mucosa, and antagonism against pathogenic bacteria (Isolauri et al., 1991; Salminen et al., 1996; Rolfe, 2000; Reid and Burton, 2002). Probiotic bacteria play an important role in reducing symptoms related to lactose intolerance and irritable bowel syndrome (IBS), may prevent diarrhea, colon cancer and allergies and may even decrease serum cholesterol levels (Gilliland, 1990; Isolauri et al., 1991; Salminen et al., 1998; Fooks et al., 1999; Kalliomaki et al., 2001; O'Mahony et al., 2005).

A number of papers have been published on bacteriocinogenic lactic acid bacteria isolated from fermented food products (Kabadjova et al., 2000; Todorov and Dicks, 2004, 2005; Todorov et al., 2006; Von Mollendorff et al., 2006). A few bacteriocins are active against a

number of food spoilage and pathogenic bacteria, including Gram-negative bacteria (Todorov and Dicks, 2004, 2005; Todorov et al., 2006; Von Mollendorff et al., 2006). Apart from bacteriocins, lactic acid bacteria produce lactic acid, hydrogen peroxide, benzoic acid, fatty acids, diacetyl and other low molecular weight compounds (Heller, 2001). Bacteriocinogenic probiotic bacteria could be beneficial when used as starter cultures, as it may prolong the shelf-life of the products and provide the consumer with a healthy dietary component at a considerable low cost (Goldin, 1998). To qualify as starter cultures lactic acid bacteria have to be present at sufficient numbers in fermented products (Heller, 2001). Furthermore, starter cultures should not enhance acidification during storage and should not have adverse effects on the taste and aroma profiles (Heller, 2001).

Most probiotic products do not have a long shelf-life, even when stored at low temperatures. The number of probiotic bacteria required to exert a beneficial effect is often too low in probiotic products (Kailasapathy and Chin, 2000). This may be due to the low pH associated with many of the probiotic products. To combat this problem, encapsulation of probiotic bacteria in hydrocolloid beads have been studied (Rao et al., 1989). Entrapment of lactic acid bacteria in calcium alginate beads increased their survival by 80 to 95% (Mandal et al., 2006). Encapsulation may thus protect the cells against harsh conditions such as acid and bile, usually associated with the gastrointestinal tract. Encapsulation has also been used to deliver lactic acid bacteria to specific targets in the gastrointestinal tract (Anal and Singh, 2007).

In this study, the probiotic and bacteriocinogenic properties of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ, isolated from boza, a cereal-based fermented beverage produced in the Balkan states, were studied. The probiotic properties investigated included tolerance to acid and bile conditions, adhesion to epithelial cells, presence of adhesion genes, aggregation and co-aggregation ability and hydrophobicity. Bacteriocinogenic properties studied included, antiviral activity, cytotoxicity and spectrum of activity. These strains were also implemented as starter cultures for the production of boza. The presence of strains JW3BZ and JW15BZ in the product after fermentation and storage was determined by performing denaturing gradient gel eletrophoresis (DGGE). Encapsulation of strains JW3BZ and JW15BZ to improve their survival under gastrointestinal conditions were also studied.

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CHAPTER 2

Cereal-based Fermented foods: A Review

1. Introduction

Fermentation of food is a very old technology, with earliest records dating back to 6000 BC (Fox et al., 1993). The methodologies and knowledge associated with the manufacturing of fermented products were handed down from generation to generation within local communities (Caplice and Fitzgerald, 1999). These communities needed products to be produced in small quantities for distribution in or around the immediate area. However, the population increase in towns and cities, due to the industrial revolution by the middle of the 19th century, resulted in a need for these products to be produced in larger quantities. This led to commercial production of fermented food. Furthermore, the blossoming of Microbiology as a science from the 1850's onwards and the development of pasteurization by Louis Pasteur towards the end of the 19th century had a major impact on our understanding of the biological basis of fermentation. According to Caplice and Fitzgerald (1999) milk, meat, cucumber and cabage are the main substrates used in the production of most familiar fermented products.

Large-scale production required products with consistent quality. Characterization of microorganisms responsible for the fermentation of various fermented products led to the isolation of starter cultures, which could be produced on a large-scale to supply factories involved in the manufacturing of these products. Defined starter cultures replaced undefined starters traditionally used in manufacturing and ensured reliable fermentation and consistent product quality (Caplice and Fitzgerald, 1999). The intensive use of starter cultures has some drawbacks and can lead to unsatisfactory strain performance (Ross et al., 2002). In the case of lactococcal fermentation, bacteriophage proliferation can affect the performance of cheese starter cultures (Klaenhammer and Fitzgerald, 1994).

The digestibility, nutritional value, organoleptic qualities and shelf-life of food are increased by fermentation (Hancioglu and Karapinar, 1997). A number of lactic acid bacteria used as starter cultures in fermented food have probiotic properties and may confer potential health benefits to the consumer.

1.1 Lactic acid bacteria in cereal-based fermented products

Cereal and cereal-legume-based fermented products are consumed in almost all parts of the world (Table 1) and form a major part of the diet in most African countries. Cereals are cultivated on more than 73 % of agricultural soil and contribute to over 60 % of the world's food production, providing vitamins, proteins, dietary fiber, energy, and minerals

Table 1

Cereal and cereal-legume-based fermented food and beverages from different regions of the world

Product	Country	Substrate	Microorganism[a]	Form in which consumed
Adai	India	Cereal/legume	*Pediococcus* sp., *Streptococcus* sp., *Leuconostoc* sp.	Breakfast or snack food
Anarshe	India	Rice	Lactic acid bacteria	Breakfast, sweetened snack food
Ang-kak (anka, red rice)	China, Southeast Asia, Syria	Rice	*Monascus prupureus*	Dry red powder as colorant
Atole	Southern Mexico	Maize	Lactic acid bacteria	Porridge based on maize dough
Bagni	Caucasus	Millet	Unknown	Liquid drink
Banku	Ghana	Maize, or maize and cassava	Lactic acid bacteria, molds	Dough as staple
Bhattejaanr	India, Sikkim	Rice	*H. anomala*, *Mucor rouxianus*	Sweet sour alcoholic paste
Bogobe	Botswana	Sorghum	Unknown	Thick, acidic
Bouza	Egypt	Wheat, malt	Lactic acid bacteria	Alcoholic thin gruel
Boza	Albania, Turkey, Bulgaria, Romania	Wheat, millet, maize and other cereals	*L.acidophilus*, *L. coprophilus*, *L. brevis*, *L. plantarum*, *L. fermentum*, *Le. mesenteroides, Le. mesenteroides* subsp. *dextranicum*, *Le. raffinolactis*, *L. rhamnosus*, *L. caryniformis*, *L. paracasei*, *L. pentosus*, *L. sanfrancisco*, *Lc. lactis* subsp. *lactis*, *P. pentosaceus*, *Le. oenos* (reclassified to *Oenococcus oeni*), *Weisella confusa* and *Weisella paramesenteroides*, *S. cerevisiae*, *C. glabrata*, *C. tropicalis*, *G. penicilatum*, *S. carlsbergensis*, *S. uvarum C. diversa, C. pararugosa, Isatchenkia orientalis, Pichia fermentans, Rhodotorula mucilaginosa, C. inconspicua, Torulaspora delbrueckii, Pichia guillermondii, and Pichia norvegensis*	Thick, sweet, slightly sour beverage
Braga	Romania	Millet	Unknown	Liquid drink
Brem	Indonesia	Rice	Unknown	Cake
Brembali	Indonesia	Rice	*Mucor indicus*, *Candida* sp.	Dark brown alcoholic drink
Burukutu	Nigeria, Benin, Ghana	Sorghum	*S. cerevisiae*, *Le. mesenteroides*, *Candida* sp.	Alcoholic beverage of vinegar-like flavor
Busa	Syria, Egypt, Turkestan	Rice or millet	*Lactobacillus* sp., *Saccharomyces* sp.	Liquid drink
Busaa	Nigeria, Ghana	Maize	*L. helveticus*, *L. salivarus*, *L. casei*, *L. brevis*, *L. plantarum*, *L. buchneri*, *S. cerevisiae*, *Penicillium damnosus*	Alcoholic beverage

Product	Country	Substrate	Microorganism[a]	Form in which consumed
Bussa	Kenya	Maize, sorghum, malt, finger millet	*C. crusei*, *S. cerevisiae*, *L. helveticus*, *L. salivarius*, *L. plantarum*	Food refreshment drink
Chee-fan	China	Soybean wheat curd	*Mucor* sp., *Aspergillus glaucus*	Cheese-like product, eaten fresh
Chicha	Peru	Maize	*Aspergillus*, *Penicillium*, yeast, bacteria	Spongy solid eaten with vegetables
Chikokivana	Zimbabwe	Maize and millet	*S. cerevisiae*	Alcoholic beverage
Chinese yeast	China	Soybeans	*Mucraceous* molds, yeast	Solid eaten fresh with rice
Chongju	Korea	Rice	*S. cerevisiae*	Alcoholic clear drink
Dalaki	Nigeria	Millet	Unknown	Thick porridge
Darassum	Mongolia	Millet	Unknown	Liquid drink
Dhokla	Northern India	Rice or wheat and Bengal gram	*Le. mesenteroides*, *St. faecalis*, *Torulopsis candida*, *T. pullulans*	Steamed cake for breakfast or snack food
Doro	Zimbabwe	Finger millet malt	Yeast and bacteria	Colloidal thick alcoholic drink
Dosa	India	Rice and Bengal gram	*Le. mesenteroides, Streptococcus faecalis, Torulopsis candida*, *T. pullulans*	Griddled cake for breakfast or snack food
Enjera	Ethiopia	Tef or other cereals	*Le. mesenteroides, P. cerevisiae*, *L. plantarum*, *S. cerevisiae*	Pancake
Gari	Nigeria	Cassava	*Leuconostoc, Alcaligenes, Corynebacterium*, *Lactobacillus* sp.	Staple, cake, porridge
Hamanatto	Japan	Wheat, soybeans	*A. oryzae, Streptococcus* sp., *Pediococcus* sp.	Raisin-like, soft, flavoring agent for meat and fish, eaten as snack
Hopper	Sri Lanka	Rice and coconut water	Yeast, lactic acid bacteria	Stake-baked pancake
Hulumur	Sudan	Red sorghum	*Lactobacillus* sp.	Clear drink
Idli	South India, Sri Lanka	Rice grits and black gram	*Le. mesenteroides*, *St. faecalis*, *Torulopsis* sp., *Candida* sp., *Tricholsporon pullulans*	Steamed cake for breakfast food
Ilambazi lokubilisa	Zimbabwe	Maize	Lactic acid bacteria, yeast and molds	Porridge as weaning food
Injera	Ethiopia	Sorghum, tef, maize or wheat	*C. guilliermondii*	Bread-like staple
Jaanr	India, Himalayas	Millet	*H. anomala*, *Mucor rouxianus*	Alcoholic paste mixed with water
Jalebies	India, Nepal, Pakistan	Wheat flour	*S. bayanus*	Pretzel-like syrup-filled confection
Jamin-bang	Brazil	Maize	Yeast, bacteria	Bread, cake-like
Kaanga-Kopuwai	New Zealand	Maize	Yeast	Soft, slimy eaten as vegetable
Kachasu	Zimbabwe	Maize	Yeast	Alcoholic beverage

Product	Country	Substrate	Microorganism[a]	Form in which consumed
Kaffir	South Africa	Malt of sorghum, maize	Lactic acid bacteria	Beer
Kaffir beer	South Africa	Kaffir corn	Yeast, lactic acid bacteria	Alcoholic drink
Kanji	India	Rice and carrots	*H. anomala*	Liquid added to vegetables
Kecap	Indonesia	Wheat, soybeans	*A. oryzae*, *Lactobacillus* sp., *Hansenula*, *Saccharomyces*	Liquid flavoring agent
Kenkey	Ghana	Maize	*L. fermentum*, *L. reuteri*, *Candida* sp., *Saccharomyces* sp., *Penicillium* sp., *Aspergillus* sp. and *Fusarium* sp.	Mush, steamed eaten vegetables
Khanomjeen	Thailand	Rice	*Lactobacillus* sp., *Streptococcu*s sp.	Noodle
Khaomak	Thailand	Rice	*Rhizopus* sp., *Mucor* sp., *Saccharomyces* sp., *Hansenula* sp.	Alcoholic sweet beverage
Kichudok	Korea	Rice	*Le. mesenteroides*, *S. faecalis*, yeast	Steamed cake
Kichudok	Korea	Rice, takju	*Saccharomyces sp*.	Steamed cake
Kishk	Egypt, Syria, Arabian countries	Wheat and milk	*L. plantarum*, *L. brevis*, *L. casei*, *B. subtilis* and yeasts	Solid, dried balls, dispersed rapidly in water
Kisra	Sudan	Sorghum, millet	*Lactobacillus* sp., *Acetobacter* sp. *S. cerevisiae*	Pancake
Koko	Ghana	Maize	*Enterobacter clocae*, *Acinetobacter* sp., *L. plantarum*, *L. brevis*, *S. cerevisiae*	Porridge as staple
Kurdi	India	Wheat	Unknown	Solid, fried crisp, salty
Kwunu-Zaki	Nigeria	Millet	Lactic acid bacteria, yeast	Paste used as breakfast dish
Lao-chao	China, Indonesia	Rice	*Rhizopus oryzae*, *R. chinensis*, *Chlamydomucor oryzae*, *Saccharomycopsis* sp.	Paste, soft juicy, glutinous consumed as such, as dessert or combined with eggs or seafood
Mahewu	South Africa	Maize and wheat flour	*St. lactice*, *Lactobacillus* sp.	Sour drink
Mangisi	Zimbabwe	Millet	Unknown	Sweet/sour non-alcoholic drink
Mantou	China	Wheat flour	*Saccharomyces sp*.	Steamed cake
Mawe	South Africa	Maize	Lactic acid bacteria, yeast	Basis for preparation of many dishes
Mbege	Tanzania	Malted millet acidic banana juice	Unkown	Food, refreshment drink
Me	Vietnam	Rice	Lactic acid bacteria	Sour food ingredient
Merissa	Sudan	Sorghum and Millet	*Saccharomyces sp*.	Alcoholic drink
Minchin	China	Wheat gluten	*Paecilomyces* sp., *Aspergillus* sp., *Cladosporium* sp., *Fusarium* sp., *Syncephalastum* sp., *Penicillium* sp. and *Trichothecium* sp.	Solid as condiment
Mirin	Japan	Rice, alcohol	*A. oryzae*, *A. usamii*	Alcoholic liquid seasoning
Miso	Japan, China	Rice and soy beans or rice other cereals such as barley	*A. oryzae*, *Torulopsis etchellisii*, *Lactobacillus* sp.	Paste use as seasoning

Product	Country	Substrate	Microorganism[a]	Form in which consumed
Mungbean starch	China, Thailand, Korea, Japan	Mungbean	*Le. mesenteroides*, *L. casei*, *L. cellobiosus*, *L. fermentum*	Noodle
Munkoyo	Africa	Kaffir corn, millet or maize plus roots of munkoyo	Unknown	Liquid drink
Mutwiwa	Zimbabwe	Maize	Lactic acid bacteria, bacteria and molds	Porridge
Nan	India, Pakistan, Afghanistan, Iran	Unbleached wheat flour	*S. cerevisiae*, Lactic acid bacteria	Solid as snack
Nasha	Sudan	Sorghum	*Streptococcus* sp., *Lactobacillus* sp., *Candida*, *S. cerevisiae*	Porridge as a snack
Ogi	Nigeria	Maize, sorghum, or millet	*L. plantarum*, *Corynebacterium* sp., *Acetobacter*, yeast	Sour porridge, baby food, main meal
Ogi	Nigeria, West Africa	Maize, sorghum or millet	*L. plantarum*, *S.cerevisiae*, *C. mycoderma*, *Corynebacterium* sp., *Aerobacter* sp., *Rhodotorula* sp., *Cephalosporium* sp., *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp.	For breakfast or weaning food for babies
Otika	Nigeria	Sorghum	Unknown	Alcoholic beverage
Papadam	India	Black gram	*Saccharomyces* sp.	Breakfast or snack food
Pito	Nigeria, Ghana	Maize, sorghum, maize and sorghum	*G. candidum*, *Lactobacillus* sp., *Candida* sp.	Alcoholic dark brown drink
Pozol	Southeasters Mexico	Maize	Lactic acid bacteria, *Candida* sp.	Spongy dough formed into balls; basic food
Puto	Philippines	Rice, sugar	*Le. mesenteroides*, *Streptomyces faecalis*, yeasts	Solid paste as seasoning agent, snack
Rabdi	India	Maize and buttermilk	*P. acidilactici*, *Bacillus* sp., *Micrococcus* sp.	Semisolid mash eaten with vegetables
Rye bread	Denmark	Rye	Lactic acid bacteria	Sandwich bread, bread
Sake	Japan	Rice	*Saccharomyces* sp.	Alcoholic clear drink
Seketeh	Nigeria	Maize	*S. cerevisiae*, *St. chevalieri*, *St. elegans*, *L. plantarum*, *Lc. lactis*, *B. subtilis*, *A. niger*, *A. flavus*, *Mucor rouxii*	Alcoholic beverage
Shaosinghjiu	China	Rice	*S. cerevisiae*	Alcoholic clear beverage
Shoyu (soy sauce)	Japan, China, Taiwan	Wheat and soybeans	*A. oryzae*, *Lactobacillus* sp., *Zygosaccharomyces rouxi*	Liquid seasoning
Sierra rice	Ecuador	Rough rice	*A. flavus*, *A. candidans*, *B. subtilis*	Brownish-yellow dry rice
Sorghum beer	South Africa	Sorghum, maize	Lactic acid bacteria, yeast	Liquid drink, acidic, weakly alcoholic
Sour bread	Germany	Wheat	Lactic acid bacteria, yeast	Sandwich bread
Soybean milk	China, Japan	Soybeans	Lactic acid bacteria	Drink
Takju	Korea	Rice, wheat	Lactic acid bacteria, *S. cerevisiae*	Alcoholic turbid drink

Product	Country	Substrate	Microorganism[a]	Form in which consumed
Talla	Ethiopia	Sorghum	Unknown	Alcoholic drink
Tao-si	Philippines	Wheat and soybeans	*A. oryzae*	Seasoning
Taotjo	East India	Roasted wheat meal or glutinous rice and soybeans	*A. oryzae*	Condiment
Tapai pulut	Malaysia	Rice	*Chlamydomucor* sp., *Enomycopsis* sp., *Hansenula* sp.	Alcoholic dense drink
Tape ketan	Indonesia	Rice or cassava	*S. cerevisiae*, *Hansenula anomala*, *Rhizopus oryzae*, *Chlamydomucor oryzae*, *Mucor* sp., *Endomycopsis fibulinger*	Soft, alcoholic solid staple
Tapekekan	Indonesia	Glutinous rice	*Aspergillus rouxii*, *E. burtonii*, *E. fibulinger*	Sweet/sour alcoholic paste
Tapuy	Philippines	Rice, glutinous rice	*Saccharomyces* sp., *Mucor* sp., *Rhizopus* sp., *Aspergillus* sp., *Leuconostoc sp.*, *L. plantarum*	Sweet/sour alcohol
Tapuy	Philippines	Rice	*Saccharomyces* sp., *Mucor* sp., *Rhizopus* sp., *Aspergillus* sp., *Leuconostoc* sp., *L. plantarum*	Sweet/sour alcoholic drink
Tarhana	Turkey	Parboiled wheat meal and yoghurt (2:1)	Lactic acid bacteria	Solid powder, dried seasoning for soups
Tauco	West Java (Indonesia)	Cereals and soybeans	*R. oligosporus*, *A. oryzae*	Seasoning
Tesgüino	Northern and North Western Mexico	Maize	Bacteria, yeast and molds	Alcoholic beverage
Thumba	Eastern India	Millet	*E. fibuliger*	Liquid drink
Tobwa	Zimbabwe	Maize	Lactic acid bacteria	Non-alcoholic drink
Torani	India	Rice	*H. anomala*, *C. quilliermondii*, *C. tropicalis*, *G. candidum*	Liquid as seasoning for vegetables
Uji	Kenya, Uganda, Tanzania	Maize, sorghum, millet or cassava flour	*Le. mesenteroides*, *L. plantarum*	Sour porridge, main meal
Vada	India	Cereal/legume	*Pediococcus* sp., *Streptococcus* sp., *Leuconostoc* sp.	Breakfast or snack food

A.=Aspergillus, B.=Bacillus, C.=Candida, E.= Endomycopsis, *G.=Geothrichum*, *H.=Hansenula*, *L.=Lactobacillus*, *Lc.=Lactococcus*, *Le.=Leuconostoc*, *P.=Pediococcus*, *R.=Rhizopus*, *S.=Saccharomyces* and *St.=Streptococcus.*

Adapted from Chavan and Kadam (1989a), Soni and Sandhu (1990), Harlander (1992), Lee (1994, 1997), Oyewole (1997), Adams (1998), Sankaran (1998) and Blandino et al. (2003).

(Charalampopoulos et al., 2002). It is therefore important to study the nutritional value and basic composition of there products. Many cereal-based products are boiled or steamed, e.g. porridges, rice, pasta and cookies. In many cases the same product is fermented, e.g. pancakes and flatbreads in Asia, sourdough bread in Europe, and a variety of fermented dumplings, porridges, and alcoholic and nonalcoholic beers in Asia and Africa (Salovaara, 2004).

By definition, fermentation is the process in which a substrate is subjected to biochemical modification resulting from the activity of microorganisms and their enzymes (Gotcheva et al., 2000). Yeast, lactic acid bacteria, fungi, or mixtures of these, are mainly responsible for natural cereal-based fermentation. Carbohydrate metabolism is mainly performed by yeast, while bacteria show proteolytic activity (Chavan and Kadam, 1989b). Fermentations by yeast and lactobacilli changes the biochemical composition of fats, minerals and vitamins contained within the cereal.

Yeasts are predominantly responsible for the production of ethanol (e.g. beers and wines), while lactic acid bacteria produce mainly lactic acid (e.g. cereals and fermented milk products). Acetic acid fermentation, responsible for the conversion of alcohol to acetic acid in the presence of excess oxygen, is mainly conducted by *Acetobacter* spp. (Blandino et al., 2003). Alkali fermentation is commonly associated with the fermentation of fish and seeds, widely used as condiment (McKay and Baldwin, 1990).

1.2 Boza, a cereal-based fermented beverage

Boza is a traditional non-alcoholic cereal-based fermented beverage from Bulgaria (Todorov and Dicks, 2004). The beverage is also consumed in other countries of the Balkan region such as the Republic of Macedonia, Serbia, Turkey, Albania and Romania (Gotcheva et al., 2000). Its origin is believed to be from the ancient populations that lived in pre-Ottoman Turkey. The Ottomans were responsible for spreading the recipe over the countries they conquered. Furthermore, the Ottoman Empire was known to feed their army with boza due to its richness in carbohydrates and vitamins A, B, C and E (http://www.veja.com.tr/english/index1.html). In Turkey, boza is served with cinnamon and roasted chickpeas and is enjoyed mainly during the winter months, whereas Bulgarians consume this beverage all year round, mainly at breakfast (http://www.vefa.com.tr/english/tariche.htm). Boza is light to dark beige, viscous and has a sweet to sour bread-like taste (Gotcheva et al., 2000, 2001). Different cereals such as millet, wheat, rye, or combinations of these are used to produce boza. These grains are composed of

an embryo (germ), an endosperm enclosed by the epidermis, and a seed coat (husk) (Gotcheva et al., 2001). The endosperm is filled with granulated starch (Hoseney, 1992). Enzymes and most of the nutrients, such as amino acids, lipids, minerals, sugars and vitamins are located in the embryo. Cellulose, minerals, pentosans and pectins are found in the husk (Nikolov, 1993). Cereal grains generally contain a range of indigenous microflora, including enterobacteria, aerobic spore formers and molds (Salovaara, 2004).

Boza is produced according to traditional family recipes. Various raw materials, at different concentrations and different fermentation processes are used, leading to differences in quality (Zorba et al., 1999). Further variations in the quality and stability may occur because of the interactions between microorganisms that cannot be controlled during fermentation. To avoid such variations, it is necessary to use starter cultures (Zorba et al., 2003). Little is known about the physical and biochemical changes that occur during boza fermentation and, therefore, future studies should focus on these variables. According to Genc et al. (2002) there is a growing interest in producing boza on a large scale and the product has to be properly characterized. The industrial preparation of boza is illustrated in Fig. 1.

Fermentation occurs by natural combinations of yeast and lactic acid bacteria (Todorov and Dicks, 2004). Only a few papers have been published on the microflora of boza. Lactic acid bacteria isolated from boza have been identified as *Lactobacillus acidophilus*, *Lactobacillus coprophilus*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Leuconostoc mesenteroides, Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc raffinolactis*, *Lactobacillus rhamnosus*, *Lactobacillus caryniformis*, *Lactobacillus paracasei*, *Lactobacillus pentosus*, *Lactobacillus sanfrancisco*, *Lactococcus lactis* subsp. *lactis*, *Pediococcus pentosaceus*, *Leuconostoc oenos* (reclassified to *Oenococcus oeni*), *Weisella confusa* and *Weisella paramesenteroides* (Gotcheva et al., 2000; Arici and Daglioglu, 2002; Todorov and Dicks, 2006a; von Mollendorff et al., 2006). The Yeasts thus far isolated are *Saccharomyces cerevisiae*, *Candida glabrata*, *Candida tropicalis*, *Geotrichum candidium* (Gotcheva et al., 2000), *Geotrichum penicilatum*, *Saccharomyces carlsbergensis*, *Saccharomyces uvarum* (Gotcheva et al., 2000; Arici and Daglioglu, 2002), *Candida diversa, Candida pararugosa, Isatchenkia orientalis, Pichia fermentans, Rhodotorula mucilaginosa, Candida inconspicua, Torulaspora delbrueckii, Pichia guillermondii,* and *Pichia norvegensis* (Botes et al., 2007). *Candida tropicalis, Geotrichum penicilatum, C. inconspicua, P. norvegensis* and *R. mucilaginosa* are considered opportunistic human pathogens (Botes et al.,

2007). Some of the lactic acid bacteria identified has been shown to exhibit probiotic properties and to produce bacteriocins (antimicrobial peptides) active against various Gram-positive and Gram-negative bacteria (Table 2), emphasizing the importance of developing them as starter cultures. A number of bacteriocins have been described for lactic acid bacteria isolated from boza (Table 2).

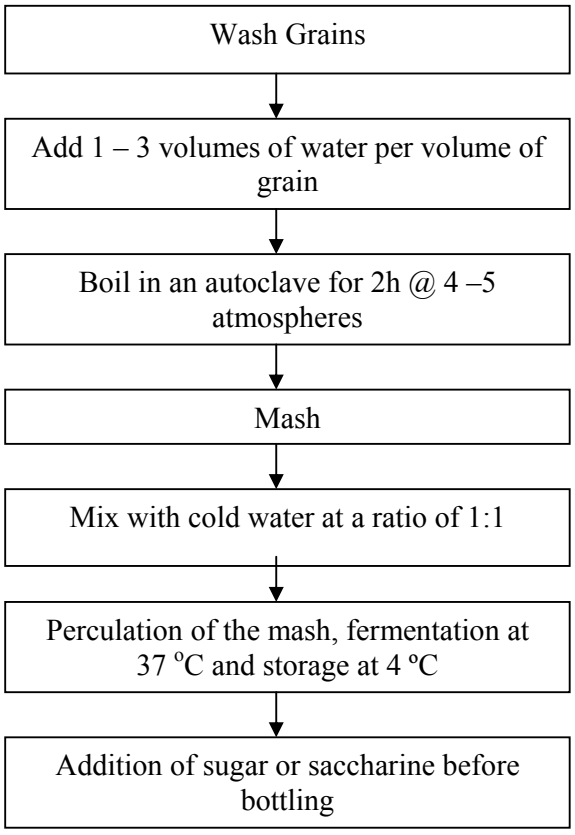

Fig. 1. Diagram summarizing the production process of boza (Gotcheva et al., 2000).

2. Probiotic lactic acid bacteria as starter cultures

Lactobacillus spp. and *Bifidobacterium* spp. are considered the genera containing the most probiotic strains (Corcoran et al., 2004). Probiotics can be defined as "live microorganisms of benefit to the host by improving its intestinal microbial balance when administered in adequate amounts" (FAO/WHO, 2001). The microbial balance is subjected to various unfavorable factors, such as stress, diet and other diseases, which may lead to a decrease in the presence of viable lactobacilli and bifidobacteria in the gastrointestinal tract (Fuller and Gibson, 1997).

Table 2

Activity spectra of bacteriocins produced by lactic acid bacteria isolated from boza. Numbers in paranthesis: number of strains inhibited/number of strains tested (ND = not determined).

Bacteriocins	Strain	Molecular mass (kDa)	Activity spectra	Reference
Pediocin ST18	*Pediococcus pentosaceus* ST18	ND	*Bacillus* spp. (1/3)*, *Carnobacterium piscicola* (1/1), *Carnobacterium divergens* (1/1), *Enterococcus faecalis* (1/1), *Lactobacillus amylophilus* (1/1), *Lactobacillus brevis* (1/1), *Lactobacillus delbrueckii* subsp. *bulgaricus* (1/1), *Lactobacillus fermentum* (1/1), *Lactobacillus helveticus* (2/2), *Lactobacillus plantarum* (7/9*), Leuconostoc mesenteroides* (5/10), *Listeria innocua* (2/2), *Listeria monocytogenes* (1/1), *Pediococcus damnosus* (1/1), *Pediococcus pentosaceus* (2/2), *Staphylococcus aureus* (1/1) and *Streptococcus thermophilus* (1/1).	(Todorov and Dicks, 2005b)
ST194BZ	*Lactobacillus plantarum* ST194BZ	3.0 and 14.0	*Enterobacter cloacae* (1/2), *Enterococcus faecalis* (2/2), *Escherichia coli* (1/2), *Lactobacillus casei* (1/1), *Lactobacillus delbrueckii* subsp. *bulgaricus* (1/1), *Lactobacillus sakei* (1/1) and *Pseudomonas* spp. (1/4).	(Todorov and Dicks, 2005a)
ST242BZ	*Lactobacillus paracasei* ST242BZ	10.0	*E. cloacae* (1/2), *E. faecalis* (2/2), *E. coli* (1/2), *Klebsiella pneumoniae* (1/1), *Lactobacillus casei* (1/1), *Lactobacillus delbrueckii* subsp. *bulgaricus* (1/1), *L. sakei* (1/1*), Pseudomonas* spp. (2/4) and *S. aureus* (7/8).	(Todorov and Dicks, 2006b)
ST284BZ	*Lactobacillus paracasei* ST284BZ	3.5	*E. cloacae* (1/2), *E. faecalis* (2/2), *E. coli* (2/2), *K. pneumoniae* (1/1), *L. casei* (1/1), *L. delbrueckii* subsp. *bulgaricus* (1/1), *L. sakei* (1/1), *Pseudomonas* spp. (3/4) and *Streptococcus* spp. (1/7).	(Todorov and Dicks, 2006b)

Bacteriocins	Strain	Molecular mass (kDa)	Activity spectra	Reference
ST414BZ	*Lactobacillus plantarum* ST414BZ	3.7	*E. cloacae* (1/2), *E. faecalis* (1/2), *E. coli* (1/2), *K. pneumoniae* (1/1), *L. casei* (1/1), *L. curvatus* (1/1) and *Pseudomonas* spp. (1/4).	(Todorov and Dicks, 2006b)
ST461BZ	*Lactobacillus rhamnosus* ST461BZ	2.8	*E. faecalis* (2/2), *E. coli* (1/2), *K. pneumoniae* (1/1), *L. casei* (1/1), *L. curvatus* (1/1), *Pseudomonas* spp. (3/4) and *Streptococcus* spp. (1/7).	(Todorov and Dicks, 2006b)
ST462BZ	*Lactobacillus rhamnosus* ST462BZ	8.0	*E. faecalis* (2/2), *E. coli* (1/2), *L. casei* (1/1), *L. delbrueckii* subsp. *bulgaricus* (1/1), *L. sakei* (1/1) and *Pseudomonas* spp. (2/4).	(Todorov and Dicks, 2006b)
ST664BZ	*Lactobacillus plantarum* ST664BZ	6.5	*E. faecalis* (2/2), *E.* (1/2), *L. casei* (1/1), *L. delbrueckii* subsp. *bulgaricus* (1/1), *L. sakei* (1/1) and *Pseudomonas* spp. (1/4).	(Todorov and Dicks, 2006b)
ST712BZ	*Lactobacillus pentosus* ST712BZ	14.0	*E. faecalis* (1/2), *E. coli* (1/2), *K. pneumoniae* (1/1), *L. casei* (1/1), *L. curvatus* (1/1) and *Pseudomonas* spp. (1/4).	(Todorov and Dicks, 2006b)
ST99	*Leuconostoc mesenteroides* subsp. *dextranicum* ST99	ND	*Bacillus* spp. (1/4), *E. faecalis*(1/1), *L. amylophilus* (1/1), *L. brevis* (1/1), *L. casei* subsp. *casei* (2/2), *L. helveticus* (2/2), *L. plantarum* (9/9), *Lactococcus lactis* subsp. *cremoris* (1/1), *L. innocua* (2/2), *L. monocytogenes* (1/1), *P. pentosaceus* (2/2), *S. aureus* (1/1) and *S. thermophilus* (1/1).	(Todorov and Dicks, 2004)
Bozacin 14	*Lactococcus lactis* subsp. *lactis* B14	5.0	*E. coli* (2/2), *Lactobacillus alimentarius* (1/1), *L. brevis* (1/3), *L. casei* (6/8), *L. curvatus* (1/2), *L. delbrueckii* subsp. *delbrueckii* (1/1), *L. plantarum* (9/18), *L. lactis* (3/3), *Leuconostoc dextranicum* (3/3), *L. mesenteroides* (3/7), *L. innocua* (2/2), *L. monocytogenes* (1/1) and *P. pentosaceus* (1/2).	(Kabadjova et al., 2000)

Bacteriocins	Strain	Molecular mass (kDa)	Activity spectra	Reference
JW3BZ	*Lactobacillus plantarum* JW3BZ	ND	*E. faecalis* (4/6), *Enterococcus mundtii* (1/1), *E. coli* (0/1), *K. pneumoniae* (0/2), *L. casei* (1/1), *L. curvatus* (0/1), *L. paracasei* subsp. *paracasei* (0/1), *L. plantarum* (0/3), *L. sakei* (2/2), *L. salivarius* (0/1), *L. lactis* subsp. *lactis* (1/1), *L. innocua* (2/2), *Pseudomonas sp.*(0/1), *S. aureus*(0/1), *Streptococcus caprinus* (0/1), *Streptococcus sp*.(0/1).	(Von Mollendorff et al., 2006)
JW6BZ	*Lactobacillus plantarum* JW6BZ	ND	*E. faecalis* (3/6), *Enterococcus mundtii* (1/1), *E. coli* (0/1), *K. pneumoniae* (0/2), *L. casei* (1/1), *L. curvatus* (0/1), *L. paracasei* subsp. *paracasei* (0/1), *L. plantarum* (0/3), *L. sakei* (2/2), *L. salivarius* (0/1), *L. lactis* subsp. *lactis* (1/1), *L. innocua* (1/2), *Pseudomonas sp.*(0/1), *S. aureus*(0/1), *S. caprinus* (1/1), *Streptococcus sp*.(1/1).	(Von Mollendorff et al., 2006)
JW11BZ	*Lactobacillus fermentum* JW11BZ	ND	*E. faecalis* (1/6), *Enterococcus mundtii* (0/1), *E. coli* (0/1), *K. pneumoniae* (0/2), *L. casei* (1/1), *L. curvatus* (0/1), *L. paracasei* subsp. *paracasei* (0/1), *L. plantarum* (0/3), *L. sakei* (2/2), *L. salivarius* (0/1), *L. lactis* subsp. *lactis* (1/1), *L. innocua* (0/2), *Pseudomonas sp.*(0/1), *S. aureus*(0/1), *S. caprinus* (1/1), *Streptococcus sp*.(1/1).	(Von Mollendorff et al., 2006)
JW15BZ	*Lactobacillus fermentum* JW15BZ	ND	*E. faecalis* (4/6), *Enterococcus mundtii* (1/1), *E. coli* (0/1), *K. pneumoniae* (1/2), *L. casei* (1/1), *L. curvatus* (0/1), *L. paracasei* subsp. *paracasei* (0/1), *L. plantarum* (0/3), *L. sakei* (2/2), *L. salivarius* (0/1), *L. lactis* subsp. *lactis* (1/1), *L. innocua* (2/2), *Pseudomonas sp.*(0/1), *S. aureus*(0/1), *S. caprinus* (1/1), *Streptococcus sp*.(1/1).	(Von Mollendorff et al., 2006)

Adapted from Von Mollendorff et al. (2006)

This decrease may result in the successive uncontrolled proliferation of pathogenic bacteria that may contribute to various clinical disorders (Fooks et al., 1999). *In vitro* studies and clinical trials with animals have shown that probiotic bacteria reduce symptoms related with irritable bowel syndrome (O'Mahony et al, 2005), diarrhea (Isolauri et al, 1991), lactose intolerance, colon cancer, allergies, and cholesterol (Gilliland, 1990; Salminen et al., 1998; Fooks et al., 1999; Kalliomaki et al., 2001). De Vrese et al. (2005) found that it also reduces the duration of the common cold.

One of the main selection criteria for probiotic lactic acid bacteria is their ability to adhere to epithelial cells or the intestinal mucosa. Adhesion is important as it is considered to play a vital role in persistence, stimulation of the immune system, enhanced healing of the damaged mucosa and antagonism against pathogenic bacteria (Isolauri et al., 1991; Salminen et al., 1996; Rolfe, 2000; Reid and Burton, 2002). Other criteria include the ability to survive at low pH and high bile salt concentrations (Mattila-Sandholm et al., 1999; Bezkorovainy, 2001).

The use of probiotic lactic acid bacteria (LAB), especially *Lactobacillus* and *Bifidobacterium* spp. as starter cultures, either alone or in combination with traditional starter cultures in various fermentation processes, is gaining significant interest. Formulated probiotic food may present consumers with a healthy dietary component at a considerable low cost (Goldin, 1998). Furthermore, it was reported that LAB may contribute to microbiological safety and/or provide one or more technological, nutritional and organoleptic advantages to a fermented product, through production of ethanol, acetic acid, aroma compounds, exopolysaccharides, bacteriocins and several enzymes (Leroy and De Vuyst, 2004).

Different developments over the years led to the concept of using starter cultures. The earliest fermented food products relied on natural fermentation through microflora present in the raw material. The load and spectrum of microorganisms populating raw material have a definite effect on the quality of the end product. Backslopping, i.e., inoculation of the raw material with a small quantity of a previously performed successful fermentation, was used to optimize spontaneous fermentation. In this case the best-adapted strain dominates. The dominant strains can be seen as a starter culture that shortens the fermentation process and reduce the risk of fermentation failure (Leroy and De Vuyst, 2004). Backslopping is still used in developing countries and even in the industrialized countries for production of sauerkraut and sourdough (Harris, 1998). The use of starter cultures in large-scale production of fermented

foods has become important for industries in the Western countries as it resulted in a control over the fermentation process and a consistent end product. However, some disadvantages do occur due to the fact that commercial starter cultures were not selected in a rational way, but rather on phage resistance and rapid acidification of the raw materials (Leroy and De Vuyst, 2004). With regard to the functionality and desired properties of the end product, these starters are not very flexible. Furthermore, it is believed that commercial starter cultures adapted to the food matrix led to a loss in genetic material (Leroy and De Vuyst, 2004). This may have contributed to the limited biodiversity of commercial starter cultures. Moreover, this leads to a product that lacks the uniqueness and characteristics that made the original product popular (Caplice and Fitzgerald, 1999).

Wild-type LAB that originates from the environment, raw material, or process apparatus serves as natural starter cultures in many of the traditionally fermented foods (Böcker et al., 1995; Weerkamp et al., 1996). Recent studies focused on the use of wild-type strains isolated from traditional products for use as starter cultures (Hébert et al., 2000; Beukes et al., 2001; De Vuyst et al., 2002). When considering LAB as a starter culture, the following factors have to be taken into account: (1) not all LAB strains have the same practical and technical importance in food fermentations; (2) *Lactobacillus* (*L. fermentum*, *L. plantarum*, *L. reuteri*), *Leuconostoc* and, to a lesser extend *Lactococcus*, *Enterococcus*, *Pediococcus* and *Weisella* spp. are usually present in traditional fermented foods; (3) not all strains of the same species are suitable as starter cultures, and (4) various industrial lactic acid fermentation processes are well-controlled despite the fact that they are spontaneous (Holzapfel, 2002). Some of these lactic acid bacteria may be classified as functional starters, due to their contribution to food safety, organlopetic properties and other nutritional advantages (Table 3).

Lactic acid bacteria are known to produce antimicrobial substances (e.g bacteriocins), polymers, sugars, sweeteners, nutraceuticals, aromatic compounds and various enzymes. This leads to a higher flexibility and wider application of LAB as starter cultures. It also represents a way by which chemical additives can be replaced by natural compounds and thus provide the consumer with new, appealing food products (Leroy and De Vuyst, 2004). Bacteriocins produced by LAB may prevent food spoilage, e.g. late spoilage of cheese by clostridia (Thomas et al., 2002). Some probiotic strains may also be used as functional starters or co-cultures in fermented food (Chandan, 1999; Ross et al., 2000; Jahreis et al., 2002).

Table 3

Examples of *Lactobacillus* spp. as functional starters or co-cultures and their role in the food industry

Advantage	Role	*Lactobacillus* spp.	References
Food preservation	Production of bacteriocins		
	-Fermented meats	*L. curvatus* *L. sakei*	Vogel et al. (1993); Hugas et al. (1995)
	-Fermented olives	*L. plantarum*	Ruiz-Barba et al. (1994)
Organoleptic	Production of exopolysaccharides	Several lactobacilli	De Vuyst and Degeest (1999); De Vuyst and Marshall (2001); De Vuyst et al. (2001)
	Production of amylase	Several lactobacilli	Mogensen (1993)
Technological	Prevention of overacidification in yoghurt	*L. delbrueckii* subsp. *bulgaricus*	Mollet (1996)
Nutritional	Production of nutraceuticals		
	-Low-calorie sugars	*L. plantarum*	Wisselink et al. (2002)
	-Production of B-group vitamins	*L. delbrueckii* subsp. *bulgaricus*	Hugenholtz and Kleerebezem (1999)
	Reduction of toxic and anti-nutritional compounds		
	-Reduction of phytic acid content, amylase inhibitors and polyphenolic compounds	*L. plantarum* *L. acidophilus*	Sharma and Kapoor (1996)

Adapted from Leroy and De Vuyst (2004)

However, when considering the use of probiotic strains as functional starters or co-cultures, it is important that they do not enhance the acidification during the shelf-life of the product, nor have adverse effects on the aroma or taste of the product (Heller, 2001).

Uncertainty still exists whether multifunctional strains possessing all desirable metabolic features would result from modern techniques and selection procedures. Therefore, recent

studies focus on the improvement of selected strains through the application of recombinant DNA technology. Application of DNA technology improves certain advantages features, e.g. health-promoting properties, accelerated acid production, wholesomeness and overproduction of specific enzymes or bacteriocins (Holzapfel, 2002). Gene disruption may be used to eliminate undesirable properties such as antibiotic and mycotoxin production by food-grade molds (Hammes and Vogel 1990; Geisen and Holzapfel, 1996). A large array of these optimized cultures is available, but is not used because of reglutaion (Holzapfel, 2002).

2.1 Cereal based probiotic foods

The concept of probiotic foods have been developed to quite an extent since its introduction to clinical nutrition and food science during the 1980's (Fuller, 1989; Shortt, 1999). Most probiotic foods available today are milk-based while a few attempts have been made using cereals. Cereal grains have a high nutritive value and are distributed world wide, making it a very suitable raw material for the development of various fermented functional foods (Angelov et al., 2006). Togwa, a lactic acid-fermented maize and sorghum gruel, inhibits the growth of some enterotoxin-producing bacteria in children under 5 years old. This suggests that togwa may possess probiotic properties (Kingamkono et al., 1998). Vogel et al. (1999) found that the lactic acid bacteria present in various lactic-fermented foods, such as sourdough, is similar or in some cases identical to species found in the gastrointestinal tract of humans and animals. *Lactobacillus plantarum* indigenous to a variety of cereal-based fermented food, is also associated with the gastrointestinal tract of humans (Ahrné et al., 1998; Molin, 2001). Colonization of the intestinal mucosa with strains of *L. plantarum* isolated from sourdough has also been reported (Johansson et al., 1993).

Barley and oats contains beta-glucan (Angelov et al., 2006), a prebiotic that reduces the levels of LDL-cholestrol by 20-30% and thus also the risk of cardiovasciluar disease (Stark and Madar, 1994; Wrick, 1994; Gallaher, 2000). For a polysaccharide or oligosaccharide to be characterized as a prebiotic, it should withstand digestion in the upper part of the gastrointestinal tract, be hydrolysable, soluble, and stimulate the growth and activity of beneficial microflora in the gut (Gibson and Roberfroid, 1995). The low glycaemic index of oats and barley is quite beneficial to diabetes in the gastrointestinal tract after ingestion as it alters the level of fat imulgation and reduces lipase acitivity (Angelov et al., 2006). Furthermore, beta-glucan stimulates the growth of beneficial bacteria associated with the colon of animals and humans (Jaskari et al, 1998; Wood and Beer, 1998).

To increase the number of beneficial bacteria in the gut, large numbers of probiotic bacteria have to be taken in by means of capsules or by using food as vector. Incorporating suitable dietary polysaccharides or oligosaccharides to the capsules may even be more effective. The latter is referred to as the prebiotic concept. Arabinoxylan is another prebiotic compound commonly found in wheat and rye (Jaskari et al., 1998; Crittenden et al., 2002; Karppinen, 2003).

Incorporation of probiotic strains in cereal-based fermented foods is possible. One such product is Yosa, a yogurt-type snack made of cooked bran fermented with lactic acid bacteria and bifidobacteria (Blandino et al., 2003). The cooked bran acts as a substrate for probiotic bacteria. This snack exhibits the postulated beneficial effects of bran and probiotic bacteria serving as an alternative to soy-based and milk-based yogurts (Salovaara 1996; Salovaara and Simonson, 2003). Oats is a suitable substrate for fermentation with probiotic lactic acid bacteria after appropriate processing (Marklinder and Lonner; 1992; Johansson et al., 1993; Salovaara 1996; Salovaara and Simonson, 2003). Cereals are high in nutrition and confer specific health benefits (Table 4).

Table 4

Possible applications of cereals or cereal constituents in functional foods

Application
Serving as fermentable substrate for growth of probiotic bacteria, particularly lactobacilli and bificobacteria
As dietary fibre, promoting several beneficial physiological effects (e.g. laxation and blood cholesterol attenuation (Spiller, 1994) and blood glucose attenuation (Bijlani, 1985))
As prebiotics due to the presence of certain non-digestible carbohydrates
As encapsulation material (vector) to enhance the stability of probiotics

Adapted from Charalampopoulos et al. (2002)

2.2 Antibiotic resistance (AR) by potential probiotic lactic acid bacteria

Some of the antibiotics currently used in the medical and veterinary field include the aminoglycosides (gentamycin, kanamycin and streptomycin), β-lactams (penicillin), glycopeptides (vancomycin), tetracycline, fluoroquinolones (ciprofloxacin), macrolides (erythromycin), chloramphenicol, sulfamethoxazole and trimethoprim (Rojo-Bezares et al., 2006). A study by Temmerman et al. (2003) reported that 68.4% of probiotic isolates

exhibited resistance against two or more antibiotics. Strains of *Lactobacillus* spp. were resistant to chloramphenicol, erythromycin, tetracycline and kanamycin (Temmerman et al., 2003). Bacteria have developed numerous antibiotic resistance mechanisms e.g. (1) enzyme inactivation of the antibiotic (Walsh, 2003), (2) extrution of the antibiotic outside the cell by active efflux pumps (Walsh, 2003), (3) alteration of the target site (Davies 1997), and (4) by directing metabolic pathways around the disrupted area (Poole, 2002). A vast number of data concerning the prevalence and mechanisms of antibiotic resistance in clinical bacteria is available. However, information on the susceptibility or the presence of antibiotic resistance genes in lactic acid bacteria and other commensal bacteria is scarce (Teuber et al., 1999; Cataloluk and Gagebakan, 2004; Flórez et al., 2005). Probiotic lactic acid bacteria colonize the gastrointestinal tract and transfer genetic material, vertically or horizontally, to indigenous microflora and *visa verca* (Mathur and Singh, 2005). This raises the question whether or not resistance genes can be transferred from LAB to other bacteria in the gastrointestinal tract (Ouwehand and Vesterlund, 2004).

Development of antibiotic resistance in bacteria can be ascribed to two factors, viz. the occurrence of resistance genes and selective pressure broad about by the use of antibiotics (Levy, 1992). Two types of resistance exist, i.e. intrinsic resistance and acquired resistance (Mathur and Singh, 2005). Intrinsic resistance is the inherent or natural resistance of a bacterial species or genus, which presents it with the ability to survive in the presence of a specific antimicrobial agent (Mathur and Singh, 2005). This type of resistance poses no risk to non-pathogenic bacteria as it is not transferred horizontally. However, some strains within a species have acquired resistance. This type of resistance can be transferred between bacteria belonging to the same or different species or genera by transposons, conjugative plasmids, the possession of insertion elements and integrons, as well as temperate and lytic phages (Davies, 1994).

Thus far, three mechanisms responsible for horizontal gene transfer have been identified: (1) natural transformation, (2) conjugation, and (3) transduction (Davis, 1994). Conjugation is thought to be the main mechanism responsible for the transfer of antibiotic resistance genes (Salyers, 1995), because many resistance genes have been located on mobile genetic elements, such as plasmids and conjugative transposons.

2.2.1 Mobile genetic elements conferring antibiotic resistance to LAB

For LAB to acquire antibiotic resistance genes they need to interact with other bacteria actively or passively by means of conjugative plasmids or transposons. Plasmids of different size, function and distribution are commonly found in LAB (Davidson et al., 1996; Wang and Lee, 1997). These plasmids have various functions, such as, metabolism of carbohydrates, citrates and amino acids, hydrolysis of proteins, production of exopolysaccharides and bacteriocins, and resistance to phages, heavy metals, and antibiotics. According to Wang and Lee (1997) at least 25 *Lactobacillus* spp. contain native plasmids. It is common for enterococci, lactococci, leuconostoc and pediococci to contain plasmids, while plasmids are less common in some strains of bifidobacteria and lactobacilli (Dellaglio et al., 1995; Sgorbati et al, 1995; Simpson 1995; Teuber, 1995). The presence of conjugative transposons in LAB has only been described in enterococci, lactococci and streptococci (Clewell, 1993; Salyers et al., 1995).

2.2.2 Plasmids encoding AR genes in lactobacilli

Plasmids encoding resistance to chloramphenicol, erythromycin, macrolide-lincomycin-streptogramin and tetracycline have been found in *L. acidophilus* (Vescovo et al., 1982), *L. fermentum* (Ishiwa and Iwata, 1980; Fons et al., 1997), *L. plantarum* (Ahn et al., 1992; Danielsen, 2002), and *L. reuteri* (Vescovo et al., 1982; Axelsson et al., 1988; Lin et al., 1996; Tannock et al., 1994). These R-plasmids vary in size, most being smaller than 10 kb. Fons et al. (1997) found a 5.7 kb plasmid in a strain of *L. fermentum* isolated from pig faeces, carrying an *erm* gene coding for erythromycin resistance. The *erm* gene shared 98.2% homology to a gene located on the enterococcal conjugative transposon Tn1545.

Lactobacillus isolates from fermented saugages contained plasmids harboring the tetracycline resistance gene, *tet* (M) (Gevers et al., 2002). The plasmids are approximately 10 kb in size, with a few exceeding 25 kb. Sequence similarities (>99.6 %) are found between the two allele types of the plasmid encoded *tet* (M) gene in *Lactobacillus* isolates and the *tet* (M) gene previously found in *Neisseria meningitidis* and *Staphylococcus aureus* MRSA101, respectively. In a similar study (Danielsen, 2002) high homology was reported between the *tet* (M) gene contained within the tetracycline resistance plasmid pMD5057 (10.9 kbp) in *L. plantarum* 5057 to sequences from *S. aureus* and *Clostridium perfringens*, respectively (Danielsen, 2002).

2.2.3 Conjugative transposons encoding AR genes in lactobacilli

Conjugative transposons are one of the main vehicles for transport of antibiotic resistance in Gram-positive bacteria. To our knowledge, no conjugative transposons encoding AR genes have been reported for lactobacilli. However, their occurrence has been reported in *Lactococcus lactis* (Tn5276, Tn5301), *E. faecalis* (Tn916, Tn918, Tn920, Tn925, Tn2702), *E. faecium* (Tn5233), *S. agalactiae* (Tn93951) and *S. pyogenes* (Tn3701). In lactococci they have been found to code for the fermentation of sucrose (*sac*) and the production of nisin (*nis*), while they confer resistance to chloramphenicol (*cat*), erythromycin (*ermAM, erm*), kanamycin (*aphA-3*) and tetracycline (*tet* (M)) in enterococci and streptococci. These transposons may be inserted, as one or multiple copies, into the chromosome or plasmids and vary in size from approximately 16 to 70 kb. Furthermore they possess the ability to mobilize chromosomal or plasmid genes. Some of these plasmids, such as the Tn916/Tn1545 family, have an extreme host range with a resistance transfer rate of 10^{-9} to 10^{-6} per donor filter matings (Mathur and Singh, 2005).

2.2.4 Conjugation in lactic acid bacteria

Lactococci are well known to possess indigenous conjugation systems (Neve et al., 1987; Gasson and Fitzgard, 1994). In contrast, information concerning native conjugation systems in lactobacilli is limited. Transfer of R-plasmids and transposons amongst LAB and from LAB to Gram-positive and Gram-negative bacteria has been reported. Enterococci are well known for its receptive nature in conjugation (Clewell and Weaver, 1989), but can also successfully act as donor for the transfer of antibiotic resistance genes to lactobacilli (Shrago and Dobrogosz, 1998) and unrelated enterococci (Rice et al., 1998). Gevers et al. (2003) reported the *in vitro* transfer of tetracycline resistance at frequencies of 10^{-4} to 10^{-6} transconjugants per recipient between seven *Lactobacillus* isolates (donors) and *E. faecalis* (recipient). Futhermore, two of these isolates were able to transfer their resistance to *Lactococcus lactis* subsp. *lactis*.

3. The genus *Lactobacillus* and its classification

The genus *Lactobacillus* alone consists of about 80 recognized species (Axelsson, 2004) and belongs to a group of generally regarded as safe (GRAS) microorganisms, collectively known as lactic acid bacteria (LAB) (Axelsson, 2004). Lactobacilli are Gram-positive, oxidase- and catalase-negative, non-sporulating, non-respiring rods that produce lactic acid as major end

product from the fermentation of carbohydrates (Kao et al., 2006). The genus is very heterogeneous, based on phenotypic, biochemical and physiological characteristics (Axelsson, 2004). This is further reflected by the wide range in G + C base composition of their DNA (Schleifer and Stackerbrandt, 1983). The reason for this heterogeneity and large number of species is due to the definition of the genus, which basically is rod shaped (Schleifer and Stackerbrandt, 1983). Orla-Jensen (1919) tried to divide this group in a similar fashion to that of cocci and classified the genus *Lactobacillus* into the subgenera *Betabacterium, Streptobacterium* and *Thermobacterium*. This division is still valid to some extent, although some alterations in the definitions have been made. This classical division was based on fermentation characteristics as summarized in Table 5 (Stiles and Holzapfel, 1997). The three groups are the (I) obligately homofermentatives, (II) facultatively heterofermentatives, and (III) obligately heterofermentatives. The presence or absence of key enzymes involved in homo- and heterofermentative sugar metabolism, i.e. aldolase, fructose-1,6-diphosphatase and phospoketolase, are one of the physiological elements used for the division. Each species in the three groups were further divided into three subgroups to reflect its position in certain phylogenetic clusters.

The classical method of differentiating between *Lactobacillus* spp. is based on growth requirements, growth at certain temperatures, arginine hydrolysis, lactic acid configuration, and patterns of carbohydrate fermentation reactions (Axelsson, 2004). These characteristics are still used as an indication, though more appropriate characterization methods are being implemented, i.e., peptidoglycan structure, DNA base composition, electrophoretic mobility of L-lactate dehydrogenase, DNA homology, species-specific PCR (derived from rRNA sequences), RAPD-PCR, PFGE (pulse field gel electrophoresis) and restriction enzyme analysis (Kandler and Weiss, 1986; Klein et al., 1998; Axelsson, 2004). Phenotypic methods together with genetic methods must be used to differentiate and characterize species, because many species are phenotypically very similar but genotypically different (Vandamme et al., 1996, Klein et al., 1998).

4. Antimicrobial compounds produced by LAB

Lactic acid bacteria produce various antimicrobial substances during fermentation, such as, organic acids, hydrogen peroxide, carbon dioxide, diacetyl, low molecular weight antimicrobial substances and bacteriocins (Blom and Mörtvedt, 1991). These specific

antimicrobial compounds act as biopreservatives in food, with records dating back to approximately 6000 B.C. (Pederson, 1971; De Vuyst and Vandamme, 1994).

Table 5

Arrangement of the *Genus Lactobacillus*

Characteristics	Group I, Obligate homofermenters	Group II, Facultative heterofermenters	Group III, Obligate heterofermenters
Fermentation of pentose	-	+	+
CO_2 from glucose	-	-	+
CO_2 from gluconate	-	+[a]	+[a]
Phosphoketolase present	-	+[b]	+
FDP aldolase present	+	+	-
	L. acidophilus	*L. casei*	*L. brevis*
	L. delbrueckii	*L. curvatus*	*L. buchneri*
	L. helveticus	*L. plantarum*	*L. fermentum*
	L. salivarius	*L. sakei*	*L. reuteri*
	L. farciminis	*L. acetotolerans*	*L. collinoides*
	L. gasseri	*L. alimentarius*	*L. fructivorans*
	L. johnsonii	*L. bifermentans*	*L. hilgardii*
	L. kefiranofaciens	*L. homohiochii*	*L. kefiri*
	L. mali	*L. paracasei*	*L. malefermentans*
		L. pentosus	*L. panis*
		L. rhamnosus	*L. parabuchneri*
			L. parakefir
			L. pontis
			L. sanfrancisco
			L. suebicus
			L. vaccinostercus
			L. vaginalis

[a]When fermented

[b]Inducible by pentoses

The bacteria listed are of importance in foods and as probiotics.

Adapted from Axelsson (2004) and Holzapfel and Stiles (1997).

The antimicrobial substances are not produced for human convenience but rather for one bacterium gaining advantage over another that competes for the same energy source (Ouwehand and Vesterlund, 2004).

4.1 Organic acid, acetaldehyde and ethanol

Various heterofermentative lactic acid bacteria produce equimolar amounts of lactic acid, acetic acid, ethanol, and CO_2 upon hexoses fermentation. Homofermentation results in the formation of lactic acid alone (Caplice and Fitzgerald, 1999). The antimicrobial effect of these organic acids formed during lactic acid fermentation is well known (Davidson, 1997). The organic acids, dissociated and undissociated, are believed to disrupt the mechanisms responsible for maintaining the membrane potential, thereby inhibiting active transport (Sheu et al., 1972; Eklund, 1989; De Vuyst and Vandamme 1994).

4.2 Hydrogen peroxide

Lactic acid bacteria produce hydrogen peroxide in the presence of oxygen through the action of NADH oxidases, flavoprotein-containing oxidases, and super oxide dismutase (Condon, 1987; Ouwehand and Vesterlund, 2004). LAB lack true catalase and therefore it is believed that hydrogen peroxide may accumulate and act inhibitory to the growth of some microorganisms (Condon, 1987). However, it is argued that hydrogen peroxide is decomposed by flavoproteins, pseudocatalases and peroxidases *in vivo* and therefore does not accumulate to significant amounts (Nagy et al., 1991; Fontaine et al., 1996). Anaerobic environments can form due to some hydrogen peroxide-producing reactions scavenging oxygen (Ouwehand and Vesterlund, 2004). Hydrogen peroxide production is important for the colonization of lactobacilli in the urogenital tract. This reduces the acquisition of gonorrhea, HIV and urinary tract infections (Vallor et al., 2001). The antimicrobial affect of hydrogen peroxide *in viv*o is being questioned (Nagy et al., 1991; Fontaine et al., 1996).

4.3 Carbon dioxide

Carbon dioxide is produced by heterolatic fermentation and contributes to an anaerobic environment that is toxic to various aerobic food microorganisms. Furthermore carbon dioxide in itself has an antimicrobial activity (Lindgren and Dobrogosz, 1990). The mechanism involved in this activity is not known, but it is believed that carbon dioxide accumulates in the lipid bilayer due to the inhibition of enzymatic decarboxylations (King and Nagel, 1975), causing disfunction of membrane permeability (Lindgren and Dobrogosz,

1990). Low levels of CO_2 have been found to promote the growth of certain microorganisms, whereas high concentrations led to growth inhibition (Lindgren and Dobrogosz, 1990).

4.4 Diacetyl

Diacetyl is produced from the fermentation of citrate and is responsible for the unique aroma and buttery flavour of various other fermented milk products (Lindgren and Dobrogosz, 1990; Cogan and Hill, 1993). Diacetyl is produced by many LAB, including the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Jay, 1982). Gram-positive bacteria are less sensitive to its antimicrobial activity than Gram-negative bacteria, molds and yeast. The mechanism responsible for this activity is the action of diacetyl on the arginine-binding protein of Gram-negative bacteria leading to interference with arginine utilization (Jay, 1982; Motlagh et al., 1991; De Vuyst and Vandamme, 1994).

4.5 Low molecular weight antimicrobial substances

Several studies have focused on the production of low molecular weight antimicrobial substances by lactic acid bacteria (Reddy and Shahani, 1971; Hamdan and Mikolajcik, 1974; Shahani et al., 1977a,b; Reddy et al., 1983; Silva et al., 1987). These substances share several characteristics, in addition to having a low molecular weight, such as being active at a low pH, soluble in acetone, thermostable and displaying a broad spectrum of activity (Axelsson, 1990). However, more in-depth studies need to be done to gain detailed information on these substances. Thus far, three low molecular weight antimicrobial substances have been properly characterized, i.e. Reuterin and Reutericyclin, both produced by *L. reuteri*, and 2-Pyrrolidone-5-carboxylic Acid, produced by *L. casei* subsp. *casei*, *L. casei* subsp. *pseudoplantarum* and *Streptococcus bovis* (Chen and Russell, 1989; Huttunen et al., 1995).

4.6 Bacteriocins

Bacteriocins are ribosomally synthesized peptides produced by various bacteria and exhibit a bacteriostatic or bacteriocidal activity against genetically closely related bacteria (Caplice and Fitzgerald, 1999; Ross et al., 2002; Chen and Hoover, 2003; Ouwehand and Vesterlund, 2004). Although bacteriocins display antibiotic properties, they differ from antibiotics in that they are synthesized ribosomally, exhibit a narrow spectrum of activity, and the organisms responsible for their production have immunity against them (Cleveland et al., 2001). Most bacteriocins from Gram-positive bacteria are produced by lactic acid bacteria (Nes et al., 1996; Ennahar et al., 2000). Previous studies have reported the antimicrobial activity of

bacteriocins produced by LAB against Gram-negative bacteria (Todorov and Dicks, 2004, 2006b; Von Mollendorff et al., 2006).

Bacteriocins are of great importance to humans as they can play a considerable role in food preservation and human therapy (Richard et al., 2006). They can be used as an alternative or replacement to various antibiotics (Richard et al., 2006). This can limit the use of antibiotics and thus reduce the development of antibiotic resistance (Ouwehand and Vesterlund, 2004). Furthermore, bacteriocins are more easily accepted by health conscious consumers, because they are naturally produced compared to chemically synthesized preservatives (Ouwehand and Vesterlund, 2004). According to Deegan et al. (2006) the ongoing study of existing bacteriocins and discovery of new bacteriocins look promising for application in the food industry.

4.6.1 Classification

Bacteriocins are divided into four main classes: (i) Class I, lantibiotics; (ii) Class II, small non-modified heat stable peptides; (iii) Class III, large heat-labile proteins; and (iv) Class IV, bacteriocins with a complex structure and glyco- and/ or lipid moieties (Tabel 6). The Class I and II bacteriocins are considered the most important due to potential commercial applications.

4.6.2 Bacteriocins of the genera Lactobacillus

Most of the bacteriocins produced by lactobacilli belong to either Class I, Class II or Class III (Ouwehand and Vesterlund, 2004). This review will mainly focus on Class II bacteriocins. Class I bacteriocins are divided into two subgroups Ia and Ib (Table 6). Class II bacteriocins are divided in to three subgroups (a, b and c). Of these, Class IIa is the most common (Table 6) Class IIa bacteriocins are small (<10 kDa) heat-stable peptides and does not contain modified amino acids. They all contain a conserved amino-terminal sequence (YGNGVXC) (Ouwehand and Vesterlund, 2004). Furthermore, they are of great interest for medical and industrial applications because of the exceptional properties they display, such as antiviral activity of enterocin CRL35 and bacteriocin ST4V and strong antilisterial activity of all Class IIa bacteriocins (O'Sullivan et al., 2002; Wachsman et al., 2003; Todorov et al., 2005). The strong antilisterial activity of Class IIa bacteriocins make them even more promising for industrial applications than that of Class I, as they have a narrow spectrum of activity and may not be active against starter cultures (O'Sullivan et al., 2002).

Table 6

Classification of bacteriocins produced by LAB

Group	Subgroups	Characteristics
Class I (lantibiotics)	Ia	Lantibiotics, peptides containing lanthionine and β-methyl lanthionine. Molecular weight 2-5 kDa. Undergo posttranslational modifications. Flexible elongated molecules. Peptide with slight positive or negative charge, or highly negative net charge.
	Ib	Globular molecules with no net charge or net negative charge.
Class II (Non-lantibiotics, unmodified bacteriocins)	IIa	Small heat-stable peptides (<10 kDa), synthesized as inactive prepeptides to get activated by posttranslational cleavage of the N-terminal leader peptide. Have a consensus sequence of YGNGV in the N-terminal.
	IIb	Two-peptide bacteriocins. Two different peptides required to form an active poration complex
	IIc	Other bacteriocins. Bacteriocins produced by the cell's general *sec*-pathway.
Class III (non-lantibiotics, large heat labile bacteriocins)		Large molecules (> 30 kDa) sensitive to heat.
Class IV		Complex bacteriocins containing lipid or carbohydrate moieties.

Adapted from Klaenhammer (1993) and Nes et al. (1996)

Some Class II bacteriocins are composed of two separate peptides. Bacteriocins displaying this property are known as the Class IIb bacteriocins (Klaenhammer, 1993). Class IIc bacteriocins used to include those activated by thiol and secreted by means of the sec-dependent pathway. However, studies on bacteriocins formerly characterized as Class IIc show that they can act with their cysteine residues being oxidized and thus can use the sec-dependent secrection pathway (Ennahar et al., 2000). Therefore, Class IIc is now used to group the other non-lantibiotic bacteriocins and the bacteriocins that do not belong to either Class IIa or IIb (Ouwehand and Vesterlund, 2004).

Class III bacteriocins are large (> 30 kDa) heat-labile proteins. Therefore, it has been proposed that this class may include bacteriolytic enzymes such as hemolysins and muramidases, which are able to imitate the physiological activities of bacteriocins (Jack et al., 1994). This group is the least well characterised and so far has only been isolated from the genus *Lactobacillus* (Klaenhammer, 1993).

4.6.3 Genetics, Biosynthesis and mode of action of Class II bacteriocins

4.6.3.1 Organization of gene clusters: genetics and biosynthesis

The organization of the gene clusters of Class II bacteriocins, such as lactococcins A, B and M (Holo and Nes, 1991; Van Belkum et al., 1991; Venema et al., 1995c), acidocin A (Kanatani et al., 1995), plantaricin A (Diep et al., 1994; 1995; 1996), mesentericin Y105 (Fremaux et al., 1995), leucocin A (Van Belkum and Stiles, 1995), divercin V41 (Métivier et al., 1998), pediocin PA-1 (Marrug et al., 1992; Motlagh et al., 1992; Bukhtiyarova et al., 1994; Venema et al., 1995a), enterocin A (Aymerich et al., 1996; O'Keeffe et al., 1999), sakacin A (Axelsson and Holck, 1995) and sakacin P (Hühne et al., 1996) have been studied. Genes that encode Class II bacteriocin production and accompanying immunity are usually found organized within operon clusters (Nes et al., 1996; Sahl and Bierbaum 1998; Ouwehand and Vesterlund, 2004). The genes responsible for the production of Class II bacteriocins usually consist of a structural gene (two genes for the two-peptide bacteriocins) encoding for the prepeptide (Rauch and de Vos, 1992), a dedicated immunity gene (Engelke et al., 1994; Klein and Entian, 1994; Diep et al., 1996; Qiao et al., 1996), an ABC-transporter gene for transport across the membrane (Klein et al., 1992), and a gene encoding an accessory protein needed for export of the bacteriocin (Net et al., 1996; Sablon et al., 2000). Furthermore, in some cases the presence of regulatory genes has been reported (Klein et al., 1993; Net et al., 1996; Sablon et al., 2000). These genes are usually associated with mobilisable elements, e.g. transposons associated with the chromosome or plasmids (Deegan et al., 2006).

4.6.3.2 Biosynthesis, post-translational modifications, activation and transport

Most class II bacteriocins are synthesized as a biological inactive prepeptide carrying an N-terminal leader peptide and a distinctive double-glycine proteolytic processing site. However, Class IIc bacteriocins differ, because they have a sec-type N-terminal signal sequence and are processed and secreted by the general secretory pathway (Leer et al., 1995; Worobo et al., 1995).

Pediocin PA-/AcH will be used as an example for further discussion of class II bacteriocins, as this is one of the most extensively studied class II bacteriocin (Ouwehand and Vesterlund, 2004). Several genera of lactic acid bacteria, e.g. *Carnobacterium*, *Lactobacillus* (*L. bavaricus*, *L. curvatus*, *L. plantarum*, *L. sake*), *Leuconostoc* and *Pediococcus*, produce pediocin-like bacteriocins (Atrih et al., 2001). In the case of Pediocin PA-1 the prepeptide and leaderpeptide (containes a double glycine cleavage site encoded by *pedA*) are synthesized simultaneously and are transported across the cell membrane via an ABC-transporter system. The N-terminal part of the ABC transporter (encoded by *pedD*) as well as an essential accessory protein (PedC) is involved in the proteolytic removal of the leader peptide that results in the formation of the mature bacteriocin (Fig. 2). The plantaricin 423 encoding region on plasmid pPLA4 has a very similar operon structure to Pediocin PA-1 (Van Reenen et al., 2003). The *plaC* and *plaD* genes of plantaricin 423 are practically identical to *pedC* and *pedD.* The prepeptide is encoded by *plaA* and consist of a mature molecule and a N-terminal leader peptide (Van Reenen et al., 2003).

Class II bacteriocins, such as sakacin A and P, plantaricins of *Lactobacillus plantarum* C11, and possibly carnobacteriocin B2, also produces an induction factor (a bacteriocin-like peptide exerting no antimicrobial activity) that activate transcription of the regulated genes. The induction factor forms part of the signal transduction system responsible for biosynthesis of class II bacteriocins. This signal transduction system consists of three components, i.e. an induction factor (IF), a histidine protein kinase and a cytoplasmic response regulator (Fig. 2.) (Nes et al., 1996). To regulate biosynthesis, a prebacteriocin and a bacterocin-like prepeptide of an induction factor are produced. The induction factor is synthesized as a prepeptide with a double-glycine leader sequence that ultimately undergoes cleavage by a dedicated ABC-transporter. Cleavage of the leader peptide of IF by the ABC-transporter coincides with externalization of the mature peptide from the cell. Following release via the ABC-transporter, the bacteriocin and IF are sensed by the membrane-bound histidine protein kinase. This leads to autophosphorylation and subsequent tranfer of the histidine residue in the extracellular domain to a conserved aspartic acid of the response regulator. This interaction triggers the response regulator to activate transcription of the genes responsible for bacteriocin production (Fig. 2) (Nes et al., 1996; Ennahar et al., 2000).

Class IIa and b bacteriocins do not experience major post-translational modification (Chen and Hoover, 2003). The prepeptide is modified by cleavage of the leader sequence that

ultimately is necessary for secretion and translocation across the cell membrane (Diep et al., 1996; Ehrmann et al., 2000). However, class IIc bacteriocins are processed during transport across the cell membrane by a signal peptidase (Chen and Hoover, 2003).

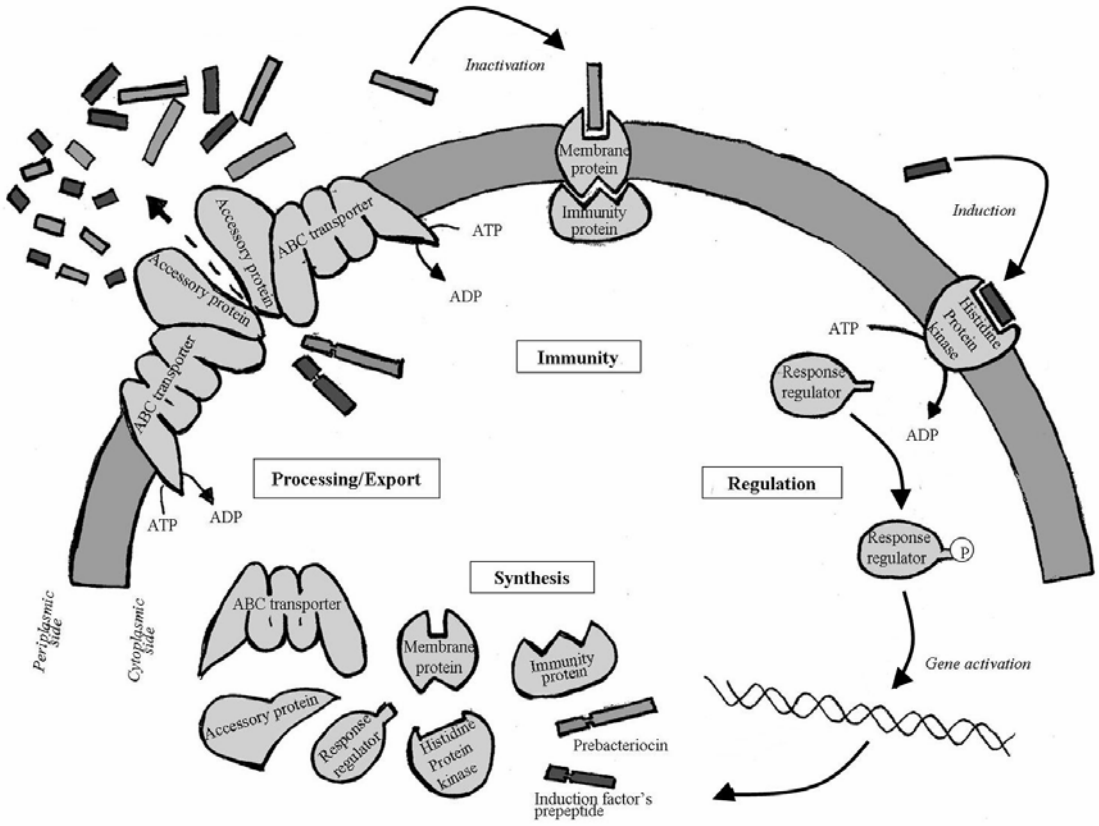

Fig. 2. Schematic diagram of the biosynthesis machinery for production of Class IIa bacteriocins: three component regulatory system, synthesis, processing, excretion and immunity (Havarstein et al., 1995; Venema et al., 1995b; Nes et al., 1996; Ennahar et al., 2000.)

4.6.3.3 Producer immunity

Bacteriocin producers need a mechanism to protect them against the action of their bacteriocins. This is accomplished by the production of immunity proteins. It is common for the immunity genes to be on the same operon as the structural bacteriocin gene and often they are found next to each other (Nes et al., 1996; Klein and Entian, 1994). Furthermore, a close genetic proximity exists between immunity genes and bacteriocin structural and processing genes (Siegers and Entian, 1995). In the case of Class II bacteriocins, one gene generally

encodes for the immunity protein. These proteins, usually consisting of 50-150 amino acid residues, are generally membrane-associated and provide complete immunity to the producer cell (Nissen-Meyer et al., 1993; Quadri et al., 1995). The mechanisms involved in immunity is poorly understood, but it has been suggested that interaction between the immunity protein and another protein, perhaps a receptor, located at the cytoplasmic side of the cell membrane of the producer, protects it against the action of the bacteriocin (Fig. 2.) (Nes et al., 1996; Ennahar et al., 2000; Sprules et al., 2004).

4.6.3.4 Mode of action

The biological targets of bacteriocins produced by LAB are the anionic lipids of the cytoplasmic membrane, which acts as the primary receptors for initiation of pore formation (Abee 1995; Montville et al., 1995; Moll et al., 1999). Previous findings suggested a protein-'receptor'-mediated activity (Bhunia et al., 1991; Chikindas et al., 1993), but recent studies focusing on the effect of class IIa bacteriocins on lipid vesicle systems indicate that protein 'receptors' are not the main requirement for pore formation (Ennahar et al., 2000). It has been suggested that these receptors act to determine specificity of class II bacteriocins (Venema et al., 1995b,c).

Pore formation ultimately results in the leakage of inorganic phosphates and an ionic imbalance (Deegan et al., 2006,). This has been demonstrated for bavaricin MN (Kaiser and Montville, 1996), mesentericin Y105 (Maftah et al., 1993) and pediocin PA-1 (Ennahar et al., 2000). The initial disturbance further causes dissipation of the proton motive force (PMF), which encompasses a complete or partial dissipation of either or both the pH gradient and the transmembrane potential (Montville and Chen, 1998). For class IIa bacteriocins complete dissipation of the pH gradient occurs readily, while only a partial dissipation of the transmembrane potential usually occurs (Moll et al., 1999; Ennahar et al., 2000). Dissipation of the proton motive force (PMF) by class IIa bacteriocins can be considered their main action to exert lethal activity (Jack et al., 1994; Abee, 1995; Venema et al., 1995b; Ennahar et al., 2000). ATP is depleted as much as 98.9% and active transport involved in the uptake of amino acids is blocked (Chikindas et al., 1993; Maftah et al., 1993). Leakage of pre-accumulated amino acids, among various other UV-absorbing materials, has been reported for mesentericin Y105 (Maftah et al., 1993) and pediocin PA-1 (Bhunia et al., 1991; Chikindas et al., 1993). This leakage may be due to the diffusion of amino acids through the pores formed by bacteriocins (Sahl et al., 1995), possibly combined with reflux via PMF transport systems

(Ennahar et al., 2000). However, in the case of mesentericin Y105 the efflux occurs at a rapid rate, suggesting that this is a result of simple leakage (Maftah et al., 1993).

In contrast to lantibiotics, class IIa bacteriocins causes no leakage of ATP. This may be due to smaller pore sizes that are formed by the action of the latter. However, ATP depletion does occur and this may result from an increased consumption of ATP in order to restore or maintain the PMF. The depletion may also be due to the efflux of inorganic phosphate that is needed to produce ATP (Chen and Montville, 1995; Ennahar et al., 2000). According to a study by Chen and Montville (1995) on the rates of pediocin-PA-1-induced ATP depletion and phosphate efflux, the depletion is most likely caused by attempts to restore the PMF, rather than a shift in the ATP hydrolysis equilibrium caused by a loss of inorganic phosphate (Ennahar et al., 2000).

Three pore formation models have been described by which bacteriocins act on the cell membranes of sensitive cells, a wedge-like model, a barrelstave-like model or a carpet mechanism (Moll et al., 1999). Class I bacteriocins may function by using a wedge-like model to induce pores, whereas Class II bacteriocins may form pores by either following the barrelstave-like model or a carpet mechanism. The carpet mechanism is accomplished by peptides orientating them parallel to the membrane, thereby interfering with the membrane structure (Moll et al., 1999). Pore formation by class IIa bacteriocins using the barrelstave-like model may be due to the peptides' putative transmembrane helices, membrane-binding ability and water solubility (Chikindas et al., 1993; Abee, 1995; Venema et al., 1995b; Ennahar et al., 2000). Thus far, two mechanisms for the initial interaction between class II bacteriocins and the membrane surface have been hypothesized, namely: (1) electrostatic binding of the bacteriocin to the membrane surface mediated by a putative receptor-type molecule bound to the membrane (Ennahar et al., 2000), and (2) binding between positively charged amino acids and anionic phospholipid heads in the membrane (Moll et al., 1999; Ennahar et al., 2000). Class II bacteriocins may rely on basically the same type of functional binding due to high structural similarities in their hydrophilic N-terminals (Ennahar et al., 2000). A crucial subsequent step in the process of pore formation is the hydrophobic interaction between the amphiphilic region of the C-terminal part of the bacteriocin and the lipid acyl chains (Kaiser et al., 1996; Fleury et al., 1996; Chen et al., 1997; Fimland et al., 1998; Chen et al., 1998). In contrast to the N-terminal domain that plays a role in the electrostatical interaction between the bacteriocin and the membrane surface, the C-terminal

is believed to be the cell-specificity determining region (Fimland et al., 1996; Fimland et al., 1998).

Structural features, such as the YGNGV motif, α-helices, disulfide bonds, and positively charged amino acids, play an important role in cell recognition and activity of class IIa bacteriocins (Moll et al., 1999). These structural features are found within different domains spanning the bacteriocin peptide, indicating its complex nature. The three-dimensional structure of leucocin A, a class IIa bacteriocin, is one of the first bacteriocins that have been fully described (Freqeau Gallagher et al., 1997). The β-sheet domain exerts antimicrobial activity, whereas the α-helix is thought to be responsible for target specificity (Moll et al., 1999). The YGNGV-motif allows for correct positioning of the bacteriocin on the membrane surface as it is recognized by a putative membrane receptor due to exposure caused by a β-turn structure (Bhugaloo-Vial et al., 1996; Fregeau Gallagher et al., 1997; Montville and Chen, 1998). The hydrophilic/amphiphilic N-termini of the β-sheet are another component involved in recognition, possibly due to its electrostatic membrane-bacteriocin interaction. However, both the YGNGV motif and N-termini of the β-sheet of class IIa bacteriocins do not determine their specificity of activity (Fig. 3.) (Ennahar et al., 2000). The central domain forms a hydrophilic or slightly amphiphilic α-helix and is believed to play a role in destabilization of the phospholipid bilayers. This mediates the insertion of the bacteriocin in the cytoplasmic membrane of the sensitive organism from an initial surface-bound state (Moll et al., 1999; Ennahar et al., 2000). However, in the case of pisciocins V1a and V1b, the central located α-helix spans almost the entire helical portion of these two bacteriocins, suggesting their role in anchoring of the bacteriocin rather than insertion into the membrane (Bhugaloo-Vial et al., 1996; Bennik et al., 1998). The C-terminal hydrophobic/amphiphilic α-helix contributes to insertion of the bacteriocin into the cytoplasmic membrane of target cells resulting in the formation of water filled pores (Moll et al., 1999; Ennahar et al., 2000). Furthermore, the C-terminal domain plays a role in the target-cell specificity due to its putative transmembrane helices. Another feature that has to be taken in consideration is the presence of disulphide bonds. All class IIa bacteriocins contain at least one disulphide bridge and have been shown to play a role in activity of the bacteriocin (Jack, et al., 1994; Eijsink et al., 1998; Montville and Chen, 1998). Studies investigating the spectra of activity of class IIa bacteriocins have shown that bacteriocins with two disulfide bonds displayed a greater and broader spectrum of activity in comparison with those containing only one bond (Richard et al., 2006).

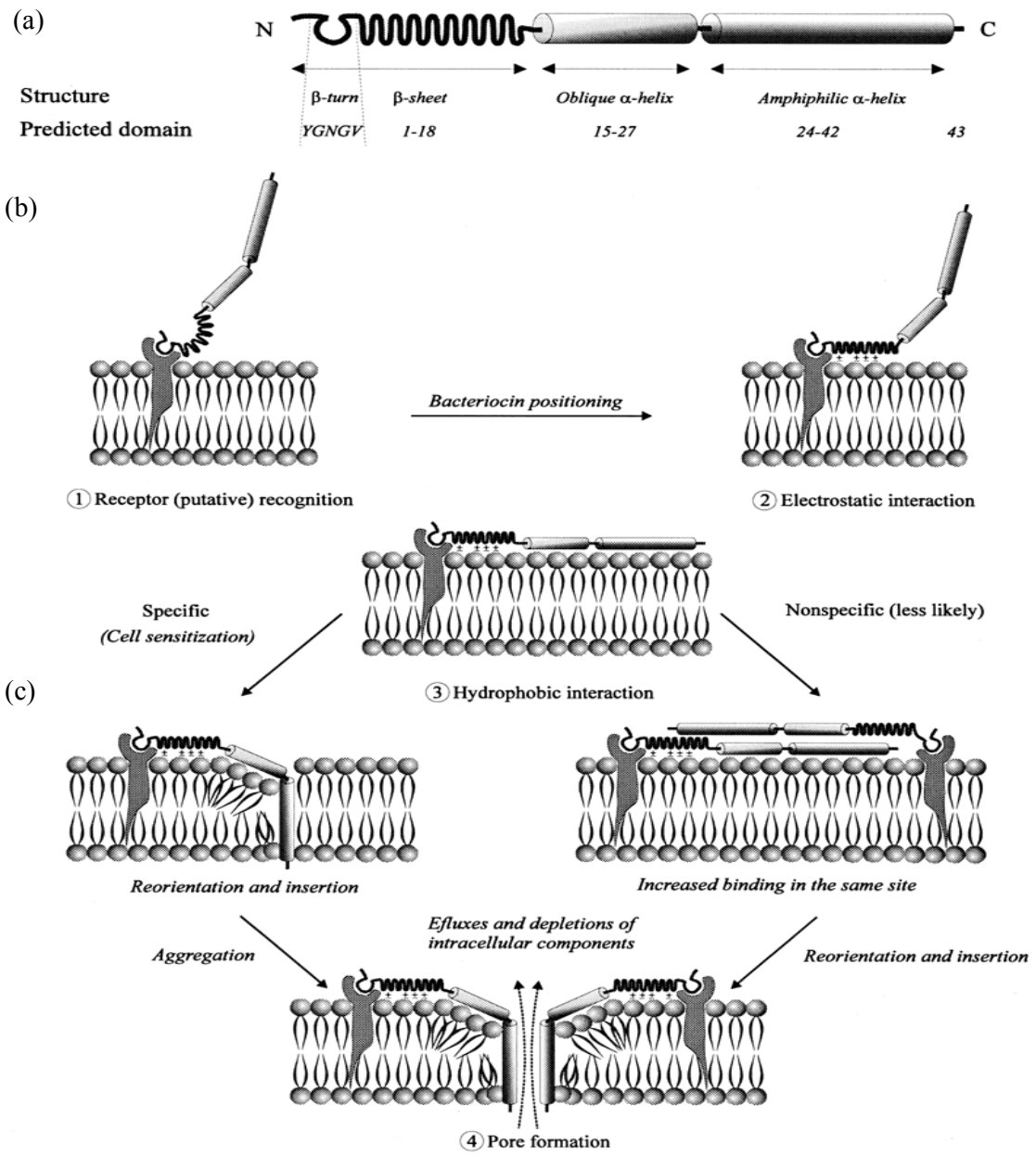

Fig. 3. Schematic representation of the structure of a model class-IIa bacteriocin and the predicted location of its domains with respect to target cell membrane: (a) predicted bacteriocin structural domains; (b) possible interactions of each domain with the membrane surface; (c) bacteriocin insertion and formation of hydrophilic pores. The hydrophobic face of the peptide is shaded dark and hydrophilic face is shaded light (Ennahar et al., 2000).

Class IIb bacteriocins are dependent on two distinct peptides for activity. They are responsible for dissipation of the transmembrane potential, while only a few affect the pH gradient (Clevelet al., 2001). These two-peptide bacteriocins (class IIb) can be divided into two

subgroups based on their ion-selectivity: (1) monovalent cation conducting bacteriocins, e.g plantaricin EF (Moll et al., 1999) and lactococcin G (Moll et al., 1998); and (2) anion conducting bacteriocins, e.g planatricin JK (Moll et al., 1999). Class IIc bacteriocins vary in their modes of action, which ultimately leads to membrane permeability, pheromone activity and specific inhibition of septum formation (Héchard and Sahl, 2002).

Minahk et al., (2004) reported on the synergy between certain antibiotics and sub-lethal concentrations of enterocin CRL35. Enterocin CRL35 increased the effectiveness of certain non-peptide antibiotics by impairment of bacterial active efflux systems, resulting in the accumulation of these toxic compounds within the cells. Future studies should focus on investigating the role played by proton gradient extrusion pumps in the synergy between certain antibiotics and sub-lethal concentrations of bacteriocins.

4.6.4 Production and modeling of bacteriocins

Consumers of food and beverages are more health conscious than in the past and the need for these products to be minimally processed and free from chemical preservatives is imperative (Ross et al., 2002). This observation directed research to exploit the occurrence of natural preservatives and their application (Chen and Hoover, 2003). In contrast to chemical preservatives, the use of LAB and/or their metabolites are generally more accepted by health conscious consumers as “natural” and “health-promoting” (Montville and Winkowski, 1997; Rodríguez et al., 2003). Class IIa bacteriocins received extensive interest as food preservatives, due to their bactericidal effect against various food-borne pathogens. Biopreservation can be defined as “the use of antaganostic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in foods to enhance food safety and extend shelf-life” (Chen and Hoover, 2003). The efficiency of bacteriocin-producing LAB in fermented foods is usually limited by various factors such as low production, genetic instability, regulatory systems, inactivation, and occurrence of resistance among target bacteria (Ennahar et al., 2000). For use of bacteriocins or bacteriocin-producing cultures in food one needs to optimize their efficiency. Recent studies focus on the heterologous expression of bacteriocins form various LAB strains to overcome these obstacles (Papagianni, 2003). Alternatively, media optimization or chemical modifications can be performed to optimize the yield of bacteriocin production (Ennahar et al., 2000; Chen and Hoover, 2003).

Heterologous expression systems are generally implemented for clarifying the role of recombinant proteins and peptides, assist in the transcriptional/translational control of recombinant gene expression, and to attain higher production levels than those of native sources (Makrides, 1996; Papagianni, 2003). Some LAB species, for example *Lactococcus lactis,* are extremely versatile in the stabilization of gene maintenance and the control of expression renders them useful for potential use as heterologous hosts (Venema et al., 1999). Constitutive production and over-expression of class-IIa-bacteriocin genes have been reported when cloned and expressed in host organisms, therefore overcoming the regulation systems of bacteriocins (Fremaux et al., 1995; McCormick et al., 1996; Biet et al., 1998; Horn et al., 1998). Various species and strains of LAB are food-grade, making them potentially useful as hosts for production of defined bacteriocins based on their properties relevant to specific food systems (Rodríguez, et al., 2003). This provide a method by which bacteriocin-producing LAB can be developed that are adapted to a specific type of food, thus preventing or decreasing problems with colonization and bacteriocin production (Ennahar et al., 2000). In a study by Chikindas et al. (1995) pediocin PA-1 was transformed with pMC117, a plasmid containing the *ped* operon under control of the lactococcal promoter P32, in *Pediococcus pentosaceus* PPE1.2. Production and secretion were achieved with production being fourfold higher than that reported for the natural producer *Pediococcus acidilactici* PAC1.0. Co-production of enterocin A and pediocin PA-1 in *Lactococcus lactis* IL 1403 has also been reported (Martinez et al., 2000). Other methods can also be implemented to achieve heterologous production of bacteriocins, namely: (1) exchanging the leader peptides and/or dedicated ABC secretion and processing systems and (2) by addition of signal peptides recognized by general secretory pathways (Rodrígues et al., 2003). Heterologous production of bacteriocins by LAB may have some drawbacks. In some cases low production levels of bacteriocins have been reported. Furthermore the use of genetically modified organisms (GMOs) for the *in situ* production of these peptides may receive disapproval from industries and health-conscious consumers (Rodrígues et al., 2003).

Many studies have focussed on optimization of media and growth conditions for increased bacteriocin production. Verellen et al. (1998) and Todorov and Dicks (2004; 2005a; 2005b, 2006a) and reported higher bacteriocin production levels for *Lactobacillus plantarum* ST194BZ, *Lactobacillus plantarum* ST13BR, *Lactobacillus plantarum* ST23LD, *L. plantarum* ST341LD, *L. plantarum* 423 in optimized growth media.

Chemical modification of bacteriocins offers the possibility for peptides to be developed with improved stability and activity. A reduction in activity of class IIa bacteriocins is generally associated with sequence modifications, including single-residue substitutions, compared to native bacteriocins (Fleury et al., 1996; Fimlet al., 1996; Quadri et al., 1997; Chen et al., 1997; Montville and Chen, 1998). However, in a study by Miller et al. (1998), pediocin PA-1 activity increased significantly after substitution of a Glu residue for Lys-11. Therefore, it is of interest to further explore the field of bacteriocin engineering.

5. Encapsulation of probiotic lactic acid bacteria

The ability of probiotic bacteria to survive and multiply in the host has a definite effect on their probiotic benefits (Gilliland 1989). Most probiotic products currently on the market do not have a long shelf-life, even when stored at low temperatures. Some of the commercial yoghurt products were found to have less viable cells than the desired level specified for probiotic effect (Kailasapathy and Chin, 2000). Some strains were inhibited or eradicated when exposed to a low pH medium. In order to combat this problem microencapsulation of probiotics in hydrocolloid beads are being investigated for improving their viability in food products and the gastrointestinal tract (Rao et al., 1989). Microencapsulation of probiotics has been a common practice for extending their shelf-life and converting them into a powder form for ease of use. Various techniques exist for encapsulating probiotic bacteria and converting them into a powdered form, such as, spray drying, fluidized bed drying and freeze drying (Krasaekoopt et al., 2003). Applying these techniques resulted in a complete release of the bacteria in the product. In this case, the released cultures are exposed to the product environment and/or the high acidic and bile conditions of the stomach and intestinal tract. Encapsulation of the cultures in hydrocolloid beads provides a physical protection barrier in such an unfavourable environment. Additional benefits of microencapsulation include: protection of cells inside the beads from bacteriophages (Steenson et al., 1987); increased survival during freezedrying and freezing; and greater stability during storage.

5.1 Encapsulation of probiotic bacteria in alginate beads for industrial application and target delivery

The process of microencapsulation involves the encapsulation of living cells within an encapsulating membrane in order to reduce cell injury or loss. The entrapment of lactic acid bacteria in calcium alginate beads has been frequently used (Sheu and Marshall, 1993). Various studies reported an increased survival of 80-95% for probiotic bacteria encapsulated

in alginate (Audet et al., 1988; Rao et al., 1989; Sheu and Marshall, 1991; Sheu and Marshall, 1993; Sheu et al., 1993; Jankowski et al., 1997; Kebary et al., 1998; Anal and Singh, 2007; Kim et al., 2008). Alginate is an accepted food additive and therefore poses no harm to the cells being immobilized (Prévost and Divies, 1988). Encapsulation using alginate as the encapsulation material can be performed by either extrusion (droplet method) and emulsion or a two-phase system (Krasaekoopt et al., 2003; Anal and Singh, 2007). Alginate extracted from several species of algae is a linear heteropolysaccharide of 1-4 links β-D-mannuronic and α-L-guluronic acids (Smidsrod et al., 1972). Alginate can vary in the proportion of these residues depending on the source of extraction (Anal and Singh, 2007). Gel formation is mainly ascribed to the preferential binding of divalent cations such as Ca^{2+} to the polymer of α-L-guluronic acid (Krasaekoopt et al., 2003; Anal and Singh, 2007).

In the extrusion technique a cell suspension of probiotic lactic acid bacteria is added to a hydrocolloid solution of alginate and mixed, where after it is extruded through a syringe needle in the form of small droplets into a calcium solution (Fig. 4.). Interfacial polymerisation occurs instantaneously with precipitation of calcium alginate. Calcium further permeates through the alginate system and causes a gradual gelation of the interior (Anal and Singh, 2007). The size of the beads mainly depends on the viscosity of the sodium alginate solution and the distance between the needles orifice and the hardening solution. The diameter of the extruder's orifice is another factor that determine the size of the beads. Smidsrod and Skjak-Braek (1990) obtained a bead size of 2-3mm using a 0.27-mm syringe. Although this method has been widely used to encapsulate probiotic lactic acid bacteria, no uniformity has been reported on the conditions of encapsulation protocols (Chandramouli et al., 2004). Encapsulation by means of extrusion is a very popular method due to its simplicity, low cost and high cell viability rates (Krasaekoopt et al., 2003).

The emulsion technique involves adding a small volume of the cell-polymer suspension (discontinuous phase) such as sunflower oil, corn oil, canola oil or soybean oil. A water-in-oil emulsion is formed after homogenization of the mixture. The resulting water-soluble polymer must then be insolubilized (cross-linked). The method used for insolubilization depends on the type of supporting material used. Various supporting materials exist, such as K-Carageenan, cellulose acetate phthalate, chitosan, gelatine and alginate. The formed beads are harvested by filtration (Fig. 4.). The beads can vary in size, between 25 µm and 2 mm, and is controlled by the speed of agitation (Krasaekoopt et al., 2003). Lactic acid bacteria used for

continuous (Audet et al., 1992) and batch fermentation (Lacroix et al., 1990) have been successfully encapsulated using this method.

Alginate has a low physical ability and this becomes a limiting factor to its use, especially in the presence of chelating agents. Gombotz and Wee (1998) reported that very high acidic conditions reduced the molecular weight of alginate in a cross-linked alginate matrix system causing it to degrade faster with release of the active ingredients. To overcome this problem, polycations, such as chitosan and poly-L-lysine were used as coating materials, thereby improving the chemical and mechanical stability of the alginate beads due to the strong complexes with alginate in the presence of Ca^{2+} chelators (Krasaekoopt et al., 2006). In a study by Krasaekoopt et al. (2004) the effect of coating alginate beads with poly-L-lysine, chitosan and an additional layer of alginate on the survival of probiotic bacteria in simulated digestive juices was investigated. Coating with chitosan provided the best protection for *Bifidobacterium bifidum*, *Lactobacillus casei* and *Lactobacillus acidophilus* (Krasaekoopt et al., 2004). Mandal et al. (2006) reported better survival of encapsulated *Lactobacillus casei* NCDC-298 compared to free cells at high bile salt concentrations, low pH and heat treatment.

5.2 Applications

Encapsulation of lactic acid bacteria may be of benefit towards applications in various food processing technologies and the delivery of the viable cells *in vivo* (Table 7). The encapsulated organisms can be used for producing dairy products such as cheese, yoghurt, frozen milk products and biomass production. Immobilized cells have been found to increase the production rate of commercially produced lactic acid. Immobilizing the cells ensures a constant high cell concentration, thereby improving lactic acid production rates, while reducing medium inhibition and requirements (Kourkoutas et al., 2005). Prévost et al. (1985) found that immobilized cells of *L. delbrueckii* spp. *bulgaricus* and *Streptococcus thermophilus* presented various advantages during the continuous manufacture of yoghurt, although it is more complicated that the traditional batch method. Using immobilized cells ensured a product with constant characteristics because the residence time, acidity and continuous inoculation of milk with a constant bacilli/cocci ratio can be controlled at a desired pH (Krasaekoopt et al., 2003). Encapsulation has also been found to protect the bacteria against bacteriophage attack due to the exclusion of phage particles form the gel matrix, making them attractive towards their use in biomass production (Steenson et al., 1987). Sheu and Marshall (1993) reported that lactobacilli entrapped in calcium alginate survived freezing

of ice cream by about 40% more compared to free cells. Survival of encapsulated bacteria significantly improved throughout storage from approximately 40% to about 60% (Kebary et al., 1998). In the case of target delivery Rao et al., (1989) found that encapsulated *Bifidobacterium pseudolongum* could survive in simulated gastric conditions at higher numbers compared to non-encapsulated bacteria.

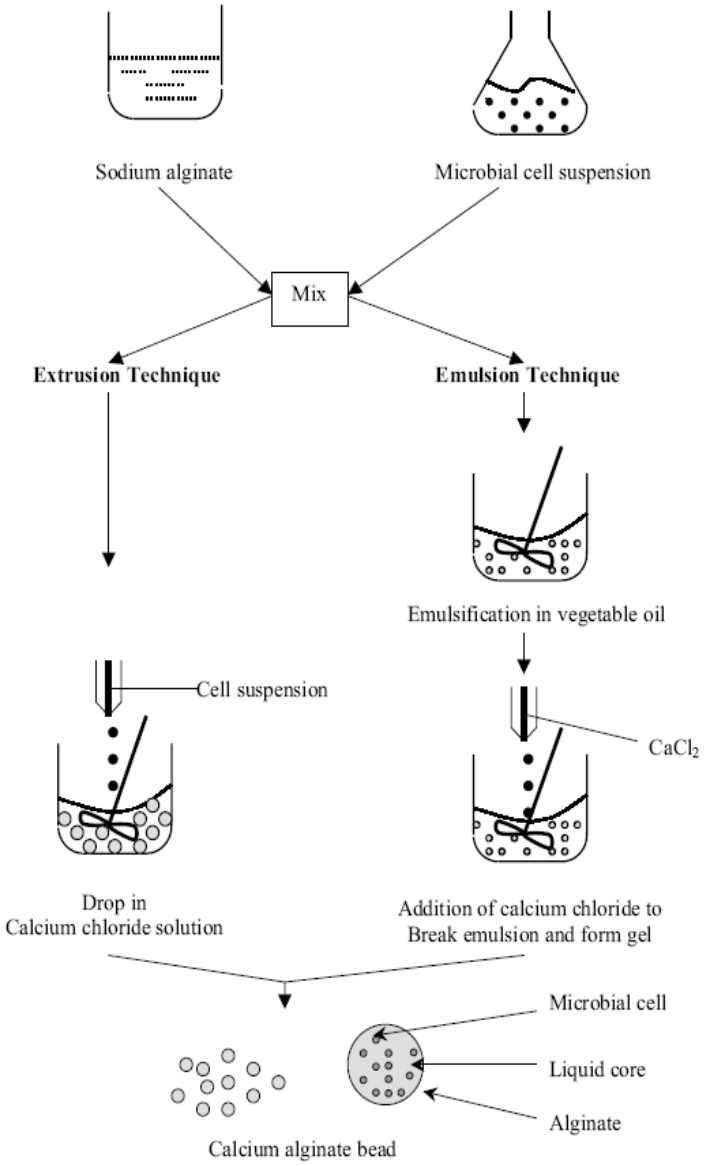

Figure 4. Schematic overview of encapsulation of bacteria by using the emulsion and extrusion technique (Krasaekoopt et al., 2003).

Table 7

Benefits of microencapulation for the production of probiotics and for their use in nutraceutical and food applications

Benefits associated with probiotic microencapsulation	
Benefit	Product
Facilitates the production of oxygen-sensitive cultures	Dried probiotic culture
Facilitates the recovery of centrifugation-sensitive cultures	Dried probiotic culture
Facilitates the recovery of high EPS-producing cultures	Dried probiotic culture
Less contamination problems	Dried probiotic culture
Cultures can be air-dried	Dried probiotic culture
Improved survival on exposure to gastric solutions	Nutraceutical
Improved survival on exposure to bile solutions	Nutraceutical
Improved stability during storage in dried form	Nutraceutical
Improved acidification rate	Dried sausages
Improved survival on heating	Biscuits, powder
Improved survival on freezing	Ice cream, milk-based medium, cranberry juice
Improved retention in the finished product	Cheese
Protection against bacteriophages	Fermented milks
Protection against yeast contaminants	Fermented milks
Improved survival during storage	Yoghurt, mayonnaise, milk

Adapted from Champagne and Fustier (2007).

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CHAPTER 3

Probiotic and bacteriocinogenic properties of *Lactobacillus plantarum* JW3BZ and *Lactobacillus fermentum* JW15BZ isolated from boza

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Probiotic and bacteriocinogenic properties of *Lactobacillus plantarum* JW3BZ and *Lactobacillus fermentum* JW15BZ isolated from boza

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Abstract

Lactobacillus plantarum JW3BZ and *Lactobacillus fermentum* JW15BZ, isolated from boza, produce bacteriocins active against a broad range of Gram-positive and Gram-negative bacteria, including pathogens isolated from contaminated food, human vaginal secretions and faeces. Strains JW3BZ and JW15BZ were resistant to low pH (pH 2.0–4.0). Both strains grew well in MRS broth with an initial pH ranging from 5.0 to 10.0. Strain JW3BZ displayed intrinsic resistance to bile salts. Strain JW15BZ, on the other hand, was sensitive to bile salts exceeding concentrations of 0.3% (w/v). Both strains are weakly hydrophobic and resistant to a broad range of antibiotics, anti-inflammatory drugs and painkillers. Only 4% of JW3BZ and JW15BZ cells adhered to Caco-2 cells and they did not compete with *L. monocytogenes* Scott A for adhesion. Strain JW3BZ contains the *MapA* adhesion gene. Both strains showed high auto- and co-aggregation. Bacteriocin (bac) JW15BZ was partially purified with ammonium sulfate, followed by separation on Sep-Pak C_{18} and reverse phase HPLC. Two separate peaks with antimicrobial activity were recorded for bacJW15BZ, suggesting that it consists of at least two antimicrobial peptides. BacJW3BZ and bacJW15BZ displayed activity against virus HSV-1 (EC_{50}=200 µg/ml). Strain JW3BZ contains operon *plnEFI*, encoding two small cationic bacteriocin-like peptides with a double-glycine leader peptide and an immunity protein. Both strains were detected in boza after 7 days of storage at 4 ºC and repressed the growth of *Lactobacillus sakei* DSM 20017, indicating that at least one of the bacteriocins is produced *in situ*. The sensory attributes of boza prepared with different starter cultures did not vary considerably, although statistical differences were observed for acidity and yeasty aroma, respectively. Strains JW3BZ and JW15BZ may be used as probiotic starter cultures to produce boza. The presence of bacJW3BZ and bacJW15BZ in the product may be of medicinal value.

Keywords: Lactobacillus plantarum, *Lactobacillus fermentum*, probiotics, starter cultures

1. Introduction

Ingestion of lactic acid bacteria in adequate numbers (10^6–10^7 cfu/ml) plays a key role in maintaining the microbial balance in the gastrointestinal tract by preventing colonization of pathogenic microorganisms (FAO/WHO, 2001). *In vitro* studies and clinical trials with animals have shown that certain strains of probiotic bacteria reduce symptoms related with irritable bowel syndrome (O'Mahony et al., 2005), lactose intolerance and allergies, reduce serum cholesterol levels, and prevent colon cancer (Gilliland, 1990; Salminen et al., 1998; Fooks et al., 1999; Kalliomaki et al., 2001) and diarrhea (Isolauri et al., 1991). It is thus not surprising that probiotic lactic acid bacteria, used either alone or in combination with traditional starter cultures are gaining significant interest in fermented foods.

The ability of lactic acid bacteria to adhere to epithelial cells or the intestinal mucosa is one of the main characteristics of probiotic bacteria (Todorov et al., 2007). Adhesion to epithelial cells plays a vital role in persistence, stimulation of the immune system, healing of damaged mucosa, and antagonism against pathogenic bacteria (Isolauri et al., 1991; Salminen et al., 1996; Rolfe, 2000; Reid and Burton, 2002). Other probiotic characteristics include survival at low pH and high bile concentrations (Mattila-Sandholm et al., 1999; Bezkorovainy, 2001).

Lactobacillus plantarum JW3BZ and *Lactobacillus fermentum* JW15BZ were isolated from boza, a cereal-based fermented beverage produced in the Balkan regions (Von Mollendorff et al., 2006). Both trains produce bacteriocins (bacJW3BZ and bacJW15BZ) active against a broad range of Gram-positive and Gram-negative bacteria, including human pathogens (Von Mollendorff et al., 2006). The aim of this study was to investigate the probiotic properties of the two strains, determine if the bacteriocins are produced in boza, and evaluate them as starter cultures.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

L. plantarum JW3BZ and *L. fermentum* JW15BZ, isolated from boza (Von Mollendorff et al., 2006), were cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 30 ^{o}C for 24 h. Indicator strains used in this study are listed in Table 1. Cultures were stored at -80 ^{o}C in growth medium, supplemented with glycerol (15%, v/v, final concentration).

2.2 Growth at different bile concentrations and pH values

MRS broth (Biolab), supplemented with 0.3, 0.6, 0.8, 1.0, 2.0 and 5.0% (w/v) oxbile (Oxoid, Basingstoke, England), respectively, were autoclaved and the pH adjusted to 6.4 with sterile 1 N NaOH or 1 N HCl. In a separate preparation, MRS broth (Biolab) was adjusted to pH 3.0, 4.0, 5.0, 7.0, 9.0, 11.0 and 13.0, respectively, with 1 N NaOH or 1 N HCl. The media were autoclaved and the pH adjusted to the original values if necessary. STERELIN^{TM} micro titer plates (Sterilin, Barloworld Scientific Ltd, United Kingdom) were filled with media (180 µl) and inoculated with a 20 µl culture (OD_{600nm}=0.3) *L. plantarum* JW3BZ or *L. fermentum* JW15BZ. Incubation was at 37 ^{o}C for 10 h. Optical density readings were recorded every hour. MRS broth (Biolab) served as control. The experiment was performed in triplicate.

2.3 Susceptibility to antibiotics and medicaments

Eighteen-hour-old cultures of strains JW3BZ and JW15BZ were embedded in MRS soft agar (1%, m/v) to a final concentration of 10^6 cfu/ml. Antibiotic disks (Table 2) were placed on the agar surface and the plates incubated at 37 ^{o}C for 24 h. Growth inhibition was recorded by measuring the zone diameter. In a separate experiment, commercial medicaments and antibiotics of known concentrations (Table 3) were dissolved in 5 ml sterile water and 10 µl spotted onto soft agar plates. Growth inhibition was determined as described before.

2.4 Hydrophobicity

The ability of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ to adhere to hydrocarbons was determined according to the method of Perez et al. (1998) with a few modifications. Stationary phase cells of JW3BZ and JW15BZ were harvested (12,000 g, 5 min, 4 ^{o}C), washed twice with 10 ml sterile 50 mM phosphate buffer (pH 6.5) and re-suspended in 10 ml of the same buffer. Three ml of each cell suspension, adjusted to OD_{560}=1.0 with sterile 50 mM phosphate buffer, was added to 0.6 ml *n*-hexadecane and vortexed for 2 min. After 1 h of incubation at 37 ^{o}C, the aqueous phase was carefully removed and the optical density (at 560 nm) determined. The percentage cell surface hydrophobicity was calculated as

$$[(A_o\text{-}A)/A_o)] \times 100,$$

where A_o and A refer to absorbance readings before and after extraction with *n*-hexadecane, respectively.

2.5 Adhesion of strains JW3BZ, JW15BZ and Listeria monocytogenes Scott A to Caco-2 cells

Caco-2 cells (Highveld Biological, Kelvin, South Africa) were grown in minimal essential medium (MEM) Earle's Base (Highveld Biological), supplemented with 100 U/ml penicillin, 10% (v/v) fetal bovine serum (Sigma, St Louis, MO, USA), and 100 U/ml streptomycin (Sigma). Incubation was at 37 ^{o}C in the presence of 5% CO_2. Caco-2 cells were seeded at 1 x 10^5 cells per well in 12-well plates (STERELIN^{TM}**)** to obtain confluence.

Strains JW3BZ and JW15BZ were grown in MRS broth (Biolab) and *L. monocytogenes* Scott A in BHI broth (Biolab) for 18 h at 37 ^{o}C. The cells were harvested (10,000 g, 10 min, 4 ^{o}C), washed twice with sterile PBS, diluted in MEM and adjusted to OD_{600}=0.5, equivalent to 1 x 10^6 cfu/ml (Todorov et al., 2007).

Competition for adherence to Caco-2 cells was studied by inoculating Caco-2 cells with 100 µl (1 x 10^5 cfu) JW3BZ or JW15BZ, and 100 µl (1 x 10^5 cfu) *L. monocytogenes* Scott A. After 2 h of incubation at 37 ^{o}C on an orbital shaker, the bacterial cells were withdrawn and the Caco-2 cells washed twice with 1 ml sterile PBS, followed by 1 ml sterile 0.5% (v/v) Triton X-100 to remove adhering cells. Ten-fold dilutions of the bacterial suspensions were prepared and plated onto MRS agar (Biolab) for enumeration of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ and on Listeria Enrichment (Merck, Darmstadt, Germany) agar to detect *Listeria monocytogenes* Scott A.

To determine whether strains JW3BZ and JW15BZ could prevent adherence of *L. monocytogenes* Scott A to Caco-2 cells, each well (1 x 10^5 Caco-2 cells) was inoculated with 100 µl of strain JW3BZ or JW15BZ (1 x 10^5 cfu) and incubated for 1 h at 37 ^{o}C. Non-adhering cells of strains JW3BZ and JW15BZ were removed by washing twice with sterile PBS. Caco-2 cells with adhered cells of JW3BZ and JW15BZ were incubated for a further 1 h in the presence of approximately 1 x 10^5 cfu of *L. monocytogenes* Scott A. The Caco-2 cells were then washed with sterile PBS and Triton X-100 (0.5%, v/v), and the number of viable cells of *L. monocytogenes* Scott A determined as described before.

In a separate experiment, the ability of strains JW3BZ and JW15BZ to displace *L. monocytogenes* Scott A from Caco-2 cells was determined by first inoculating the cells (1 x 10^5 cfu per well) with *L. monocytogenes* Scott A (1 x 10^5 cfu). After 1 h at 37 ^{o}C, non-adhering Scott A cells were washed from the wells, as described before, and the Caco-2 cells

inoculated with either strain JW3BZ or JW15BZ (1 x 10^5 cfu) and the plates incubated for a further 1 h. The wells were then washed and the number of viable cells of strains JW3BZ and JW15BZ, and *L. monocytogenes* Scott A released from Caco-2 cells determined as described before.

2.6 PCR amplification of genes encoding adhesion of lactic acid bacteria

Genomic DNA of strains JW3BZ and JW15BZ was isolated using the method of Dellaglio et al. (1973). Primers MapA-F (5'-GTACCAACGAAGTTCGATTC-3'), MapA-R (5'-TCAGTAA CGTTACCACCAAA-3'), Mub-F (5'-TGTCCAACACTACCACTGAA-3') and Mub-R (5'-GGTAATAACCTTGAGAA TGC-3'), designed from sequences of the adhesion genes *MapA* and *Mub* of *L. plantarum* WCFS1 (accession number AL935261.1) and *L. acidophilus* NCFM (accession number CP000033.2), respectively, were used to amplify the genomic DNA of strains JW3BZ and JW15BZ. The following conditions were used for all PCR reactions: Denaturation at 94 ^{o}C for 5 min, followed by 30 cycles at 94 ^{o}C for 45 s, annealing at 55 ^{o}C for 1 min and polymerization at 72 ^{o}C for 1 min. Final elongation was 10 min at 72 ^{o}C. The amplicons were sequenced using BigDye Terminator Cycle Chemistry (Biosystems, Warrington, UK) on an ABI Genetic Analyzer 3130XL Sequencer (Biosystems).

2.7 Aggregation

Auto-aggregation and co-aggregation were studied using the method of Malik et al. (2003). *L. plantarum* JW3BZ, *L. fermentum* JW15BZ, *Enterococcus faecium* HKLHS, T8 and ST5HA, *Enterococcus mundtii* ATCC PTA 7278 (ST4SA), *L. plantarum* AMA-K, ST8KF, ST303Co, ST202CO and ST101CO, *Lactobacillus sakei* DSM 20017, *Lactobacillus salivarius* 271, *Listeria innocua* LMG 13568, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119*, Pediococcus pentosaceus* ST3HA and *Streptococcus macedonicus* ATCC BAA-249 were cultured in 10 ml MRS broth (Biolab) or BHI broth (Biolab) for 24 h, harvested (300 g, 2 min, 25 ^{o}C), washed with 10 ml sterile distilled water and resuspended in sterile distilled water to OD_{660nm}=0.3. After 60 min of incubation at 25 ^{o}C, the cells were harvested (300 g, 2 min, 25 ^{o}C) and the optical density (at 660 nm) of the culture supernatants determined. The percentage auto-aggregation was calculated using the equation:

$$\%\text{Auto-aggregation} = [(OD_0 - OD_{60}) / OD_0] \times 100,$$

where OD_0 refers to the initial optical density of the organism and OD_{60} to the optical density recorded after 60 min at 25 ^oC.

In a separate experiment, the strains were cultured and harvested as described before and suspended in sterile water to OD_{660nm}=0.3. Strains JW3BZ and JW15BZ (500 µl of each) were paired with 500 µl of each of the other strains and incubated at 25 ^oC for 60 min. The strains were harvested (300 g, 2 min, 25 ^oC) and optical density values of the culture supernatants recorded at 660 nm. The percentage co-aggregation was calculated using the equation:

$$\% \text{ Co-aggregation} = [(OD_{Tot} - OD_S)/ OD_{Tot}] \times 100,$$

where OD_{Tot} refers to the optical density immediately after pairing of isolates; and OD_S to the optical density of the supernatant after 60 min of incubation at 25 ^oC.

All experiments were performed in triplicate.

2.8 Bacteriocin assay

L. plantarum JW3BZ and *L. fermentum* JW15BZ were grown in 10 ml MRS broth at 30 ^oC for 24 h. The cultures were harvested (8,000 g, 10 min, 4 ^oC) and the pH of the supernatants adjusted to 6.0 with sterile 1 N NaOH to prevent the inhibitory effect of lactic acid (Todorov and Dicks, 2005). Antimicrobial activity was determined using the agar spot-test described by Schillinger and Lücke (1989). The target strains are listed in Table 1. Activity was expressed as arbitary units (AU) per ml. One arbitary unit is defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator.

2.9 Testing for antiviral activity

The method described by Wachsman et al. (1999) was used. Monkey kidney Vero cells grown to confluence in 24-well culture plates for 48 h were infected with herpes simplex virus type 1 (HSV-1) strain F, obtained from the American Type Culture Collection (ATCC, Rockville, USA), at a MOI (multiplicity of infection) of 1. Virus stocks were prepared in monolayers of Vero cells grown in minimum essential medium (MEM), supplemented with 5% inactivated calf serum and 50 µg/ml gentamycin. After 1 h of adsorption at 37 ºC, the cells were covered with maintenance medium MM (MEM, supplemented with 2% inactivated calf serum),

containing different concentrations of bacJW3BZ and bacJW15BZ, respectively. Incubation was for 24 h. Infected cultures were subjected to two cycles of freeze-thawing, followed by centrifugation at low speed (1,000 *g*). Supernatants were diluted and titers determined according to a plaque formation assay (Wachsman et al., 1999). Antiviral activity was expressed as EC_{50}, i.e. the peptide concentration required to yield a 50% reduction in viral activity, compared to untreated (control) cultures.

2.10 Cytotoxicity tests

Cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, St. Louis) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) was used to assay growth and cell survival (Wachsman et al., 1999). Briefly, Vero cells grown in 96-well culture plates for 24 h were treated with serial dilutions of bacJW3BZ and bacJW15BZ for 24, 48 or 72 h. After this time cell monolayers were washed with Hanks solution and 50 µl of MTT (0.1 µg/ml) were added to each well. Following 2 h incubation at 37^{o}C, 4% CO_2 and 100% relative humidity, supernatants were removed and 200 µl of ethanol 96% were added to solubilise the formazan. The optical density of each well was measured on an Eurogenetics MPR-A 4i microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm (Wachsman et al., 1999).

2.11 Bacteriocin purification

Strain JW15BZ was cultured in 1 L MRS broth (De Man et al., 1960), without Tween 80, for 24 h at 30 ^{o}C. Cells were harvested (10,000 g, 15 min, 4 ^{o}C) and the cell-free culture supernatant incubated for 10 min at 80 ^{o}C to inactive proteolytic enzymes. Proteins were precipitated by gradually adding ammonium sulfate to the cell-free culture supernatant (60% saturation), stirred for 4 h at 4 ^{o}C and then centrifuged at 20,000 g for 1 h at 4 ^{o}C. The pellet was re-suspended in 25 mM ammonium acetate (pH 6.5) and loaded on a Sep-Pak C_{18} column (Waters Millipore, Milford, Ireland). The column was equilibrated with 20% (v/v) iso-propanol in 25 mM ammonium acetate (pH 6.5) and the proteins eluted with 40% and 60% iso-propanol in 25 mM ammonium acetate (pH 6.5). The eluted samples were dried under vacuum (Speed-Vac; Savant, France) and dissolved in 0.1% (v/v) trifluoracetic acid (TFA). The active fractions were pooled and subjected to reverse-phase HPLC on a C_{18} Nucleosil (Waters) column (50 x 4.6 mm). TFA (0.1%) in water (eluent A) and 10% TFA (0.1%) in 90% acetronitrile (eluent B) were used as eluents. A linear gradient from 0 to 100% B was applied over 65 min and kept at 100% B for 10 min. Absorbance readings were taken at

220 nm. Fractions were collected, dried under vacuum, dissolved in 1 ml sterile de-ionized water and stored at -20 ^{o}C. Activity was tested using the agar-spot test method (Schillinger and Lücke, 1989). Fractions displaying the highest activity from the first separations were pooled and again injected.

2.12 Amplification of plantaricin genes with different primers

Primers were designed from DNA sequences encoding bacteriocins of *L. plantarum* (Table 4). Genomic DNA of strain JW3BZ was amplified in a Gene Amp 9700 PCR system (Applied Biosystems, California, USA) as follows: Denaturation at of 94 ^{o}C for 3 min, followed by 30 cycles of denaturation at 94 ^{o}C for 1 min, annealing for 1 min (see Table 4 for temperatures), elongation at 72 ^{o}C for 30 s, and final extension at 72 ^{o}C for 5 min. Fragments were separated in a 2% agarose gel and 0.5 x TAE buffer.

In a separate experiment, genomic DNA of *L. plantarum* JW3BZ was amplified using primers designed from the *plnEFI* operon encoding two small cationic bacteriocins (Diep et al., 1996). Primers used for PCR amplification are listed in Table 5. PCR amplification was performed as follows: Denaturation at 94 ^{o}C for 3 min, followed by 35 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 53 ^{o}C for 1 min, elongation at 72 ^{o}C for 30 s, and extension at 72 ^{o}C for 5 min. Products were subjected to electrophoresis using 1% (w/v) agarose gels and 0.5 x TAE buffer. DNA fragments were extracted and purified from the gels using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, USA) and sequenced using an ABI Genetic Analyzer 3130XL Sequencer (Applied Biosystem). Sequence alignments were performed with the DNA sequence of the operon *plnEFI* (Genbank accession number X94434) using DNAMAN for Windows® Sequence Analysis Software (Lynnon BioSoft, Quebec, Canada).

2.13 Production of boza

Boza was prepared by adding 525 g barley to 3.5 liters water and boiled for 20 min. The mixture was stirred continuously. Cold water (500 ml) was added, the mixture homogenized with a blender and filtered through cheesecloth. Sugar (175 g) was added, the volume adjusted to 3.5 l with cold water, and divided into 250 ml samples. One sample was inoculated with 75 mg commercial bakers' yeast (*Saccharomyces cerevisiae*). Six samples were inoculated with 75 mg bakers' yeast and 0.1% (v/v) starter culture. All starter cultures, including *L. sakei* DSM20017, were grown to 1 x 10^{8} cfu/ml in 10 ml MRS broth before use. The starter cultures were (i) *L. plantarum* JW3BZ, (ii) *L. plantarum* JW6BZ,

(iii) *L. fermentum* JW11BZ, (iv) *L. fermentum* JW15BZ, (v) *Enterococcus mundtii* ST4SA, and (vi) commercial boza. A duplicate set of 7 samples inoculated as described here, received 0.1% (v/v) *L. sakei* DSM 20017 as target strain. All samples were fermented for 6 h at 37 ^{o}C and then stored at 4 ^{o}C for 7 days. The number of viable lactic acid bacteria in each sample was determined by plating onto MRS agar (Biolab), supplemented with 50 mg/l Natamycin (Gist Brocades, B.V., Delft, Netherlands) to prevent yeast growth.

2.14 Isolation of lactic acid bacteria from boza and DNA extraction

Lactic acid bacteria were isolated from boza on days 1, 5 and 7 after fermentation. Serial dilutions were made in sterile physiological saline, plated onto MRS agar (Biolab) and incubated at 30 ^{o}C for 24-48 h. Cells were harvested from the surfaces of the plates with 10 ml sterile physiological saline and sterile glass beads. Cell suspensions (2 ml each) were transferred to sterile eppendorf tubes and DNA isolated according to the method of Dellaglio et al. (1973).

2.15 Denaturing gradient gel electrophoresis

Primers 341FGC (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG –3') and 534R (5' ATTACCGCGGCTG CTGG –3') were used to amplify approximately 200 bp, representing the V3 variable region of the 16S rRNA gene (Garrity et al., 2001). The PCR reaction mix contained 5 µl of 10 x buffer, 3 µl of 25 mM MgCl2, 5 µl of 5 µM 341FGC, 5 µl of 5 µM 534R, 10 µl of 1 mM DNTP's, 0.5 µl *Taq* polymerase (Takara), and 1 µl DNA (50 ng/µl). Each reaction was adjusted to a final volume of 50 µl with sterile Milli-Q water and amplified in a Gene Amp 9700 PCR system (Applied Biosystems, California, USA). Initial denaturation was at 94 ^{o}C for 5 min, followed by 30 cycles of denaturation at 94 ^{o}C for 30 s per cycle, annealing at 56 ^{o}C for 30 s and elongation at 72 ^{o}C for 1 min. Final elongation was at 72 ^{o}C for 8 min (Theunissen et al., 2005). All PCR products were subjected to electrophoresis in 2% (w/v) agarose and 0.5 x TAE electrophoresis buffer. The amplified fragments were separated by denaturing gradient gel electrophoresis (DGGE) using the Bio-Rad Dcode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, USA). PCR products (40 µl) were separated in 8% (w/v) polyacrylamide gels (37.5 acrylamide: 1.0 bisacrylamide) and 0.5 x TAE buffer with a linear gradient from 40% to 60%. Electrophoresis was performed at a constant 100 V for 12 h at 60 ^{o}C. The gels were stained with ethidium bromide (0.5 mg/l) for 30 min. DNA fragments amplified from

L. plantarum JW3BZ, *L. plantarum* JW6BZ, *L. fermentum* JW11BZ, *L. fermentum* JW15BZ, *L. sakei* DSM 20017 and *E. mundtii* ST4SA were used as markers.

2.16 Detection of starter cultures in boza with species- specific PCR

Species-specific PCR was performed to confirm the results obtained by DGGE using species-specific primers for *L. plantarum* (planF 5'-CCGTTTATGCGGAAC ACC TA-3' and pREV 5'-TCG GGA TTA CCA AAC ATC AC-3') (Torriani et al., 2001), *L. sakei* (Ls 5'-ATGAAACTATTAAATTGGTAC-3' and 16 5'-GCTGGATCACCTCCTTTC-3') (Berthier and Ehrlich, 1998), *L. fermentum* (FERM1: 5'-GTTGTTCGCATGAACAACGCTTAA-3' and LOWLAC: 5'-CGACGACCATGA ACCACCTGT-3') (Chagnaud et al., 2001), and *Enterococcus sp.* (Ent1 5'-TACTGACAAACCATTCATGATG-3' and Ent2 5'-AACTTCGTCACCAA CGCGAAC-3') (Ke et al., 1999). Pure cultures of *L. plantarum* JW3BZ and JW6BZ, *L. fermentum* JW11BZ and JW15BZ, and *L sakei* DSM 20017 were used to optimize the PCR amplifications. Laboratory prepared boza samples were tested for the presence of these microorganisms to confirm the results obtained by the PCR-based DGGE analysis.

2.17 Sensory analysis

Descriptive sensory analysis was performed on boza produced with different combinations of lactic acid bacteria as starter cultures. The sensory panel consisted of nine trained panelists, trained according to the consensus method of Lawless and Heymann (1999). A 100 mm unstructured line scale was used for attribute intensity evaluation, with the left side of the scale corresponding to the lowest intensity (zero) and the right side corresponding to the highest intensity (100).

A consensus list of attributes that described boza included yeasty aroma, yeasty flavor, bitterness, sweetness and acidity (Table 6). The panelists were seated in individual booths in a temperature-controlled (21 ^{o}C) and light-controlled (artificial daylight) room. Samples were presented in a complete randomized order in four sessions. Distilled water and unsalted fat-free crackers were given to the panelists between samples. Data were subjected to the appropriate analyses of variance (ANOVA) using SAS version 8.2 Statistical software (SAS, 1999). Shapiro-Wilk tests were performed to test for non-normality (Shapiro and Wilk, 1965).

3. Results

3.1 Growth at different bile concentrations and pH values

Growth of *L. plantarum* JW3BZ in MRS broth (Biolab) supplemented with 0.3% (w/v) bile was similar to values obtained when grown in MRS broth (Biolab). Growth in the presence of 0.6% (w/v) bile was slow and completely suppressed in the presence of 0.8 to 5.0% (w/v) bile (not shown). Growth of *L. fermentum* JW15BZ was repressed in the presence of 0.3% (w/v) bile and completely inhibited in the presence of 0.6 to 5.0% (w/v) bile (not shown).

Optimal growth for strains JW3BZ and JW15BZ was recorded at pH 5.0, with little difference in growth between pH 6.0 and 10.0 (not shown). Growth of both strains was repressed in MRS broth with initial pH set at 4.0 and below (not shown).

3.2 Susceptibility to antibiotics and medicaments

L. plantarum JW3BZ and JW15BZ were resistant to a number of antibiotics (Table 2). However, growth of both strains was inhibited by Cefepime, Ceforoxime, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Clindamycin, Erythromycin, Neomycin, Ofloxacin, and Tetracycline (Table 2). In addition, strain JW3BZ was inhibited by Ceftazidime, Cephazolin, Fusidic acid, Nitrofurantion, Oxacillin, Tobramycin and Vancomycin, while strain JW15BZ was inhibited by Amikacin and Rifampicin (Table 2).

Growth of both strains was repressed by Cipro (a commercial preparation of Ciprofloxacin) and Thioridazin, with Thioridazine HCl as active substance (Table 3). Dolocyl, an anti-inflammatory drug, prevented the growth of strain JW3BZ, but only slightly inhibited the growth of strain JW15BZ (Table 3). Diuretidin (Triamterenum, hydrochlorothiazidum) and Proalgin (Metamizole sodium) completely inhibited the growth of strain JW3BZ, but not the growth of strain JW15BZ (Table 3).

3.3 Hydrophobicity

Low Hydrophobicity values were recorded for *L. plantarum* JW3BZ (3.4 %) and *L. fermentum* JW15BZ (7.8 %).

3.4 Adhesion of strains JW3BZ, JW15BZ and L. monocytogenes *Scott A to Caco-2 cells*

Only 4% cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ adhered to Caco-2 cells during the first hour of incubation. No increase in adhesion was observed after a further 1 h of

incubation. Pre-colonization of Caco-2 cells with strains JW3BZ and JW15BZ (1 x 10^5 cfu) for 1 hour at 37 ^oC did not prevent *L. monocytogenes* Scott A from adhering to the cells. Similar results were obtained for adhesion of strains JW3BZ and JW15BZ to Caco-2 cells when the cells were pre-colonized with *L. monocytongenes* Scott A.

3.5 Amplification of genes involved in adhesion

A DNA fragment corresponding to the size of *MapA* was amplified from the DNA of strain JW3BZ but not from the DNA of strain JW15BZ. *Mub* was not detected in any of the two strains. Sequencing of the PCR product amplified from strain JW3BZ revealed 82% homology to *MapA* of *L. plantarum* WCFS1 (accession number NC 004567).

3.6 Auto-aggregation and Co-aggregation

Different values of auto-aggregation were recorded for *L. plantarum* JW3BZ, *L. fermentum* JW15BZ and all other strains studied. Overall high levels of auto-aggregation were observed for strains JW3BZ (58.2%) and JW15BZ (67.3%). Auto-aggregation values ranged from 34.6 to 77.4% for partner strains.

Strains JW3BZ and JW15BZ co-aggregated with all 17 partner strains (not shown). Strain JW3BZ displayed overall high rates of aggregation with *L. ivanovii* subsp. *ivanovii* ATCC 19119 (60.3%), *E. faecium* HKLHS (60.0%), *L. plantarum* ST202Co (59.2%), *S. macedonicus* ATCC BAA-249 (63.4%), *L. sakei* DSM 20017 (70.5%), and to a lesser extent with *E. mundtii* ATCC PTA 7278 (ST4SA) (54.3%), *L. plantarum* AMA-K (50.5%), *L. plantarum* ST101Co (53.9%), *L. plantarum* ST303Co (53.6%) and *L. innocua* LMG 13568 (54.7%). *L. ferme*ntum JW15BZ displayed the highest level of aggregation with *L. plantarum* ST101Co (50.3%) and *L. sakei* DSM 20017 (65.5%) (not shown).

3.7 Bacteriocin bioassay

L. plantarum JW3BZ and *L. fermentum* JW15BZ produced bacteriocins active against a broad spectrum of Gram-positive and Gram-negative bacteria (Table 1).

3.8 Antiviral activity and cytotoxicity of bacteriocins

BacJW3BZ and bacJW15BZ displayed antimicrobial activity (EC_{50}=200 µg/ml) against virus HSV-1 (not shown). Different levels of cytotoxicity were recorded, ranging from CC_{50}-values of 1377 µg/ml for bacJW3BZ to 2594 µg/ml for bacJW15BZ.

3.9 Bacteriocin purification

Proteins of strain JW15BZ collected from the Sep-Pak C_{18} column displayed antimicrobial activity against *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *E. faecium* HKLHS. Re-injected fractions collected from the 40% iso-propanol separation yielded an active peak at a retention time of 48 min (Fig. 1 (a)). Separation of active fractions by HPLC (elution with 73% acetonitrile), yielded a single peak with antimicrobial activity at a retention time of 48.5 min (Fig. 1 (b)). Re-injected fractions collected from the 60% iso-propanol separation yielded an active peak at a retention time of 52.5 min (Fig. 2 (a)). Separation of active fractions by HPLC (elution with 57% acetonitrile) yielded a single active peak at a retention time of 45.0 min (Fig. 2 (b)).

3.10 Amplification of genes encoding plantaricins

DNA fragments amplified from the genome of *L. plantarum* JW3BZ corresponded in size to *plnD*, *plnEF*, *plnI* and *plnG*. Comparison of the sequences revealed 96% homology to *plnD*, 95% to *plnEF*, 95% to *plnI*, and 98% to *plnG.* No fragments were amplified with primers that have been designed for *plnA*, *plnB* and *plnC, plnJ, plnK*, *plnN*, *plnNC8* and *plnS.*

DNA fragments amplified from the genome of *L. plantarum* JW3BZ corresponded in size to the *plnEFI* operon of *L. plantarum* C11. Comparison of the sequence revealed 97% homology with the sequence of *plnEFI* (Genbank accession number X94434).

3.11 DGGE

PCR amplification of DNA isolated from laboratory prepared boza samples produced fragments of 200 bp. DGGE fingerprints obtained are shown in Figs. 3-5. Species were identified by specific bands in the DGGE fingerprint. *L. plantarum* JW3BZ, used as single starter culture, or in combination with *L. sakei* DSM 20017, remained present in boza for at least 7 days (Fig. 3), whereas *L. sakei* could not be detected. An unknown DNA band was detected in all samples during storage (Figs. 3-5). *L. fermentum* JW15BZ remained present in boza for at least 7 days (Fig. 4), whereas *L. sakei* DSM 20017 could not be detected. These results were confirmed by species-specific PCR (not shown). Boza prepared with commercial bakers' yeast or boza as a starter culture were also studied over a period of 7 days (Fig. 5). Strains of *L. plantarum* and *L. fermentum* naturally present in the commercial boza starter culture were recorded (Fig. 5). The presence of *L. plantarum* in boza produced with commercial bakers' yeast was confirmed by PCR with species-specific primers (not shown).

L. sakei DSM 20017 as starter culture remained in boza for at least 5 days when inoculated in combination with commercial bakers' yeast (Fig. 5.).

3.12 Sensory analysis

Sensory analysis of the six boza preparations is shown in Fig. 6. No significant difference ($p \leq 0.05$) was observed for yeasty aroma, sweetness and bitterness. No significant difference was observed for samples fermented with starter cultures JW3BZ, JW6BZ, JW11BZ and *E. mundtii* ST4SA or commercial boza. However, the yeasty aroma of boza prepared with strain JW15BZ was slightly lower compared to boza prepared with the latter starter cultures. The acid taste of all samples was relatively low; with the lowest recorded for boza prepared with *E.mundtii* ST4SA. No correlation was found between the different sensory attributes tested.

4. Discussion

Approximately 2.5 l gastric juice (pH ± 2.0) is secreted daily in the stomach (Charteris et al., 1998), forming an environment which inhibits the growth of most microorganisms (Kimoto et al., 1999). Growth of strains JW3BZ and JW15BZ were repressed at pH 2.0 to 4.0, but not completely inhibited, suggesting that they have some intrinsic resistance to acid and may survive conditions in the small intestine. Similar results have been reported for *L. plantarum* 423, *L. salivarius* 241, *L. curvatus* DF38 and *Lactococcus lactis* subsp. *lactis* HV 219 (Brink et al., 2006; Todorov et al., 2007). The ability of probiotic bacteria to survive passage through the stomach is variable and strain-dependent (Clark et al., 1993; Charteris et al., 1998, Zavaglia et al., 1998). Loss in viability has been reported for lactococci in simulated gastric juice (Kimoto et al., 2000).

Bile concentrations in normal healthy individuals range from 0.3 to 0.5% (Zavaglia et al., 1998; Dunne et al., 1999). *L. plantarum* JW3BZ displayed good intrinsic resistance to bile concentrations of 0.3 to 0.6%, whereas *L. fermentum* JW15BZ displayed poor resistance to bile concentrations of 0.3 to 5.0%. Similar levels of bile resistance have been recorded for *S. thermophilus* A4, *L. delbrueckii* subsp. *bulgaricus* AB1 (Vinderola and Reinheimer, 2003), *L. plantarum* 423, *L. salivarius* 241, *L. curvatus* DF38 and *L. lactis* subsp. *lactis* HV219 (Brink et al., 2006; Todorov et al., 2007). The relative good intrinsic resistance of strain JW3BZ to bile may promote survival in the gastrointestinal tract. However, according to Marteau and et al. (1997) sensitivity to bile is not necessarily a disadvantage, as lysis of cells during passage

through the gastrointestinal tract may result in the release of β-galactosidase and improve the digestion of lactose in the small intestine.

Strains JW3BZ and JW15BZ displayed resistance to a broad range of antibiotics and commercial neuroleptic, analgetic, anti-inflammatory, diuretic and antitussive medicaments Dodocyl, an anti-inflammatory medicament with Ibuprofen as active substance, completely inhibited the growth of strain JW3BZ, while strain JW15BZ was only slightly inhibited. However, no growth inhibition was recorded with Adco-Ibuprofen, also containing Ibuprofen. Similar results were recorded for *Lactococcus lactis* subsp. *lactis* HV219 (Todorov et al., 2007). Inhibition by Ibuprofen seems to be concentration dependent, since the only difference in the latter two medicaments is the concentration of Ibuprofen. Resistance to antibiotics and anti-inflammatory drugs may be of advantage when the strains are used as probiotics. Minahk et al. (2004) reported a synergy between certain antibiotics and sub-lethal concentrations of enterocin CRL35. Enterocin CRL35 increased the effectiveness of certain non-peptide antibiotics by impairment of bacterial active efflux systems, resulting in accumulation of antibiotics in the cytoplasm. In a more recent paper Knoetze et al. (2007) suggested to treat multi-drug resistant strains with a combination of the broad-spectrum antimicrobial peptide ST4SA, produced by *Enterococcus mundtii* ST4SA, and certain antibiotics. Peptide ST4SA dissipates the proton-motive force, which may disrupt efflux pumps and lead to increased intracellular levels of antibiotics (Knoetze et al., 2007). Further studies are needed to determine the effect of sub-lethal concentrations of bacteriocins on proton gradient extrusion pumps.

Several mechanisms are essential for the adhesion of microbial cells to the intestine, including the cell surface (Rosenberg et al., 1980; Savage, 1992; Kiely and Olson, 2000). Cells of high hydrophobicity adhere stronger to epithelial cells (Naidu et al., 1999). However, previous studies have shown that, in some cases, no relationship was found between the surface hydrophobicity of the bacterial cells and the extent to which the cells adhere to either hydrophobic or hydrophilic substrates (Mattos-Guaraldi et al., 1999; Coquet et al., 2002). Strains JW3BZ and JW15BZ are weakly hydrophobic, suggesting that they may not adhere strongly to epithelial cells. Similar low hydrophobicity values were recorded for *Lactobacillus delbrueckii* subsp. *bulgaricus* Hb2 (Vinderola and Reinheimer, 2003) and *L. lactis* subsp. *lactis* HV219 (Todorov et al., 2007). *Lactobacillus acidophilus* strains A3, A9, 08, 53, 5 and CSL displayed high hydrophobicity values (38.1 – 50.2%) (Vinderola and Reinheimer, 2003).

Similar results have been recorded for *Bifidobacterium bifidum* A12, BBI and Bb12 (46.7-54.7% hydrophobicity), *Lactobacillus casei* A13, A14, LB and BRA (12.0-24.1% hydrophobicity) and *L. rhamnosus* A15, A16 and LS (10.9 – 19.9% hydrophobicity) (Vinderola and Reinheimer, 2003). Various other factors also play a role in adhesion, e.g. the expression of surface-associated proteins, variation in growth conditions, and the physiological state of the cells.

Only 4% of JW3BZ and JW15BZ cells adhered to Caco-2 cells during the first hour of incubation. An additional 1 h of incubation had no effect on adhesion. Similar results were reported for *L. lactis* subsp. *lactis* HV219 (Todorov et al., 2007). Based on these findings, one may argue that strains JW3BZ and JW15BZ will not have sufficient time to colonize the small intestine within the first 2 h after the food has been ingested. However, colonization also depends on the food matrix and intestinal conditions (Todorov et al., 2007). Pre-colonization of Caco-2 cells with strains JW3BZ and JW15BZ did not prevent *L. monocytogenes* Scott A from adhering to the cells. Similar results were obtained when Caco-2 cells were first colonized with *L. monocytogenes* Scott A. Concluded from these results; strains JW3BZ, JW15BZ and Scott A do not compete for the same receptors on Caco-2 cells.

Only a few strains harbor genes encoding production of adhesion proteins. The mucus-binding protein (Roos and Jonsson, 2002) and mucus adhesion-promoting protein (*MapA*; GenBank accession number AJ 293860) produced by *Lactobacillus reuteri* are the best-studied. *MapA* of strain JW3BZ displayed 82% homology to *MapA* of *L. plantarum* WCFS1 (Genbank accession number NC 004567). *Mub* was not detected in strain JW3BZ. Neither *MapA* nor *Mub* was detected in strain JW15BZ. The presence of *MapA* in strain JW3BZ suggests that, if expressed, the protein may facilitate adhesion to mucus in the intestinal tract. It would, however, be interesting to know if the gene is up- or down regulated in the presence of mucus. In the case of *L. plantarum* 423, *Mub* and *MapA* were up-regulated in the presence of mucus, proportional to increasing concentrations (Ramiah et al., 2007). *MapA* in *L. plantarum* 423 was up-regulated in the presence of 3.0 g/l bile and 3.0 g/l pancreatin at pH 6.5, whereas *Mub* was down-regulated (Ramiah et al., 2007). Certain mucus genes may thus be up-regulated under stressful conditions, ensuring adhesion of the cells to the gastrointestinal tract (Ramiah et al., 2007).

Auto-aggregation and co-aggregation of microorganisms play an integral role in the development of biofilms and render cells the advantage of colonizing complex ecosystems

such as the gastrointestinal tract (Kolenbrander, 2000). Auto-aggregation is enhanced by increased hydrophobicity and has a tendency to be stronger than co-aggregation (Rickard et al., 2004). Auto-aggregation varied between strains JW3BZ and JW15BZ, and also for each of the other strains tested. No direct correlation could be found between auto-aggregation and hydrophobicity of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ. *L. plantarum* JW3BZ displayed overall high rates of co-aggregation with *L. ivanovii* subsp. *ivanovii* ATCC 19119, *E. faecium* HKLHS, *L. plantarum* ST202Co, *S. macedonicus* ATCC BAA-249, *L. sakei* DSM 20017, and to a lesser extent with *E. mundtii* ATCC PTA 7278 (ST4SA) *L. plantarum* strains AMA-K, ST101Co, ST202Co and ST303Co, and *L. innocua* LMG 13568. The high levels of auto-aggregation observed for strains JW3BZ and JW15BZ may facilitate adherence to the gastrointestinal tract. Co-aggregation values recorded for strain JW15BZ was lower than that recorded for strain JW3BZ. Highest co-aggregation of JW15BZ was recorded with *L. plantarum* ST101Co and *L. sakei* DSM 20017. Co-aggregation interactions may facilitate a closer proximity of strains JW3BZ and JW15BZ to sensitive pathogens enhancing the bactericidal effect. However, co-aggregation may also occur between potential human and foodborne pathogens not sensitive to strains JW3BZ and JW15BZ.

The broad spectrum activity of bacteriocins JW3BZ and JW15BZ (Table 1) is unique for bacteriocins of lactic acid bacteria (Todorov and Dicks, 2006). Activity of bacteriocin JW15BZ against *Klebsiella pneumoniae* is not a common phenomenon and has only been reported for bacteriocins ST242BZ, ST284BZ produced by *Lactobacillus paracasei* ST242BZ and ST284BZ, ST414BZ produced by *L. plantarum* ST414BZ, ST461BZ produced by *Lactobacillus rhamnosus* ST461BZ and ST712BZ produced by *Lactobacillus pentosus* ST712BZ (Todorov and Dicks, 2006).

To our knowledge only a limited number of papers reported on antiviral activity of lactic acid bacteria i.e. enterocin CRL35 produced by *Enterococcus faecium* CRL35 (Wachsman et al., 1999, 2003), bacteriocin 1043 produced by *Lactobacillus delbrueckii* 1043 (Serkedjieva et al., 2000) and bacteriocin ST4V produced by *Enterococcus mundtii* ST4V (Todorov et al., 2005). The mode of action of bacteriocins against viruses is not known. Possible explanations could be the aggregation of virus particles, blockage of receptor sites on the host cell, or inhibition of key reactions in the multiplication cycle (Wachsman et al. 2003). Both bacteriocins (bacJW3BZ) and bacJW15BZ) displayed antiviral activity (EC_{50}) values of 200 µg/ml (not

shown). HSV-1 causes primary and recurrent infections of mucous membranes, orofacial and genital lesions, and encephalitis (Whitley and Roizman 2001).

Bacteriocin JW3BZ with a CC_{50}-value of 1377 µg/ml is less cytotoxic than bacteriocin JW15BZ with a CC_{50}-value of 2594 µg/ml. Bacteriocin ST4V isolated from *E. mundtii*, and enterocin CRL35 isolated from *E. faecium*, yielded similar CC_{50}-values (1600 µg/ml and 2500 µg/ml, respectively; Wachsman et al. 1999; Todorov et al. 2005).

Differences in retention times recorded when bacteriocin JW15BZ was eluted with different concentrations of acetronitrile suggests that activity may be due to more than one peptide. Electron spray mass-spectrophotometry and amino acid sequencing of these fractions need to be performed to proof this hypothesis. To our knowledge, no amino-acid or nucleotide sequence has been published for bacteriocins produced by *L. fermentum*.

L. plantarum JW3BZ contains genes homologues to *plnD* (96%), *plnEF* (95%), *plnG* (98%), and *plnI* (95%) of *L. plantarum* C11 (Diep et al., 1996). *PlnE*, *plnF* and *plnI* belongs to operon *plnEFI* of *L. plantarum* C11, encoding two small cationic bacteriocin-like peptides, plantaricin E and plantaricin F, with double-glycine-type leader peptides and its respective immunity proteins. Operon *plnJKLR*, also present in *L. plantarum C11*, is similar to operon *plnEFI* (Diep et al., 1996). Genes *plnA*, *plnB* and *plnC* of the regulatory operon *plnABCD* were not detected in strain JW3BZ, suggesting that the genes are either missing from the regulatory operon, or that other regulatory genes are involved. Only *plnD*, the negative regulator of the signal-transducing pathway, was detected in strain JW3BZ. The presence of *plnG* in *L. plantarum* JW3BZ, which forms part of the operon *plnGHSTUV* (Omar et al., 2006) encoding an ABC transporter system, suggests that strain JW3BZ may use this transport system to secrete peptides containing double-glycine N-terminal leaders. The bacteriocin activity displayed by strain JW3BZ may possibly be ascribed to the production of plantaricin E and F. The size of plantaricin E and F corresponds to the approximate size determined by SDS-page for the bacteriocins produced by *L. plantarum* JW3BZ (von Mollendorff et al., 2006).

DGGE analysis of the boza samples containing yeast and no bacterial starter cultures confirmed the presence of natural microflora in boza. This is to be expected, as strains JW3BZ and JW15BZ were originally isolated from commercial boza. *L. sakei* DSM 20017

was either absent or present at low cell numbers after 1, 3 and 7 days of storage at 4 ºC. This is not surprising, as *L. sakei* DSM 20017 is sensitive to bacJW3BZ and bacJW15BZ (von Mollendorff et al., 2006). The unidentified band present in the DGGE profile of all the boza samples, could possibly be a bacterial contaminant in the products or was part of the natural microflora present in the raw ingredients used for the production of boza.

The sensory attributes of the boza samples prepared with different starter cultures did not vary considerably, although statistical differences ($p \geq 0.05$) were observed for acidity and yeasty aroma. These differences, although statistically significant, are so small and would probably not be detected by the consumers. Fermentation of the product contributes to the acidity of the product (Zorba et al., 2003).

Results obtained in this study suggest that strains JW3BZ and JW15BZ will survive conditions of the gastrointestinal tract and adhere to mucus and epithelial cells. Production of broad-spectrum bacteriocins with antiviral activity and no changes in the organoleptic profile of boza fermented with these strains is an added advantage. Detailed *in vivo* studies in animals need to be done before the strains can be classified as probiotic.

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Table 1

Indicator strains and growth media

Strain	Medium	Bacteriocin	
		JW3BZ	JW15BZ
E. faecium ST211Ch, ET12	MRS	+	+
Enterococcus faecalis BFE 1071	MRS[a]	-	-
E. faecalis E77, E92	MRS	+	+
E. faecalis E88, E90	MRS	+	++
E. faecalis FA2	BHI	+	+
E. faecalis P21	BHI[b]	-	-
Enterococcus faecium ET88	MRS	-	+
E. faecium HKLHS	BHI	+	+
Enterococcus mundtii ATCC PTA 7278 (ST4SA)	MRS	+	++
Escherichia coli P40, RPEC1	BHI	-	-
Klebsiella pneumoniae P30	BHI	-	+
*K. pneumonia*e P39	BHI	-	-
Lactobacillus acidilactici 4356	MRS	-	-
Lactobacillus casei LHS	BHI	+	+
L. casei Shirota	MRS	-	-
Lactobacillus curvatus DF38	MRS	-	-
L. curvatus ET31	MRS	-	-
Lactobacillus fermentum ET35	MRS	-	+
Lactobacillus paracasei subsp. *paracasei* ST11BR	MRS	-	-
Lactobacillus plantarum 423, LMG 13556, ST8KF, ST101Co, ST202Co, ST303Co, ST216Ch, ST202Ch	MRS	-	-
Lactobacillus sakei DSM 20017, LMG 13558	MRS	+	+
L. sakei ST22CH, ST154CH, ST153CH	MRS	-	+
Lactobacillus salivarius 241	MRS	-	-
Lactococcus lactis subsp. *lactis* HV219	MRS	+	++
Listeria innocua LMG 13568, F	BHI	+	+

Listeria ivanovii subsp. *ivanovii* ATCC 19119	BHI	+	+
Pediococcus acidilactici ET34	MRS	-	-
Pseudomonas sp. P25	BHI	-	-
Staphylococcus aureus P36, RPSA1	BHI	-	-
Streptococcus caprinus ATCC 700065, ATCC 700066	MRS	-	+
Streptococcus pneumoniae P29, P48	BHI	-	-
Streptococcus sp. TL1	BHI	+	+
Streptococcus sp. TL2R	MRS	-	+
Streptococcus sp. TL2W	BHI	+	+

[a] De Man, Rogosa and Sharpe. (Biolab)

[b] Brain Heart Infusion (Biolab, Biolab Diagnostics Ltd, South Africa)

- = no activity; + = inhibition zone ≤ 10 mm in diameter; ++ inhibition zone > 10 mm in diameter.

BFE = Bundesforschungsanstalt für Ernährung, Karlsruhe, Germany; LMG = Laboratorium voor Mikrobiologie, Ghent, Belgium; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ATCC = American Type Culture Collection; ET = Escola Superior de Biotecnologia, Porto, Portugal.

Table 2

Susceptibility of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ to antibiotics

Antibiotics	Inhibition zone (diameter in mm)	
	JW3BZ	JW15BZ
Amikacin (30 µg/disk)	-	++
Cefepime (30 µg/disk)	++	++
Ceforoxime (30 µg/disk)	++	++
Cefotaxime (30 µg/disk)	++	++
Ceftazidime (30 µg/disk)	++	-
Ceftriaxone (30 µg/disk)	++	++
Cephazolin (30 µg/disk)	++	-
Chloramphenicol (10 µg/disk)	++	++
Ciprofloxacin (5 µg/disk)	++	++
Clindamycin (2 µg/disk)	++	++
Erythromycin (5 µg/disk)	++	++
Fusidic Acid (10 µg/disk)	++	-
Neomycin (30 µg/disk)	++	++
Nitrofurantoin (300 µg/disk)	++	-
Ofloxacin (5 µg/disk)	++	++
Oxacillin (1 µg/disk)	++	-
Rifampicin (5 µg/disk)	-	++
Tetracycline (30 µg/disk)	++	++
Tobramycin (10 µg/disk)	++	-
Vancomycin (30 µg/disk)	++	-
Furazolidone (50 µg/disk), metronidazole (5 µg/disk), nalidixic Acid (30 µg/disk), sulphafurazole (25 µg/disk), sulphamethoxazole (25 µg/disk), sulphamethoxazole/trimethoprim (25 µg/disk), sulphonamides (300 µg/disk), trimethoprim (25 µg/disk)	-	-

- = no activity; + = inhibition zone ≤ 10 mm in diameter; ++ inhibition zone > 10 mm in diameter.

Table 3

Effect of commercially available medicaments on the growth of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ

Commercial name	Concen-tration (mg/ml)	Active substance	Inhibition zone (diameter in mm)	
			JW3BZ	JW15BZ
Acetylcystein 600 Stada ® Tabs (anti-infammatory)	120	Acetylcystein	-	-
Adco-Ibuprofen (anti-inflammatory)	40	Ibuprofen	-	-
Ambro (anti-inflammatory)	20	Ambroxol	-	-
Analgin (antibiotic)	100	Metamizole sodium	-	-
Atarax (Anti-inflammatory)	5	Hidroxizina dichlorhidrato	-	-
Bisalax (treatment of constipation)	1	Bisacodylum	-	-
Cerucal (anti-inflammatory)	205	Metoclopramide hydrochloride	-	-
Cipro (antibiotic)	100	Ciprofloxacin	++	++
Codterpin (antibiotic)	50	Codeinum urum, Natrii hydrogen-cabonas, Terpini hydras	-	-
Dehydratin Neo (Moderate diuretic)	5	Hdrochlorothiazidin	-	-
Dimenhydrinat (antihistaminic)	10	Dimenhydrinat	-	-
Diuretidin (Moderate diuretic)	7.5	Triamterenum, hydrochlorothiazidum	++	-
Dolocyl (anti-inflammatory)	40	Ibuprofen	++	+
Espumisan	8	Simethicon, methyl-4-	-	-

(Simethicon) (painkiller)		hydroxybenzoat		
Famotidine (anti-acid medicament)	4	Famotidine	-	-
Mefenacid (anti-inflammatory)	100	Acidum mefenamcium	-	-
Novphyllin (antiasthmatic medicament)	10	Dimenhydrinate	-	-
Oleum Jecoris (hepatic and pancreatic remedy)	7.5	Retinol palmitas (Vit A), Ergocaliferolum (Vit D2), Oleum Jecoris Aselli	-	-
Paracetamol (anti-inflammatory)	100	Paracetamol	-	-
Proalgin (anti-inflammatory)	100	Metamizole sodium	++	-
Thioridazin (neuroleptic medicament)	2	Thioridazine hydrochloride, starch, colourant E110	++	++
Voltaren (anti-inflammatory)	10	Sodium diclofenac	-	-

- = no activity; + = inhibition zone ≤ 10 mm in diameter; ++ inhibition zone > 10 mm in diameter.

Table 4

PCR primers used for detection of genes involved in plantaricin production

Target gene	Primers sequence 5' to 3'	Annealing temp. (^{o}C)	Amplicon size (bp)
plnA	F: GTACAGTACTAATGGGAG	50.5	450
	R: CTTACGCCAATCTATACG		
plnB	F: TTCAGAGCAAGCCTAAATGAC	54	165
	R: GCCACTGTAACACCATGAC		
plnC	F: AGCAGATGAAATTCGGCAG	53	108
	R: ATAATCCAACGGTGCAATCC		
plnD	F: TGAGGACAAACAGACTGGAC	55	414
	R: GCATCGGAAAAATTGCGGATAC		
plnEF	F: GGCATAGTTAAAATTCCCCCC	53	428
	R: CAGGTTGCCGCAAAAAAAG		
plnI	F: CTCGACGGTGAAATTAGGTGTAAG	57	450
	R: CGTTTATCCTATCCTCTAAGCATTGG		
plnJ	F: TAACGACGGATTGCTCTG	51	475
	R: AATCAAGGAATTATCACATTAGTC		
plnK	F: CTGTAAGCATTGCTAACCAATC	53	246
	R: ACTGCTGACGCTGAAAAG		
plnG	F: TGCGGTTATCAGTATGTCAAAG	54	453
	R: CCTCGAAACAATTTCCCCC		
plnN	F: ATTGCCGGGTTAGGTATCG	56	146
	R: CCTAAACCATGCCATGCAC		
Plantaricin NC8 structural gene	F: GGTCTGCGTATAAGCATCGC R: AAATTGAACATATGGGTGCTTTAAATTCC	56	207
Plantaricin S structural gene	F: GCCTTACCAGCGTAATGCCC R: CTGGTGATGCAATCGTTAGTTT	54	320
Plantaricin W structural gene	F: TCACACGAAATATTCCA R: GGCAAGCGTAAGAAATAAATGAG	44	165

Adapted from: Omar et al. (2006)

Table 5

PCR primers for amplification of operon EFI.

Primer set	Primer sequence 5' to 3'	Amplicon size (bp)
plnEF2-F	ATACCACGAATGCCTGCAAC	1197
plnG-R	CCTCGAAACAATTTCCCCC	
plnEF-F	GGCATAGTTAAAATTCCCCCC	428
plnEF-R	CAGGTTGCCGCAAAAAAAG	
plnD-F	TGAGGACAAACAGACTGGAC	1721
plnEF2-R	CACGGATAGTTCAAGCCATC	

Table 6

Descriptors for the respective sensory attributes

Sensory Characteristics	Attribute	Definition	Scale anchors
Aroma	Yeasty aroma	Aroma associated with yeast dough	No yeasty aroma/ strong yeasty aroma
Flavor	Yeasty flavor	A flavor associated with uncooked yeast dough	No yeasty flavor/ strong yeasty flavor
Taste	Bitterness	A prominent bitter flavor associated with black label	No bitter taste/ prominent bitter taste
	Sweetness		No sweet taste/ prominent sweet taste
	Acidity	A flavor associated with Bulgarian yogurt	Low acidity/ very high acidity

*Scale ranges from 0 (minimum) to 100 (maximum) for all attributes.

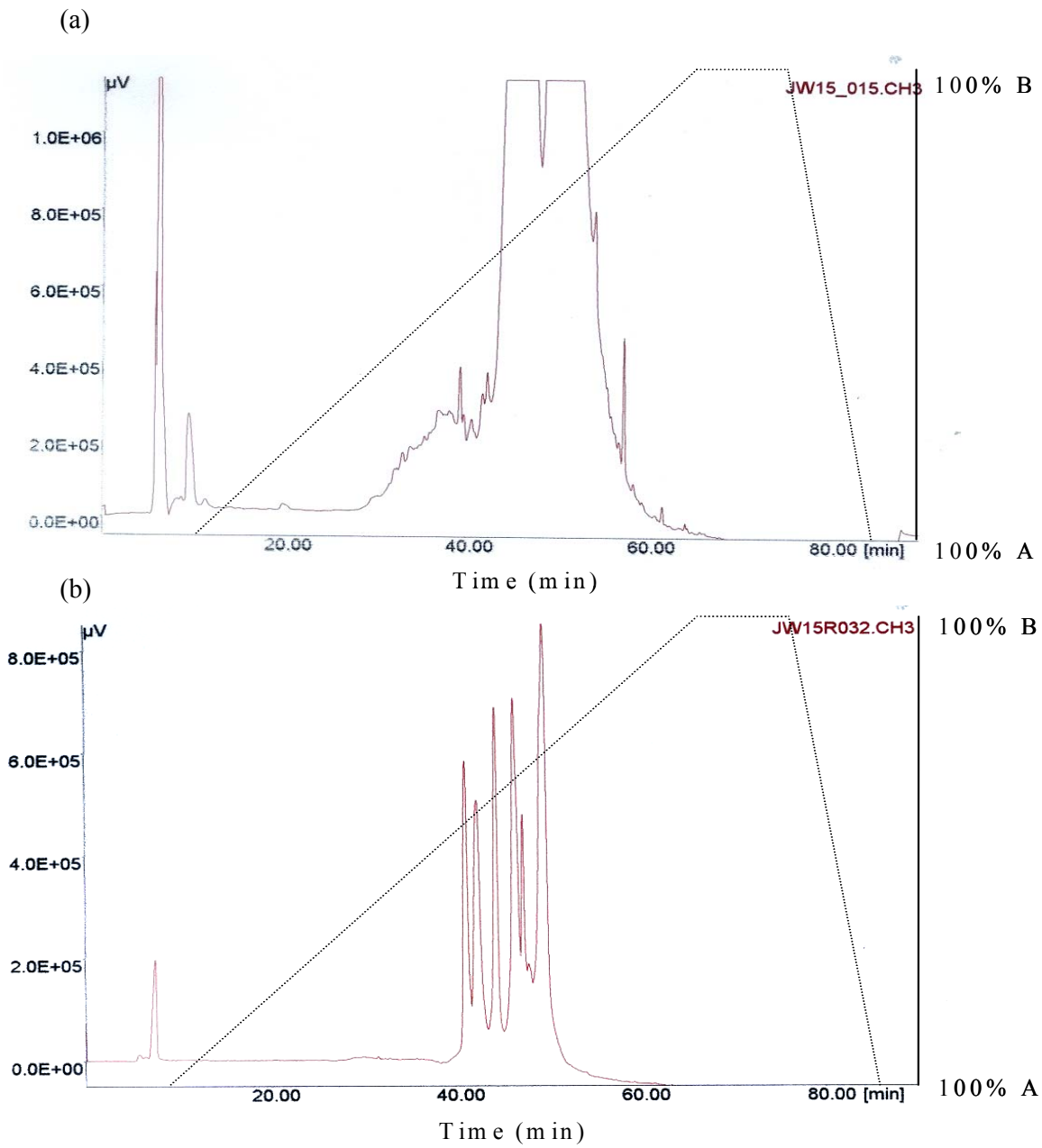

Fig. 1. Bacteriocin JW15BZ, eluted with 40% iso-propanol in 25 mM ammonium acetate through a Sep-Pak C_{18} column, separated by reverse-phase HPLC on a C_{18} Nucleosil column. Eluents were trifluoroacetic acid (TFA; 0.1%) in water (eluent A) and 10% TFA (0.1%) in 90% acetronitrile (eluent B). A gradient from 0 to 100% eluent B was applied over 65 min and kept at 100% eluent B for 10 min. Active fractions from the first seperation (a) were pooled and re-injected which produced a single active peak at a retention time of 48.5 min (b).

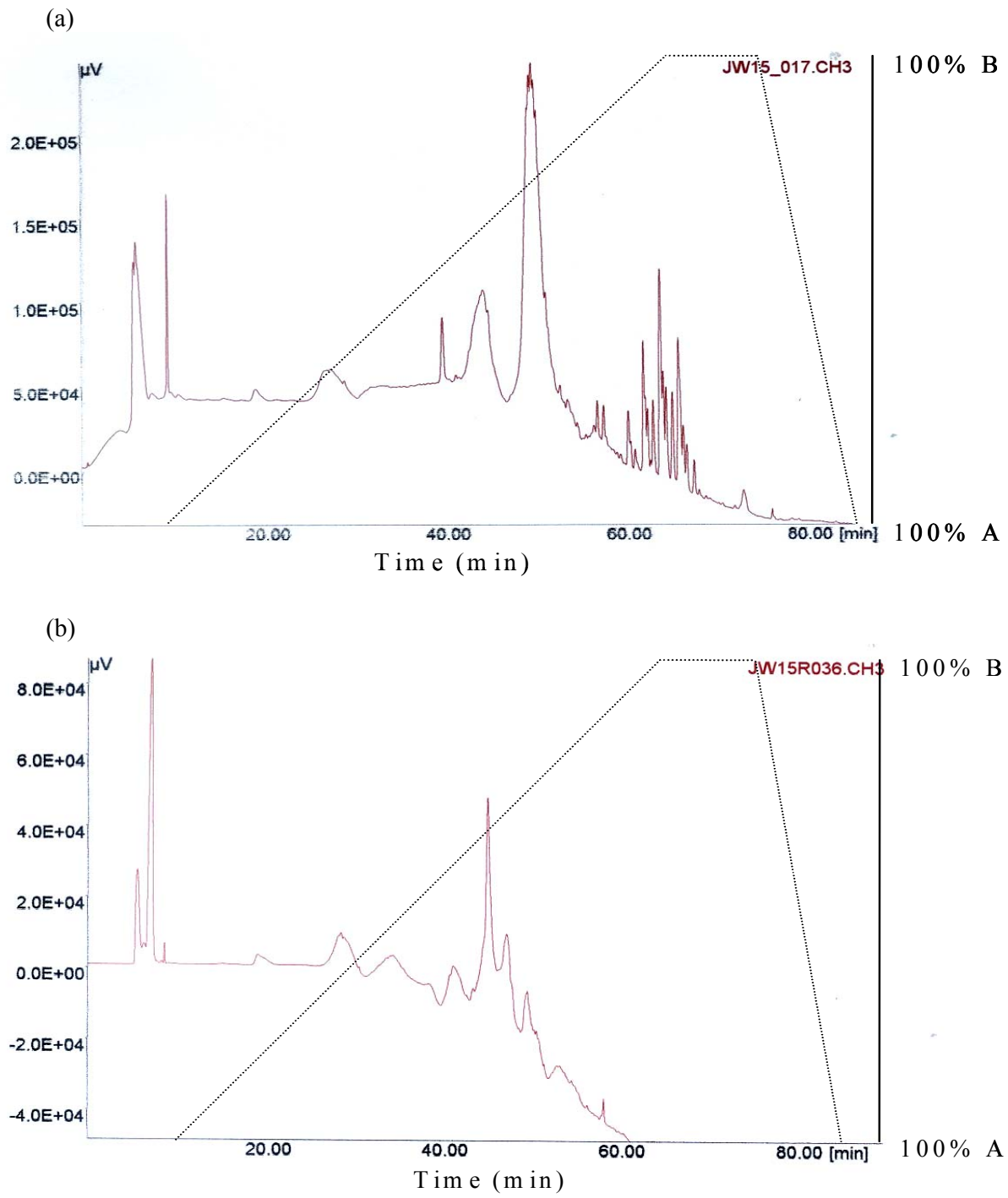

Fig. 2. Bacteriocin JW15BZ, eluted with 60% iso-propanol in 25 mM ammonium acetate through a Sep-Pak C_{18} column, separated by reverse-phase HPLC on a C_{18} Nucleosil column. Eluents were trifluoroacetic acid (TFA; 0.1%) in water (eluent A) and 10% TFA (0.1%) in 90% acetronitrile (eluent B). A gradient from 0 to 100% eluent B was applied over 65 min and kept at 100% B for 10 min. Active fractions from the first seperation (a) were pooled and re-injected which produced a single active peak at a retention time of 45 min (b).

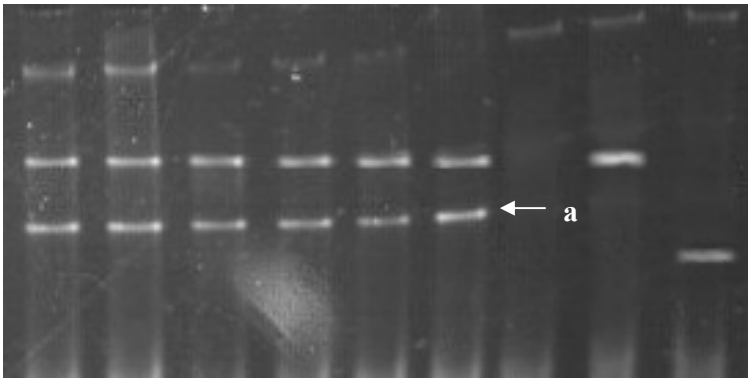

Fig. 3. PCR-based DGGE fingerprints of the microbial population present in boza prepared with *L. plantarum* JW3BZ alone (lanes A1 = day 1, A2 = day 3 and A3 = day 7) and in combination with *L. sakei* DSM 20017 (lanes A4 = day 1, A5 = day3 and A6 = day 7) on days 1, 3 and 7 days of fermentation. Lane A7 = no DNA, Lane M1 = *Lb. plantarum* JW3BZ control DNA and Lane M2 = *L. sakei* DSM 20017 control DNA. Band "a" was unidentified.

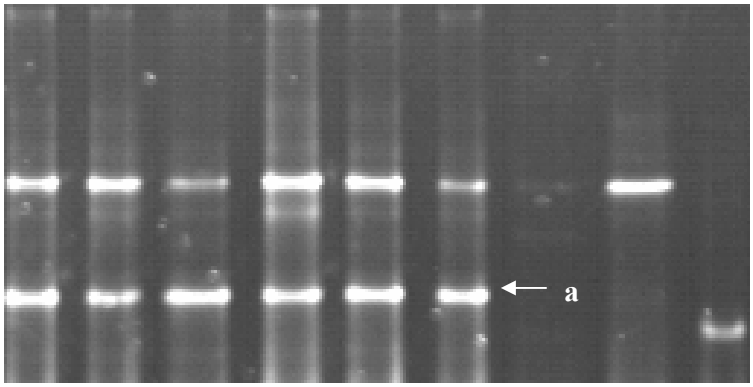

Fig. 4. PCR-based DGGE fingerprints of the microbial population present in boza prepared with *L. fermentum* JW15BZ alone (lanes B1 = day 1, B2 = day 3 and B3 = day 7) and in combination with *L. sakei* DSM 20017 (lanes B4 = day 1, B5 = day 3 and B6 = day 7) on days 1, 3 and 7 after fermentation. Lane B7 = no DNA, Lane M1 = *L. fermentum* JW15BZ control DNA and Lane M2 = *L. sakei* DSM 20017 control DNA. Band "a" was unidentified.

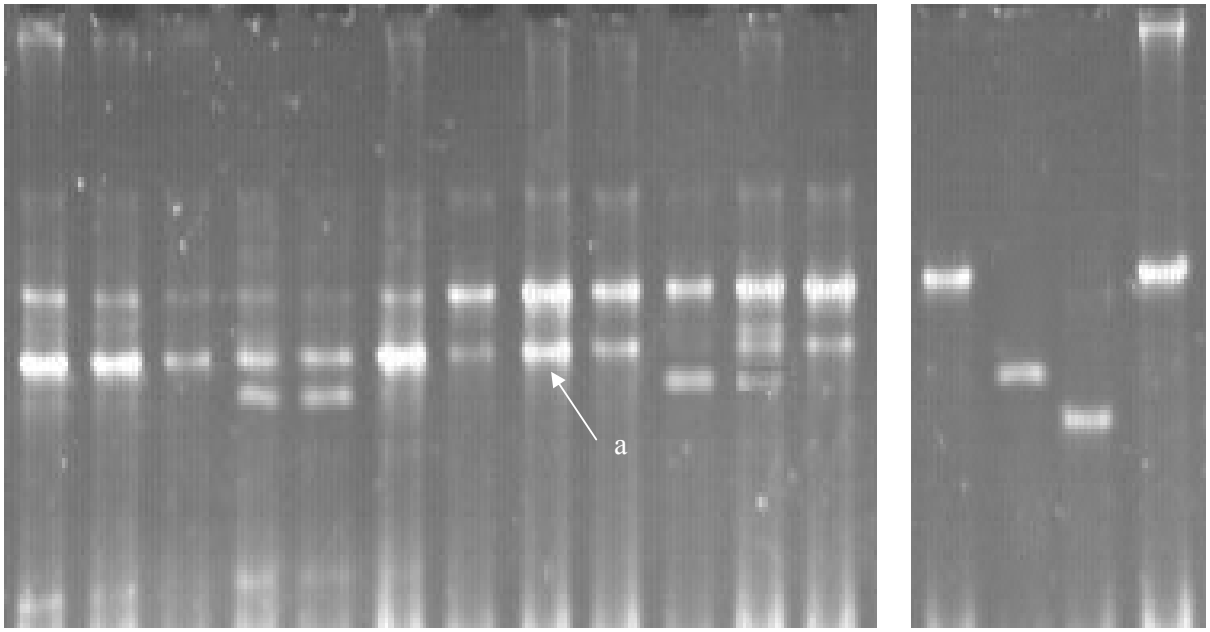

Fig. 5. PCR-based DGGE fingerprints of the microbial population present in boza prepared with no starter culture (lanes C1 = day 1, C2 = day 3 and C3 = day 7), *L. sakei* DSM 20017 (lanes C4 = day 1, C5 = day 3 and C6 = day 7), and boza obtained from a local Bulgarian market alone (lanes C7 = day 1, C8 = day 3 and C9 = day7) and in combination with *L. sakei* DSM 20017 (lanes C10 = day1, C11 = day 3 and C12 = day 7) on days 1, 3 and 7 after fermentation. Lane M1 = *L. fermentum* JW15BZ control DNA, Lane M2 = *L. sakei* DSM 20017 control DNA, Lane M3 = Enterococcus spp. control DNA and Lane M4 = *L. plantarum* JW3BZ. Band "a" was unidentified.

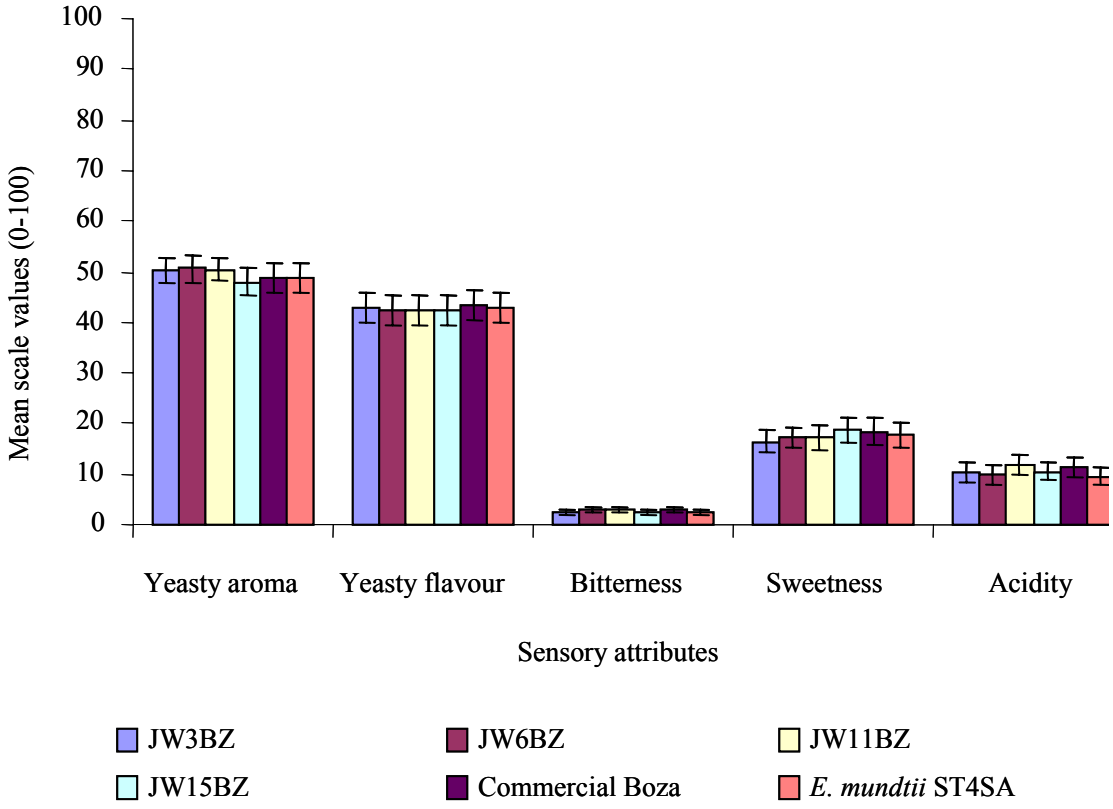

Fig. 6. Sensory attributes (means±SE) of boza samples prepared with different starter cultures.

CHAPTER 4

Survival of *Lactobacillus plantarum* JW3BZ and *Lactobacillus fermentum* JW15BZ in alginate beads and their release at conditions simulating the human gastrointestinal tract

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Survival of *Lactobacillus plantarum* JW3BZ and *Lactobacillus fermentum* JW15BZ in alginate beads and their release at conditions simulating the human gastrointestinal tract

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Abstract

Encapsulation of *Lactobacillus plantarum* JW3BZ and *Lactobacillus fermentum* JW15BZ in 2% sodium alginate protected the cells from low pH (1.6) and 2.0% (w/v) bile. The rate at which cells were released from the matrix varied, depending on the conditions. Better survival of of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ encapsulated in 2% (w/v) alginate was observed during 9 h in a gastrointestinal model. Highest release was observed at conditions simulating colonic pH (pH 7.4), starting from 56-65% during the first 30 min, followed by 87%. Complete (100%) release was recorded after 2.5 h at these conditions. Protection of encapsulated strains *L. plantarum* JW3BZ and *L. fermentum* JW15BZ against the harsh conditions of the gastrointestinal tract presents them with possible applications in various food processing technologies and for the delivery of viable cells *in vivo*.

Keywords: Lactobacillus plantarum, Lactobacillus fermentum, encapsulation

1. Introduction

Probiotics are live microorganisms that exert health benefits when consumed at levels of 10^6-10^7 cfu per gram product (Guarner & Schaafsma, 1998; Ouwehand & Salminen, 1998; Shah, 2000; FAO/WHO, 2001). Probiotic benefits include suppressing the growth of pathogens, controlling serum cholesterol levels, beneficially influencing the immune system, improvement of lactose digestion and immune system, synthesises of vitamins, increasing the bio-availability of minerals and possible anti-carcinogenic properties (Modler, 1990; Shah and Jelen, 1990; Gilliland, 1990, 1991; Gomes et al., 1999; Kailasapathy and Chin; 2000; Chan and Zhang, 2005). Probiotic properties are, however, affected by the ability of the strains to survive and multiply in the host (Gilliland 1989, Todorov et al, 2007).

Most probiotic products currently on the market do not have a long shelf-life, even when stored at low temperatures. Some of the yoghurt products commercially available have less viable cells than the level required to have a probiotic effect (Kailasapathy and Chin, 2000). Once consumed, the strains are exposed to the harsh conditions in the intestinal tract, lowering the viable cell numbers even further. One possible method to protect probiotic strains from high acids and bile would be to encapsulate the cells in hydrocolloid beads (Rao et al., 1989). Alginate, a natural polymer extracted from several species of algae, is the most widely used matrix for encapsulation (Kailasapathy, 2002) and is an accepted food additive (Prevost and Divies, 1988). Previous studies reported an 80-95% increase in survival when probiotic strains were encapsulated in alginate (Audet et al., 1988; Rao et al., 1989; Sheu and Marshall, 1991; Sheu and Marshall, 1993; Sheu et al., 1993; Jankowski et al., 1997; Chandramouli et al., 2004; Mandal et al., 2006).

Lactobacillus plantarum JW3BZ and *L. fermentum* JW15BZ, isolated from boza, have probiotic properties (von Mollendorff et al., 2006), and produce bacteriocins JW3BZ and JW15BZ with antimicrobial activity against a number of food spoilage and pathogenic bacteria (von Mollendorff et al., 2006). This study was performed to compare the survival of encapsulated cells to plantonic cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ in conditions simulating the human gastrointestinal tract.

2. Materials and Methods

2.1 Encapsulation of bacterial cells

The encapsulation method of Sheu et al. (1993) was used. Sodium alginate suspensions of 2%, 3% and 4% were prepared in 100 ml distilled water and autoclaved (15 min at 120 ^{o}C). *Lactobacillus plantarum* JW3BZ and *Lactobacillus fermentum* JW15BZ were grown in 10 ml MRS (De Man et al., 1960) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) to an optical density (OD_{600nm}) of 3.0 (approximately 1 x 10^9 cfu/ml) and harvested (2,000 *g*, 10 min, 4 ^{o}C). The cells were washed twice with 5 ml sterile 0.1% (w/v) peptone water. Four millilitres of this cell suspension was added to 20 ml alginate suspension, vortexed to homogeneity and transferred to a sterile syringe fitted with a 0.45 mm diameter needle. The alginate cell suspension was slowly ejected and allowed to drop into sterile 100 ml 0.05 M $CaCl_2$, supplemented with 0.1% (v/v) Tween 80. The alginate beads were allowed to stabilise for 30 min and were then washed three times with sterile 0.1% (w/v) peptone water. The beads were harvested (350 *g*, 10 min, 4^{o}C) and collected by filtration through Whatman filter paper (No. 1). The beads were stored in sterile 0.1% (w/v) peptone water at 4 ^{o}C for a maximum of 2 days.

2.2 Survival of encapsulated cells at different pH and bile concentrations

In the first experiment, 1 g of alginate beads with entrapped cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ, respectively, were transferred to 10 ml 0.08 M HCl supplemented with 0.2% (w/v) NaCl (pH 1.6) and incubated for 3 h at 37 ^{o}C. The beads were harvested after 1 h and 3 h of incubation and depolymerised in 10 ml phosphate buffer (0.1 M, pH 7.4). Ten-fold-dilutions of the released cells were prepared in 9 ml sterile distilled water and then plated onto MRS (Biolab) agar. The plates were incubated at 37 ^{o}C and the number of viable cells determined after 48 h. The experiment was repeated by transferring the alginate beads to 10 ml of simulated colonic solution (0.1 M KH_2PO_4, pH 7.4). The beads were gently mixed and incubated at 37 ^{o}C on an orbital shaker (60 rpm). Samples were taken after 0.5 h, 1 h, 2 h and 3 h and viable counts determined as described before.

In the second experiment, 1 g encapsulated cells was exposed to 10 ml 1% and 2% (w/v) bile salt (Oxoid Ltd, Basingstoke, England), respectively. After 3 h and 12 h of incubation at 37 ^{o}C, the beads were depolymerised and the number of viable cells determined by plating onto MRS agar (Biolab), as described before.

In both experiments, viable cell numbers were also determined immediately after encapsulation. Cells that have not been encapsulated were also subjected to the treatments described in both experiments. The percentage cells released from the beads and the percentage survival of free cells were calculated as follows:

Percentage release (survival) = [cfu/ml$_{t0}$ / cfu/ml$_{tn}$] x 100, where cfu/ml$_{t0}$ refers to the initial cfu/ml and cfu/ml$_{tn}$ to the cfu/ml at the various time intervals.

2.3 Release of encapsulated cells at conditions simulating different sections of the gastrointestinal tract

Alginate beads with encapsulated cells (10 g) were transferred to 250 ml sterile (autoclaved) stomach solution (12.5 g MRS from Biolab, 0.78 g NaCl, 0.28 g KCl, 0.03 g $CaCl_2$ and 0.15 g $NaHCO_3$, pH adjusted to 4.0 with 1 M HCl). After 2.5 h at 37 ^oC, the stomach solution was adjusted to pH 3.0 with sterile 1 M HCl and viable cell numbers were determined as described before. The stomach solution was then removed, the beads washed twice with large volumes of sterile 0.1% (m/v) peptone water and transferred to 250 ml stomach solution, supplemented with 125 ml pancreatic solution [1.5 g $NaHCO_3$, 0.11 g Pancreatin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 0.08 g Oxgall (Oxoid, Hampshire, England), sterilized by autoclaving (121 ^oC, 15 min)]. The pH was adjusted to 6.5 with sterile 1 N NaOH and kept at this for the first 4 h to represent conditions in the duodenum and ileum. The pH was then adjusted to 6.0 with sterile 1 N HCl and kept at this value for the remaining 2.5 h to represent conditions in the jejunum and first section of the colon. Incubation was at 37 ^oC. Samples (1 ml) were withdrawn after 2 h (representing conditions in the duodenum), 4 h (representing conditions in the jejunum), 6 h (representing conditions in the ileum) and 6.5 h (representing conditions in the first section of the colon). Cell numbers were determined as described before. The percentage cells released from the beads and the percentage survival of free cells were determined as described before.

The experiment was repeated with non-encapsulated (planctonic) cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ to serve as control. A cell suspension (10 ml of 1 x 10^8 cfu/ml) was transferred to 250 ml of simulated stomach solution (pH 4.0) and incubated at 37 ^oC for 2.5 h. The stomach solution was then adjusted to pH 3.0 with sterile 1 M HCl and the number of viable cells determined as described before. Simulated pancreatic solution (125 ml) was added to the stomach solution and the pH of the mixture gradually adjusted to 6.5

with sterile 1 M NaOH. The pH of the media was kept at 6.5 for 4 h (representing conditions in the duodenum and jejunum) and then adjusted to 6.0 for the remaining 2.5 h (representing conditions in the ileum and first section of the colon). Incubation was at 37 ^{o}C. Samples were collected at the relevant time intervals and viable cell numbers determined as described before. The percentage cells released from the beads and the percentage survival of free cells were determined as described before.

All experiments in this study were performed in triplicate.

3. Results

3.1 Survival of encapsulated and free cells in the presence of high acid and bile

Encapsulated cells of JW3BZ and JW15BZ survived exposure to simulated gastric conditions (pH 1.6) at 1 x 10^7 cfu/g to 1 x 10^8 cfu/g. (not shown). Strains JW3BZ and JW15BZ encapsulated in 2% and 3 % alginate survived 1 h of exposure to pH 1.6 at 1 x 10^3 cfu/g to 1 x 10^5 cfu/g (not shown.), while planctonic cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ were completely sensitive to these conditions (not shown).

Cell numbers recorded for encapsulated cells of *L. plantarum* JW3BZ and *L. fermentum* JW15 exposed to bile are shown in Figs. 1 and 2. (a,b,c). Encapsulation of strains JW3BZ and JW15BZ in 2-4% (w/v) alginate increased the viability of the cells when exposed to bile salt concentrations of 1-2% (w/v) (Fig. 2 (a,b,c)). Planctonic cells, on the other hand, were inhibited after after 12 h at 1% and 2% (w/v) bile (Fig. 1). The viability of the encapsulated cells after exposure to 1% (w/v) and 2% (w/v) bile for 12 h ranged between 1 x 10^6 cfu/g to 1 x 10^7 cfu/g for strain JW3BZ and strain JW15BZ. No direct correlation between the viability of encapsulated cells with an increase in alginate concentrations could be observed, with the exception of encapsulated strain JW15BZ in the presence of 2% bile after 3 h and 12 h (Fig. 2 (c)).

3.2 Release of encapsulated cells in simulated colonic pH solution

The initial release was at 56-65% during the first 30 min, followed by 87%, and eventually complete (100%) release after 2.5 h (not shown).

3.3 Release of encapsulated cells in a simulated gastro-intestinal model

No cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ were released from the alginate

beads after 2.5 h exposure to simulated stomach solution at pH 3.0 (Fig. 3. (a,b)). Approximately 86 % cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ were released after 2 h at conditions simulating the duodenum (Fig. 3 (a,b)). A further increase in the number of viable cells were observed after 2 h exposure to conditions simulating the jejunum, followed by 2 h at conditions simulating the ileum and first section of the colon. However, only 75% of non-encapsulated *L. plantarum* JW3BZ and *L. fermentum* JW15BZ survived after 2.5 h of exposure to simulated stomach solution (pH 3.0) (Fig. 3 (a,b)). No increase in cell numbers for strain JW3BZ was observed after 2 h exposure to simulated duodenum conditions, whereas JW15BZ showed a slight increase (Fig. 3 (a,b)). A further increase in the number of viable cells of non-encapsulated *L. plantarum* JW3BZ and *L. fermentum* JW15BZ were observed after 4 h, 6 h and 6.5 h at conditions simulating the small intestine and first part of the large intestine. The number of viable cells obtained for non-encapsulated *L. plantarum* JW3BZ and *L. fermentum* JW15BZ were significantly lower than recorded for their encapsulated counterparts (Fig. 3 (a,b)).

4. Discussion

Alginate at 2 % provided the best protection to strains JW3BZ and JW15BZ against the harsh conditions in the gastrointestinal tract. These results are in contrast to the report of Mandal et al. (2006) that showed an increase in viability of *L. casei* NCDC-298 with an increase in alginate concentration. Lee and Heo (2000) also reported a decrease in the death rate of cells of *Bifidobacterium longum* proportional with an increase in alginate concentration.

Varying results were observed after exposure of non-encapsulated cells of JW3BZ and JW15BZ to 1% (w/v) and 2% (w/v) bile. Encapsulated cells displayed more tolerance to 1% (w/v) and 2% (w/v) bile than free cells. A direct correlation was observed between an increase in cell viability and an increase in alginate concentration for encapsulated cells of *L. fermentum* JW15BZ, but not for *L. plantarum* JW3BZ. In a similar study, Mandal et al. (2006) reported an increase in cell viability with an increase in alginate concentration. The increase in viability of encapsulated cells compared to free cells of *L. fermentum* JW15BZ is in accordance with other researchers (Lee and Heo, 2000; Krasaekoopt et al., 2003; Chandramouli et al., 2004). In contrast, Trindade and Grosso (2000) reported a decrease in viability of immobilized *B. bifidum* and *L. acidophilus* after exposure to 2% (w/v) and 4% (w/v) bile salts.

Fifty-six to 65% cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ were released from alginate beads at simulated colonic pH solution from an initial count of approximately ~ 1 x 10^8 cfu/g (results not shown). Highest release was observed at conditions simulating colonic pH (pH 7.4), starting from 56-65% during the first 30 min, followed by 87%, and eventually complete (100%) release after 2.5 h (results not shown). Similar results were reported for whey protein-based microcapsulated cells of bifidobacteria when tested at simulated intestinal conditions (Picot and Lacroix, 2004).

The viability of released cells from 2% (w/v) alginate capsules containing either *L. plantarum* JW3BZ or *L. fermentum* JW15BZ was higher compared to free cells of strains JW3BZ and JW15BZ after 2 h in simulated duodenum conditions. Better results observed for the encapsulated strains may be ascribed to the alginate matrix acting as a barrier against the high acidic conditions associated with the stomach, followed by the subsequent release of the encapsulated cells at a pH of 6.5. A further increase in the number of viable cells of non-encapsulated *L. plantarum* JW3BZ and *L. fermentum* JW15BZ were observed after 4 h, 6 h and 6.5 h at the various conditions simulating the small intestine. The number of viable cells obtained for non-encapsulated *L. plantarum* JW3BZ and *L. fermentum* JW15BZ were significantly lower than that recorded for the strains in the encapsulated form. These results suggest that encapsulated strains may survive the harsh conditions of the gastrointestinal tract, resulting in higher viable cell numbers in the small intestine and large intestine. An efficient number of viable and metabolically active cells in the colon are essential for the growth and colonization of probiotic bacteria (Suita-Cruz and Goulet, 2001).

Concluded from this study, encapsulated cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ may be used in functional foods. Encapsulation provided protection against stressful conditions in the gastrointestinal tract. Further studies on the survival of the encapsulated bacteria in various food processing technologies and the delivery of the viable cells *in vivo* have to be conducted.

Acknowledgements

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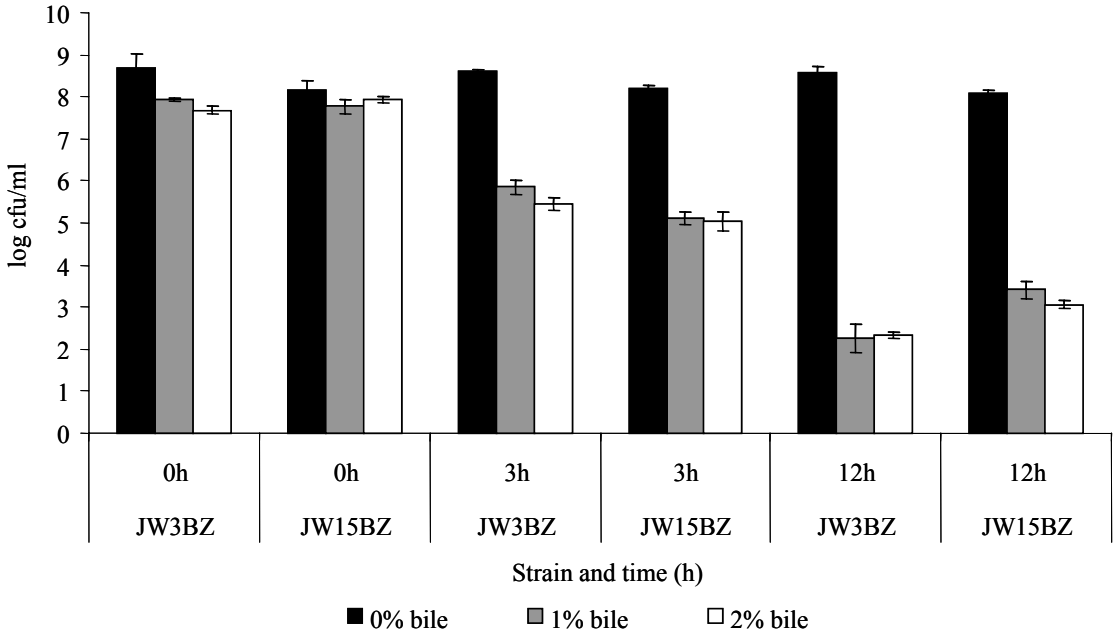

Fig. 1. Effect of bile salt (1% and 2%, w/v) on planctonic cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ.

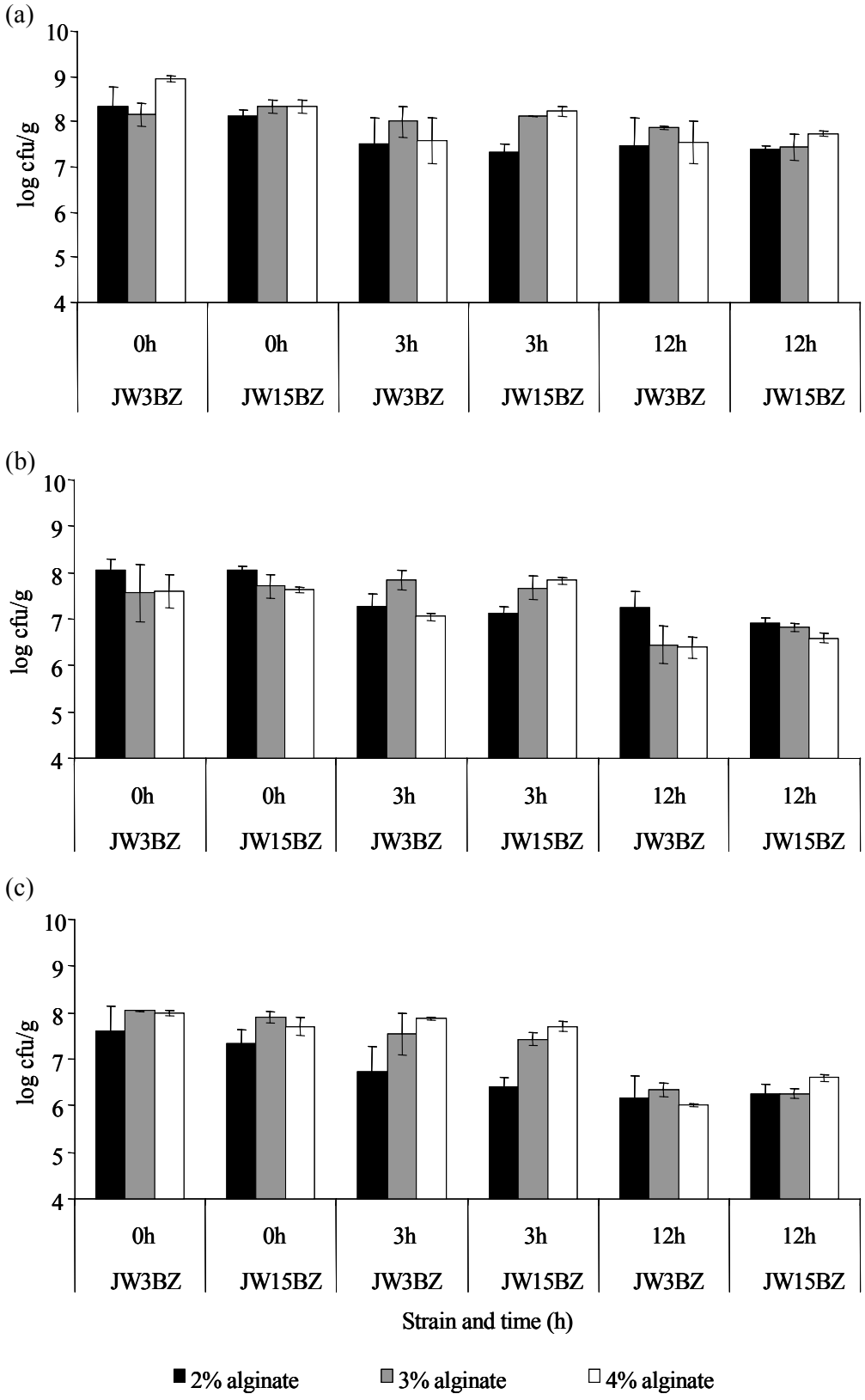

Fig. 2. Effect of bile salt on encapsulated cells *L. plantarum* JW3BZ and *L. fermentum* JW15BZ.: (a) controle (0 % bile); (b) 1 % bile and (c) 2 % bile.

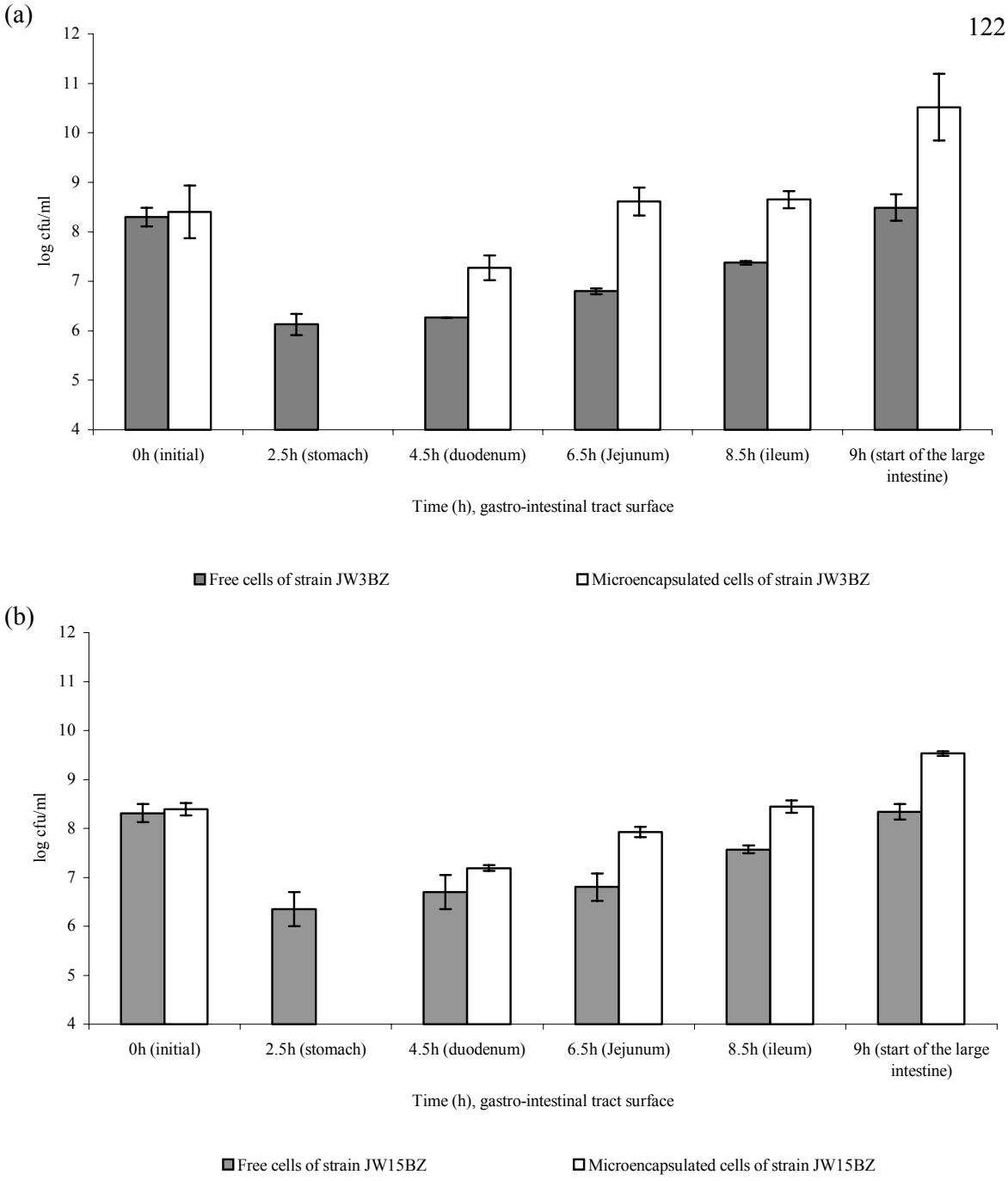

Fig. 3. Release of encapsulated and survival of non-encapsulated cells of (a) *L. plantarum* JW3BZ and (b) *L. fermentum* JW15BZ in a simulated gastrointestinal model.

CHAPTER 5

General Discussion and Conclusions

General Discussion and Conclusions

The use of lactic acid bacteria, especially *Lactobacillus* and *Bifidobacterium* spp. as starter cultures for the production of various fermented probiotic products are gaining significant interest. Formulated probiotic foods (functional foods) may present the consumer with a healthy dietary component at a considerable low cost (Goldin, 1998). Various lactic acid bacteria isolated from fermented food and beverages have been described. However, to use these strains as starter cultures in the production of functional foods, various parameters have to be studied. These include probiotic and bacteriocinogenic properties of the strains, survival in a fermented product and the influence of the strains on the sensory attributes of the final product.

In this study the probiotic and bacteriocinogenic properties of two lactic acid bacteria, i.e. *Lactobacillus plantarum* JW3BZ and *Lactobacillus fermentum* JW15BZ, isolated form boza were studied.

The main selection criteria for probiotic lactic acid bacteria are their ability to adhere to epithelial cells or the intestinal mucosa and to survive at low pH and high bile salt concentrations (Mattila-Sandholm et al., 1999; Bezkorovainy, 2001). Colonization is important, as it plays an important role in survival of the strains, stimulation of the immune system, repair of the damaged mucosa, and antagonism against pathogenic bacteria (Isolauri et al., 1991; Salminen et al., 1996; Rolfe, 2000; Reid and Burton, 2002).

Lactobacillus plantarum JW3BZ and *L. fermentum* JW15BZ produced bacteriocins JW3BZ and JW15BZ active against a broad range of Gram-positive and Gram-negative bacteria, including human pathogens (von Mollendorff et al., 2006). Activity against *Klebsiella pneumoniae* is not a common phenomenon and has only been reported for bacteriocins ST242BZ, ST284BZ produced by *Lactobacillus paracasei* ST242BZ and ST284BZ, ST414BZ produced by *L. plantarum* ST414BZ, ST461BZ produced by *Lactobacillus rhamnosus* ST461BZ and ST712BZ produced by *Lactobacillus pentosus* ST712BZ (Todorov and Dicks, 2006).

Strains JW3BZ and JW15BZ were able to grow at pH 2.0-4.0, but at a considerable slower rate compared to growth at pH 6.0 – 10.0. Strain JW3BZ displayed good intrinsic resistance

to bile salts, whereas poor resistance was recorded for strain JW15BZ. Resistance to bile and acids are important for survival in the gastrointestinal tract. Probiotic lactic acid bacteria with low intrinsic bile resistance, such as strain JW15BZ may be lysed during passage through the gastrointestinal tract, resulting in the release of β-galactosidase, which could increase the rate of lactose digestion in the small intestine (Marteau et al., 1997).

Strains JW3BZ and JW15BZ displayed resistance to a number of antibiotics and commercially available neuroleptic, analgetic, anti-inflammatory, deuretic and antitussive medicaments, suggesting that they could be used as probiotics in conjunction with these medicaments. Future studies need to investigate the role played by proton gradient extrusion pumps in the synergy between certain antibiotics and sub-lethal concentrations of bacteriocins. Minahk et al. (2004) reported on the synergy between certain antibiotics and sub-lethal concentrations of enterocin CRL35. Enterocin CRL35 increased the effectiveness of certain non-peptide antibiotics by impairment of proton efflux systems, resulting in the accumulation of toxic compounds within the cells. Knoetze et al. (2007) suggested the use of bacteriocin ST4SA from *Enterococcus mundtii* ST4SA as an alternative to antibiotics in the treatment of otitis media. Peptide ST4SA dissipates the proton-motive force, which may disrupt efflux pumps and lead to increased intracellular levels of antibiotics (Knoetze et al., 2007).

Strains JW3BZ and JW15BZ are weakly hydrophobic. Cells of high hydrophobicity adhere stronger to epithelial cells (Naidu et al., 1999). However, previous studies indicated that, in some cases, no relationship could be found between surface hydrophobicity and the extent at which cells adhere to a hydrophobic or hydrophilic substrate (Coquet et al., 2002; Mattos-Guaraldi et al., 1999). Only 4% of cells of strains JW3BZ and JW15BZ adhered to Caco-2 cells. One may question if strains JW3BZ and JW15BZ will have sufficient time to colonize the small intestine, with an average 2 h digestion time (Kutchai, 1988). However, various other factors such as the food matrix and intestinal conditions also influence the rate of colonization (Todorov et al., 2007). Strain JW3BZ contains a homologue of *MapA*, a gene found in *L. plantarum* WCFS1 known to play a role in adhesion (Ramiah et al., 2007). Furthermore, strains JW3BZ and JW15BZ displayed high auto-aggregation, as well as co-aggregation with other lactic acid bacteria and potential pathogenic bacteria. Co-aggregation may facilitate a closer proximity of strains JW3BZ and JW15BZ to target pathogens,

enhancing their bactericidal effect. Auto-aggregation may facilitate the formation of biofilms contributing to colonization of the gastrointestinal tract.

L. plantarum JW3BZ contains operon *plnEFI* found in *L. plantarum* C11, coding for two small cationic bacteriocin-like peptides with double-glycine-type leader peptides and their respective immunity proteins (Diep et al., 1996). *PlnD,* the negative regulator of the signal-transducing pathway encoded by the operon *plnABCD,* was detected in strain JW3BZ. The lack of *plnA*, *plnB* and *plnC* suggest that these genes are missing from the regulatory operon, or that other regulatory genes are involved. The approximate size of bacteriocin JW3BZ, as previously determined by SDS-page, corresponds to the molecular size of plantaricin E and F (von Mollendorff et al., 2006).

Purification of bacteriocin JW15BZ using reverse phase HPLC resulted in two active substances, suggesting that *L. fermentum* JW15BZ may produce two different antimicrobial peptides. Electron spray mass-spectrometry and amino acid sequencing of these peptides need to be performed to proof this hypothesis. To our knowledge, no amino acid or nucleotide sequences have been published for bacteriocins produced by *L. fermentum*, emphasizing the need for sequencing these peptides.

Bacteriocins JW3BZ and JW15BZ displayed different levels of cytotoxicity, with bacteriocin JW3BZ being the least cytotoxic. Similar results were reported for bacteriocins CRL35, isolated from *E. faecium* and ST4V, isolated from *E. mundtii* (Wachsman et al., 1999; Todorov et al., 2005).

Bacteriocins JW3BZ and JW15BZ displayed similar antiviral activity (EC_{50}=200 μg/ml) against HSV-1. The virus causes primary and recurrent infections of mucous membranes, orofacial and genital lesions, and encephalitis (Whitley and Roizman 2001). The mechanism responsible for the antiviral activity of bacteriocins is not known, emphasizing the need to explore this field of research. Possible explanations could be the aggregation of virus particles, blockage of receptor sites on the host cell, or inhibition of key reactions in the multiplication cycle (Wachsman et al. 2003).

DGGE results reported the presence of strain JW3BZ and JW15BZ in boza over one week. Strains JW3BZ and JW15BZ inhibited the growth of the sensitive strain (*Lactobacillus sakei*

DSM 20017) in boza for at least 7 days, suggesting that the strains could be used as starter cultures to extend the shelf-life of boza. The use of strains JW3BZ and JW15BZ in combination with commercially available yeast as starter for the production of boza did not immensely effect the products sensory attributes compared to boza prepared using commercial boza as starter culture.

Alginate encapsulation improved the survival of strains JW3BZ and JW15BZ in the presence of 1% and 2% (w/v) bile compared to non-encapsulated cells. The increase in viability of encapsulated cells compared to free cells of *L. plantarum* JW3BZ and *L. fermentum* JW15BZ is in accordance with other researchers (Lee and Heo, 2000; Krasaekoopt et al., 2003; Chandramouli et al., 2004). Strains JW3BZ and JW15BZ immobilized in 2% (w/v) alginate survived simulated gastric conditions (pH 1.6). From these results, it is evident that encapsulation renders the strain protection against the harsh conditions associated with the gastrointestinal tract. Encapsulated strains JW3BZ and JW15BZ survived and proliferated better under simulated gastrointestinal conditions compared to their non-encapsulated counterparts. Further studies on the survival of encapsulated bacteria in various food processing technologies and the delivery of cells *in vivo* to target sites are needed.

Results obtained from this study suggested that strains JW3BZ and JW15BZ may survive conditions of the gastrointestinal tract and adhere to mucus and epithelial cells. Encapsulation of strains JW3BZ and JW15BZ in an alginate matrix were found to further enhance their survival. Production of broad-spectrum bacteriocins with antiviral activity and no changes in the organoleptic profile of boza fermented with these strains is an added advantage. Future research should focus on the possible medical applications of bacteriocins JW15BZ and JW3BZ and detailed *in vivo* studies in animals need to be done before the strains can be classified as probiotic.

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