BACTERIOCINS AND BACTERIOCIN PRODUCERS PRESENT IN KEFIR AND KEFIR GRAINS

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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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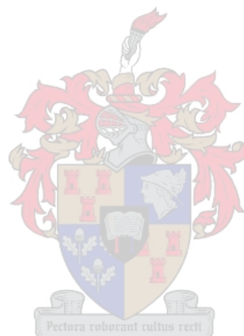
Jillian Elizabeth Powell Date



ABSTRACT

Kefir is a traditional fermented milk that is carbonated, has a sharp acidic taste, yeasty flavour and contains a low percentage alcohol (less than 2% (v/v)). The beverage is manufactured by fermenting milk with Kefir grains, comprised of microorganisms, polysaccharides and milk proteins. The microbial population of Kefir grains primarily include lactic acid bacteria (LAB), namely lactococci and lactobacilli, yeasts, *Acetobacter* and filamentous fungi.

Kefir exhibits antimicrobial activity *in vitro* against some fungi, and Gram-positive and Gram-negative bacteria. Although the exact cause of this inhibition in Kefir is not known, the ability of LAB to inhibit the growth of closely related bacteria is well known. This inhibition of pathogenic and spoilage microbes may be due to the production of organic acids, hydrogen peroxide, acetaldehyde, diacetyl, carbon dioxide or bacteriocins. Acid is not the only contributor to the antimicrobial activity of Kefir and Kefir grains, and bacteriocins may play a role in the inhibitory activity.

The bacteriocin producer *Lactobacillus plantarum* ST8KF, isolated from Kefir and Kefir grains, produces a bacteriocin 3.5 kDa in size. The mode of activity of bacteriocin ST8KF (bacST8KF) is thought to be bacteriostatic in exponential cultures of *Enterococcus faecalis* E88, *Lactobacillus casei* LHS, *Lactobacillus curvatus* DF38, *Lactobacillus sakei* DSM 20017, *Lactobacillus salivarius* 241 and *Listeria innocua* F and LMG 13568. The peptide is sensitive to proteolytic enzymes and does not adsorb to the surface of the producer cell. The bacteriocin is stable between pH 2.0 and 10.0, and for 20 min at 121°C. Maximum bacteriocin activity was observed in modified MRS medium supplemented with glucose or saccharose, meat extract, KH_2PO_4, glycerol, thiamine or cyanocobalamin, or in modified MRS medium without tri-ammonium citrate.

Maximum levels of adsorption of bacST8KF (80%) to *Lb. casei* LHS and *Lb. sakei* DSM 20017 were recorded. Adsorption (80%) of the bacteriocin to *Lactobacillus paraplantarum* ATCC 700211^T and *Streptococcus caprinus* ATCC 700066, which are not sensitive to the bacteriocin was also recorded. Optimal adsorption to *E. faecalis* E88 was recorded at 25°C at pH 2.0, and to *L. innocua* LMG 13568 at 4°C, 10°C and 25°C at pH 6.0. Potassium ions, $MgCl_2$, Tris, NH_4-citrate, Na-acetate, Na_2CO_3, EDTA and SDS led to decreased adsorption to both

sensitive strains, while NaCl and β-mercaptoethanol resulted decreased adsorption to *E. faecalis* E88, but not to *L. innocua* LMG 13568. Methanol resulted in lower levels of adsorption to *L. innocua* LMG 13568 but not to *E. faecalis* E88. Triton X-100 and Triton X-114 increased the adsorption of bacST8KF by 40%, and ethanol and chloroform had no effect on bacteriocin adsorption. The growth of *Lb. plantarum* ST8KF and *L. innocua* LMG 13568 in a mixed culture resulted in an increase of bacST8KF production. Cells treated with bacST8KF secreted DNA and β-galactosidase. As bacST8KF remains stable under a variety of conditions, the bacteriocin may have application, if awarded GRAS (generally regarded as safe) status, in various food products as a natural additive or preservative.

The genes encoding bacteriocin production are located on a 3.9 kilo base (kb) plasmid. Curing of the plasmid resulted in a mutant strain of *Lb. plantarum* ST8KF, and the *Lb. plantarum* strains ST8KF(+) and ST8KF(-) differed with regards to antibiotic resistance and carbohydrate fermentation reactions. The wild type and the cured strain were incorporated into Kefir grains during mass cultivation. The survival of the bacST8KF sensitive *Enterococcus mundtii* ST4SA added to the milk during Kefir production using the enriched mass cultured grains was monitored using fluorescent *in situ* hybridization. *Enterococcus mundtii* ST4SA was present in higher numbers in the ST8KF(-) Kefir system when compared to the ST8KF(+) system. It can, therefore, be concluded that *Lb. plantarum* ST8KF(+) contributes to the antimicrobial activity of Kefir through the production of bacteriocin ST8KF.

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UITTREKSEL

Kefir is ’n tradisioneel-gefermenteerde en gekarboneerde melk drankie. Dit het ’n skerp suur smaak, gis aroma en het ’n lae alkohol inhoud (minder as 2% v/v). Die drankie word vervaardig deur melk met Kefir korrels te fermenteer. Die korrels bestaan uit mikro-organismes, polisakkariede en melk proteïene. Die mikrobiese populasie van Kefirkorrels sluit hoofsaaklik melksuur bakterieë (LAB), naamlik lactococci en lactobacilli, giste, *Acetobacter* en miseliêre fungi in.

Kefir toon antimikrobiese aktiwiteit, *in vitro*, teen sommige fungi, en Gram-positiewe en -negatiewe bakterieë. Alhoewel die presiese oorsaak vir hierdie inhibisie onbekend is, is die vermoë van LAB om groei in naverwante baketerieë te inhibeer bekend. Die produksie van organiese sure, waterstofperoksied, asetaldehied, diasetiel, koolstofdioksied en/of bakteriosiene kan lei tot die inhibisie van patogene en bederf mikrobes. Dit is gewys dat suur nie die enigste bydrae tot die antimikrobale aktiwiteit van Kefir en Kefir korrels lewer nie. Bakteriësiene mag ook ’n rol in die inhibisie speel.

Die bakteriosien produseerder, *Lactobacillus plantarum* ST8KF, is geïsoleer uit Kefir en Kefirkorrels, en produseer ’n bakteriosien (bacST8KF) van 3.5 kDa. Dit word gedink dat hierdie bakteriosien bakteriostaties is, en dus effektief teen eksponënsie kulture van *Enterococcus faecalis* E88, *Lactobacillus casei* LHS, *Lactobacillus curvatus* DF38, *Lactobacillus sakei* DSM20017, *Lactobacillus salivarius* 241 en *Listeria innocua* F en LMG 13568 is. BacST8KF is sensitief vir proteolitiese ensieme en absorbeer nie aan die oppervlakte van die produseeder sel nie. Die bakteriosien is stabiel tussen pH 2.0 en 10.0, en teen 121°C vir 20 min. Maksimum bakteriosien aktiwiteit is gevind in gemodifiseerd MRS-medium wat verryk is met glukose of sukrose, vleis-ekstrak, KH_2PO_4, gliserol, tiamien of sianokobalamien, of in gemodifiseerd MRS-medium sonder tri-ammonium sitraat.

Maksimum vlakke van adsorpsie van bacST8KF (80%) aan *Lb. casei* LHS en *Lb. sakei* DSM 20017 is gevind. Absorpsie (80%) van die bakteriosien aan *Lactobacillus paraplantarum* ATCC 700211^T en *Streptococcus caprinus* ATCC 700066, wat nie sensitief teenoor die bakteriosien is nie, was ook gevind. ‘n pH van 2.0 en 20°C het gelei tot die beste adsorpsie aan *E. faecalis* E88, en 4°C, 10°C en 25°C by pH 6.0 was die beste vir absorpsie aan *L. innocua* LMG 13567.

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Kalium ione, $MgCl_2$, Tris, NH_4-sitraat, Na-asetaat, Na_2CO_3, EDTA en SDS het swakker absorpsie tot gevolg gehad, terwyl NaCl en β-merkapto-ehanol 'n afname in absorpsie aan *E. faecalis* E88, maar nie tot *L. innocua* LMG 13568, getoon het. Metanol het swakker adsorpsie aan *L. innocua* LMG 13568 getoon, maar nie aan *E. faecalis* E88 nie. Triton X-100 en Triton X-114 het die adsopsie van bacST8KF met 40% verhoog, terwyl etanol en chloroform geen effek op bakteriosien adsorpsie getoon het nie. Die behandeling van *L. innocua* E88 en *E. faecalis* LMG 13568 met bacST8KF het 'n daling van twee log siklusse tot gevolg gehad, en die groei van *Lb. plantarum* ST8KF en *L. innocua* LMG 13568 in 'n gemengde kultuur het 'n toename in bacST8KF produksie tot gevolg gehad. Die venietiging van *L. innocua* LMG 13568 en *E. faecalis* E88 is bevestig deur atoomkrag mikroskopie, en selle behandel met bacST8KF het DNA en β-galaktosidase uitgeskei. Aangesien bacST8KF stabiel is onder 'n verskeidenheid van toestande, mag die bakteriosiene, as dit GRAS (generally regarded as safe) status bekom, natuurlike byvoegsel of preserveermiddel, op verskeie voedselprodukte van toepassing wees.

Die gene wat vir bakteriosien produksie kodeer is op 'n 3.9 kb plasmied geleë. Bereiding van die plasmied het gelei tot 'n mutante *Lb. plantarum* ST8KF stam. Die ST8KF (+) en ST8KF(-) *Lb. plantarum* stamme het verskil ten opsigte van antibiotiese weerstand en ook koolhidraat fermentasie reaksies. Die wilde en bereide stamme is in Kefir korrels geïnkorporeer gedurende massa kweking. Oorlewing van die bacST8KF sensitiewe *Enterococcus mundtii* ST4SA, wat by die melk gevoeg is tydens Kefir produksie vanaf massa gekweekte korrels, is gemonitor deur van fluoreserende *in situ* hibridisasie gebruik te maak. *Enterococcus mundtii* ST4SA was teenwoordig teen hoër vlakke in die ST8KF(-) Kefir sisteem as in die ST8KF(+) sisteem. Daar kan dus afgelei word dat *Lb. plantarum* ST8KF(+) wel bydra tot die antimikrobiese aktiwiteit van Kefir deur die produksie van bakteriosien ST8KF.

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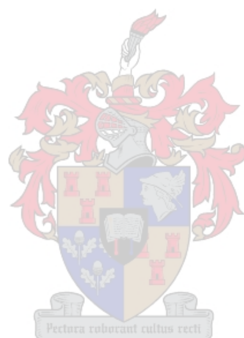

dedicated to my parents

for their endless support

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

Fermentation is one of the oldest and most widespread methods of preserving food, particularly milk. The knowledge of milk preservation using fermentation processes has been handed down from generation to generation (Alm, 1991). All fermented milk products involve the inoculation of milk with microbial cultures (Koroleva, 1988) and many traditional fermented products involve yeast-lactic fermentations (Marshall, 1987).

Kefir is a refreshing, self-carbonated fermented milk with a slightly acidic taste (Saloff-Coste, 1996), yeasty flavour and creamy consistency (Obermann, 1985; Duitschaever, 1989). The beverage originated in the Caucasian Mountains in Russia (Duitscheaver, 1989) and is commonly manufactured by fermenting milk with Kefir grains (Kwak *et al*., 1996). The grains resemble small cauliflower florets (Loretan *et al*., 2003) and are comprised of actively growing and reproducing bacteria and yeasts (Farnworth, 2003), milk protein, polysaccharides and other products of bacterial metabolism (Hertzler & Clancy, 2003). The microbial population of Kefir grains include lactic acid bacteria (LAB), namely lactococci and lactobacilli, and yeasts (both lactose fermenting and non-lactose fermenting) (Kwak *et al*., 1996), *Acetobacter* and filamentous fungi (Duitschaever, 1989).

Many health benefits have been attributed to Kefir, including the enhancement of the immune system and improved digestive health, particularly with regard to lactose digestion (Hertzler & Clancey, 2003). Kefir has broad antitumour, antibacterial and antifungal properties (Saloff-Coste, 1996) and exhibits antimicrobial activity *in vitro* against Gram-positive and Gram-negative bacteria and some fungi (Saloff-Coste, 1996; Garrote *et al*., 2000). The exact cause of the inhibition is not known, but may be due to the production of organic acids, hydrogen peroxide (Shahani & Chandan, 1979, Juven *et al*., 1992), acetaldehyde, diacetyl, carbon dioxide or bacteriocins (Helander *et al*., 1997). Acid production is not the only contributor to the antimicrobial activity of Kefir and Kefir grains, and bacteriocins may play a role in the inhibitory activity (Balasubramanyam *et al*., 1994; Morgan *et al*., 2000; Gulmez & Guven, 2003). Bacteriocins are ribosomally synthesised polypeptides with activity against

genetically closely related bacteria (Klaenhammer, 1988). The peptides generally vary with regards to their mode of action, molecular weight, genetic origin, biochemical properties and spectrum of activity. Bacteriocins produced by LAB display a high degree of heterogeneity.

Bacteriocins have attracted much interest as those with GRAS status are safe for human consumption and can be used in the preservation of food products, without any implications on consumer health (Olasupo, 1996). The demand by consumers for a decrease in the use of chemical additives in food has led to research on the use of natural antimicrobial substances secreted by food fermentative bacteria to inhibit undesirable microorganisms (Berry *et al.*, 1991; Schillinger *et al.*, 1991). Bacteriocins produced by Kefir and Kefir grain microbial populations may have potential use in the preservation of food products, and in the inhibition of food spoilage and pathogenic bacteria.

The main objective of this study was to determine whether bacteriocins produced by LAB contribute to the antimicrobial activity of Kefir and Kefir grains. The first aim was to isolate a bacteriocin-producing strain from Kefir and Kefir grains. The second aim was to investigate the parameters affecting the adsorption of the isolated bacteriocin to a variety of sensitive strains, and this was followed by determining whether the antimicrobial effect of Kefir can be contributed, at least in part, to bacteriocin production.

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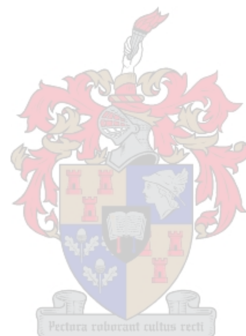

CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

The history of Kefir is shrouded by legend, with the drink dating back many centuries (Saloff-Coste, 1996; Hetzler & Clancy, 2003). The word Kefir is derived from the Turkish word "keif" which means "good-feeling" (Kaufmann, 1997) and the drink originated in the Caucasian mountains (Saloff-Coste, 1996; Garrote *et al*., 1998; Hetzler & Clancy, 2003; Loretan *et al*., 2003) of Russia, between the Black Sea and the Caspian Sea. The Kefir grains, considered to be a gift from the prophet Mohammed (Koroleva, 1988a), were passed from generation to generation among the Moslem tribes. These people considered the grains a source of family and tribal wealth, and guarded the secret process of Kefir making (Kaufmann, 1997).

Kefir is not a curdled product and is produced by the addition of Kefir grains to fresh milk (Loretan *et al*., 2003). Traditionally, the milk was placed in goatskin leather bags and fermented for 24 h at room temperature (Duitschaever *et al*., 1987; Kaufmann, 1997). The content was tied off in one corner of the leather bag (where most of the grains were retained), and the Kefir separated from the grains by pouring the beverage off. This produced a foaming drink, creamy in consistency and texture, with an alcohol content of approximately 0.08 – 2.0% (v/v) (Anfiteatros, 2004). During the 24 h fermentation, the Kefir grains change the milk into a thick, astringent tasting beverage. During cold weather, the leather bag was placed in the sun during the day, or hung near a fireplace at night. It was also custom to hang the bag near a doorway, whereby visitors would give the bag a gentle rock as they passed by (Koroleva, 1988b).

Kefir was also regularly subjected to a secondary fermentation, during which a mixture of fresh Kefir, fresh milk and the root of Snow Rose (*Rhododendron caucasicum*) was poured into wooden barrels or clay crocks (Koroleva, 1988b; Anfiteatros, 2004). The container was then sealed airtight and

the content fermented for some days. This produced a highly carbonated beverage, with possibly a slightly higher alcohol content (Anfiteatros, 2004).

Today, Kefir is still manufactured in Russia and Europe under a variety of names, such as Kephir, Kiaphur, Kefer, Kepi and Kippi (Kwak *et al*., 1996). It is also popular in Eastern European countries and is produced in small quantities in the former Czechoslovakia, Poland, Hungary, Finland, Sweden, Norway and Germany (Marshall & Cole, 1985; Koroleva, 1988b; Libudzisz & Piatkiewicz, 1990). It is also available in the United States and is growing in popularity in Japan (Saloff-Coste, 1996).

B. KEFIR BEVERAGE

Kefir is a refreshing, self-carbonated fermented milk with a slightly acidic taste (Saloff-Coste, 1996), yeasty flavour and creamy consistency and when agitated, the beverage foams and fizzes (Obermann, 1985; Duitschaever, 1989). This led to Kefir being named "the champagne of cultured dairy products" (Kaufmann, 1997). The beverage is produced from cow, goat, sheep, camel, buffalo or soya milk (Abraham & De Antoni, 1998; Loretan *et al*., 2003), and the milk can be unpasteurised, pasteurised, whole fat, low fat, skimmed or fat free. The higher the fat content, the thicker and creamier the Kefir. Pasteurised milk is recommended since bacteria in raw milk may influence the microbial balance of the Kefir grains (Kaufmann, 1997).

The nutritional composition and flavour of Kefir vary significantly and depend on a variety of factors, including the source and fat content of the milk, composition of the grains, and fermentation conditions (Saloff-Coste, 1996). Kefir has a pH of 4.2 – 4.6 (Odet, 1995), an ethanol content of 0.5 – 2.0% (v/v), a lactic acid content of 0.8 – 1.0% (m/v), a carbon dioxide content of 0.08 – 0.2% (v/v), and contains formic, succinic and propionic acids, as well as trace amounts of isoamyl alcohol, acetone and diacetyl (Duitschaever, 1989; Libudzisz & Piatkiewicz, 1990). Lactose is reduced (Saloff-Coste, 1996) and β-galactosidase increases during fermentation (Zubillaga *et al*., 2001). There is also a small increase in proteolysis, leading to an increase in free amino acids (Koroleva, 1988b).

As the Kefir grains become active and are allowed to grow, the microorganisms are shed into the milk (Mann, 1985). Once present in the milk, the microbes continue to multiply (Garrote *et al*., 1998) and according to Kurmann *et al*. (1992) one millilitre of a good-quality Kefir contains 10^9 lactococci, $10^7 – 10^8$ leuconostocs, $10^7 – 10^8$ thermophilic lactobacilli, $10^4 – 10^5$ yeasts and $10^4 – 10^5$ acetic acid bacteria. Kefir is therefore a natural probiotic and contains live active cultures that out-compete pathogenic microorganisms, repopulate the digestive tract and aid in digestion (Otes & Cagindi, 2003).

C. KEFIR GRAINS

Kefir is obtained from the fermentative activity of Kefir grains (Garrote *et al*., 2000). The grains are insoluble in water and common solvents (Liu & Moon, 1983), gelatinous, and irregular in size (Kaufmann, 1997), varying from 0.3 – 3.5 cm in diameter (Garrotte *et al*., 1997). They are white to yellow in colour and resemble small cauliflower florets. Sheet-like structures and scroll-like forms of Kefir grains are easily distinguished from cauliflower-like forms. Kefir grains probably evolved through the curling of the sheet-like structures, with subsequent folding and re-folding into a globular structure (Marshall *et al*., 1984).

Kefir grains are comprised of a mass of actively growing and reproducing bacteria and yeasts (Farnworth, 2003), polysaccharides and other products of bacterial metabolism, together with milk protein (Hertzler & Clancy, 2003). When added to milk, the grains swell and form a jelly-like product, Kefiran (Obermann, 1985; Loretan *et al*., 2003). Microbial cells account for the major part of the grain, together with autolysis products, curd proteins and carbohydrates such as Kefiran (Libudzisz & Piatkiewicz, 1990). Kefiran is water-soluble (Rodrigues *et al*., 2005) and facilitates the formation of aggregates (Loretan *et al*., 2003).

The grains are formed during the process of Kefir making, and as far as is known, only from existing grains. The grains are initially very small but increase in size during fermentation (Steinkraus, 1996) as the microorganisms multiply and Kefiran accumulates (Saloff-Coste, 1996). Despite intensive research to produce Kefir grains from pure and mixed cultures, no successes have been reported (Libudzisz & Piatkiewicz, 1990; Rea *et al*., 1996). This can be ascribed to the fact that very little is known about the mechanism of grain formation, and a

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combination of different factors may have an influence on the biomass increase of the Kefir grains. These factors include the renewal of the milk at regular intervals, the cultivation temperature, grain washing, and the presence of essential nutrients in the correct concentration in the growth medium (Shoevers & Britz, 2003).

A variety of methods by which Kefir grains may be stored have been developed, and each method affects the activity of the grains differently. Grains stored in water can only be kept for 8 – 10 days, air-dried or lyophilized Kefir grains can be kept for 12 – 18 months, with no loss of activity (Marth & Yousef, 1991; Garrote *et al*., 1997). Frozen grains stored at -20°C maintained microbial activity for 7 – 8 months, whereas refrigerated grains showed a decrease in activity after 10 days (Oberman & Libudzisz, 1998). Preservation of Kefir grains at -80°C has less of an effect on the microbial composition of the grains than preservation of the grains at -4° or -20°C (Garrote *et al*., 1997).

The average chemical composition of Kefir grains is 890 – 900 $g.kg^{-1}$ water, 3 $g.kg^{-1}$ lipids, 32 $g.kg^{-1}$ protein, 60 $g.kg^{-1}$ sugars and 7 $g.kg^{-1}$ ash (Ottogalli *et al*., 1973; Garrote *et al*., 2001). The dry mass (10 – 16% (w/m)) of a fresh grain consists of 30% (w/m) protein and 25 – 50% (w/m) carbohydrates (Libudzisz & Piatkiewicz, 1990).

D. KEFIR GRAIN MICROBIAL POPULATIONS

Kefir grains primarily include lactic acid bacteria (LAB), namely lactococci and lactobacilli, and yeasts (both lactose fermenting and non-lactose fermenting) (Kwak *et al*., 1996), *Acetobacter* and filamentous fungi. The organisms embedded in Kefir grains are responsible for a lactic acid-alcoholic fermentation, which gives Kefir its typical organoleptic properties (Duitschaever, 1989). Although the grains contain a relatively stable and specific balance of microorganisms which exist in a symbiotic relationship (Garrote *et al*., 1998), the exact combination of bacteria and yeasts may vary. The Kefir grain microbial population depends on the origin of the grains (Otogalli *et al*., 1973; Lin *et al*., 1999), on the local culturing conditions and on the storage conditions (Garrote *et al*., 2001).

Rea *et al*. (1996) examined the interior and exterior of Kefir grains using a scanning electron microscope and found considerable variations between the

different areas of the grains. The peripheral part of the grain is almost exclusively populated by bacteria, while the center of the grain is dominated by yeasts (Bottazzi & Bianchi, 1980; Lin *et al.*, 1999). The areas between the center and outer grain contains a balance of bacteria and yeasts, which changes progressively according to the distance from the core (Bottazzi & Bianchi, 1980). The microbial species associated with Kefir and Kefir grains are listed in Table 1.

E. KEFIRAN

Kefiran constitutes between 24 – 25% (m/m) of the dry weight of the Kefir grain, and is a matrix of fibrillar amorphous material that consists largely of polysaccharides (Kooiman, 1968; Marshall *et al.*, 1984; Duitshcaever *et al.*, 1988; Saloff-Coste, 1996). This fibrillar matrix surrounds the bacteria and yeast in Kefir grains, and holds the grains together (Santos *et al.*, 2003).

The structure of Kefiran is a branched glucogalactan (Micheli *et al.*, 1999) consisting of approximately equal amounts of D-glucose and D-galactose residues (Kooiman, 1968). In addition to D-glucose and D-galactose, Kefiran also contains a repeating unit of 6-0 substituted galactose (Mukai *et al.*, 1988). A number of Kefiran-producing homofermentative LAB have been isolated from Kefir grains (Koroleva, 1991), including *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, *Leuconostoc mesenteroides* and *Lactococcus lactis* subsp. *cremoris*. Kefiran production by homofermentative LAB is stimulated by CO_2, ethanol, or unknown metabolites produced by heterofermentative LAB, lactose fermenting yeasts, and non-lactose fermenting yeasts present in Kefir grains (Adachi *et al.*, 1990). It is unknown which LAB are responsible for production of Kefiran. *Lactobacillus kefiranofaciens* has been

Table 1 Microorganisms associated with Kefir and Kefir grains

Microorganism	***Reference***
Lactic acid bacteria	
Enterococcus durans	***Marshall, 1993***
Lactobacillus acidophilus	Marshall, 1987; Libudzisz & Piatkiewicz, 1990; Kwak *et al*., 1996
Lactobacillus brevis	Marshall, 1987; Libudzisz & Piatkiewicz, 1990; Kwak *et al*., 1996
Lactobacillus buchneri	Iwasawa *et al*., 1981
Lactobacillus casei	
subsp. *alactosus*	Libudzisz & Piatkiewicz, 1990; Kurmann *et al*., 1992; Marshall, 1993; Kwak *et al*., 1996
subsp. *rhamnosus*	Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990; Marshall, 1993
subsp. *pseudoplantarum*	Angulo *et al*., 1993
subsp. *tolerans*	Angulo *et al*., 1993
Lactobacillus cellobiosus	Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Kwak *et al*., 1996
Lactobacillus delbrueckii	
subsp. *bulgaricus*	Koroleva, 1988a; Marshall, 1993; Kwak *et al*., 1996
subsp. *lactis*	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
Lactobacillus fermentum	Angulo *et al*., 1993
Lactobacillus gasseri	Angulo *et al*., 1993
Lactobacillus helveticus	
subsp. *jugurti*	Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990; Kwak *et al*., 1996
subsp. *lactis*	Marshall, 1993
Lactobacillus kefir	Marshall, 1987; 1993; Kwak *et al*., 1996; Pintado *et al*., 1996
Lactobacillus kefiranofaciens	***Marshall, 1993; Kwak* et al.*, 1996; Pintado* et al.*, 1996***
Lactobacillus kefis	Pintado *et al*., 1996
Lactobacillus kefirgranum	Takizawa *et al*., 1994
Lactobacillus lactis subsp. *lactis*	Kwak *et al*., 1996
Lactobacillus parakefir	Takizawa *et al*., 1994
Lactobacillus plantarum	Kwak *et al*., 1996
Lactococcus lactis	
subsp. *cremoris*	Koroleva, 1988a; Marshall, 1993; Libudzisz & Piatkiewicz, 1990; Kwak *et al*., 1996
subsp. *lactis*	Marshall, 1993; Kwak *et al*., 1996; Pintado *et al*., 1996
subsp. *diacetylactis*	Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Kwak *et al*., 1996
Lactococcus filant	Kwak *et al*., 1996
Leuconostoc kefir	Kwak *et al*., 1996
Leuconostoc mesenteriodes	
subsp. *cremoris*	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
subsp. *dextranicum*	Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990; Kwak *et al*., 1996
subsp. *mesenteriodes*	Marshall, 1993; Kwak *et al*., 1996
Streptococcus durans	Marshall, 1987; Libudzisz & Piatkiewicz, 1990; Kwak *et al*., 1996
Streptococcus filant	Libudzisz & Piatkiewicz, 1990
Streptococcus salivarius	
subsp. *thermophilus*	Libudzisz & Piatkiewicz, 1990; Marshall, 1993; **Kwak *et al*., 1996**

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Table 1 **Cont.**

Microorganism	**Reference**
Weissella viridescens	Angulo *et al*., 1993
Acetic acid bacteria	
Acetobacter aceti	Koroleva, 1988a; Kurmann *et al*., 1992; Marshall, 1993
Acetobacter rasens	Koroleva, 1988a; Marshall, 1993
Acetobacter rancens	Koroleva, 1988a; Saloff-Coste, 1996
Yeasts	
Brettanomyces anomalus	*Mann, 1979; Garrote* et al*., 1997; Lin* et al*., 1999*
Candida friedricchii	Mann, 1979; Garrote *et al*., 1997; Lin *et al*., 1999
Candida holmii	Mann, 1979; Marshall, 1993; Brialy *et al*., 1995; Garrote *et al*., 1997; Lin *et al*., 1999;
Candida kefir	Marshall, 1987; Libudzisz & Piatkiewicz, 1990; Marshall, 1993
Candida pseudotropicalis	***Libudzisz & Piatkiewicz, 1990; Marshall, 1993*** Pintado *et al*., 1996
Candida tenuis	Pintado *et al*., 1996
Candida valida	Mann, 1979; Garrote *et al.,* 1997*;* Lin *et al.,* 1999
Cryptococcus kefyr	Kaufmann, 1997; Liu & Moon, 1983; Koroleva, 1988a; Kwak *et al*., 1996;
Kluyveromyces bulgaricus	Libudzisz & Piatkiewicz, 1990; Marshall, 1993
Kluyveromyces fragilis	Kwak *et al*., 1996; Liu & Moon, 1983; Kaufmann, 1997
Kluyveromyces lactis	Libudzisz & Piatkiewicz, 1990; Marshall, 1993; Kwak *et al*., 1996
Kluyveromyces marxianus	
subsp. *marxianus*	Koroleva, 1988a; Kwak *et al*., 1996; Marshall, 1993
subsp. *bulgaricus*	Liu & Moon, 1983; Kwak *et al*., 1996; Kaufmann, 1997
Pichia fermentans	Mann, 1979; Garrote *et al*., 1997; Lin *et al*., 1999
Saccharomyces carlsbergensis	Libudzisz & Piatkiewicz, 1990; Kwak *et al*., 1996; Pintado *et al*., 1996
Saccharomyces cerevisiae	Marshall, 1987; Koroleva, 1988a; Kwak *et al*., 1996
Saccharomyces delbrueckii	Streinkraus, 1996
Saccharomyces florentinus	Libudzisz & Piatkiewicz, 1990; Brialy *et al*., 1995
Saccharomyces globosus	Libudzisz & Piatkiewicz, 1990
Saccharomyces kefir	Kwak *et al*., 1996
Saccharomyces lactis	Pintado *et al*., 1996
Saccharomyces lipolytic	Mann, 1979; Garrote *et al*., 1997; Lin *et al*., 1999
Saccharomyces unispores	Libudzisz & Piatkiewicz, 1990
Torula kefir	Kwak *et al*., 1996
Torulaspora delbrueckii	Marshall, 1987; Koroleva, 1988a; Libudzisz & Piatkiewicz, 1990
Torulopsis holmii	Streinkraus, 1996
Mycelial fungi	
Geotrichum candidum	Marshall, 1987; Roginski, 1988

identified as the main polysaccharide producer (Fujisawa *et al*., 1988; Rea *et al*., 1996). However, this is controversial as it was found that milk whey is required in the growth medium for Kefiran production and the polysaccharide is only produced in small quantities (Yokai *et al*., 1991). *Lactobacillus kefir* is considered to be a minor polysaccharide producer (Kandler & Kunath, 1983) and *Lactobacillus brevis* rapidly looses its ability to produce Kefiran once isolated from Kefir grains (La Riviére *et al*., 1967). *Lactococcus lactis* and *Leuc. mesenteroides* also have the ability to produce extracellular polysaccharides (Marshall *et al*., 1984).

The Kefiran polysaccharide has antibacterial, antimycotic and antitumour activity (Micheli *et al*., 1999). In a recent study by Rodrigues *et al*. (2005), Kefiran inhibited the growth of seven bacterial strains and one yeast species. Kefiran also exhibits antitumour activity against Ehrlich carcinoma and Sarcoma 180 solid tumor (Shiomi *et al*., 1982), and anti-metastatic activity against Lewis lung carcinoma and the highly metastatic B16 melanoma in mice (Furukava, 2001; Schoeman, 2001). Kefiran may thus be a good antimicrobial, anti-inflammatory and cicatrising agent for the use in a variety of infections (Rodrigues *et al*., 2005).

F. KEFIR PRODUCTION

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The traditional method of Kefir making involves the direct addition of Kefir grains (2 – 10% (m/v)) to milk that has been pasteurised and cooled to between 20° and 25ºC. The milk and grains are then subjected to a 24 h fermentation at room temperature. After this, the grains are removed by filtration, and the beverage is ready for consumption (Saloff-Coste, 1996). Fermenting for a shorter period produces a milder, sweeter tasting Kefir, while a longer fermentation produces a sour beverage (Anfiteatros, 2004).

A second method, known as the “Russian method”, involves two fermentations and is used for the production of Kefir on a larger scale. The first fermentation allows for the preparation of the starter culture and is achieved by incubating milk with grains (2 – 3% (m/v)). The grains are then removed by filtration and the mixture is added to milk (1 – 3% (m/v)) as a starter. This milk is then subjected to a second 12 – 18 h fermentation (Saloff-Coste, 1996). Although these methods have been used for many years, traditional methods produce only small volumes of Kefir and require several steps, with each additional step

increasing the risk of contamination. The shelf-life of traditional Kefir is very short, usually less than three days, but if kept in glass bottles the beverage can be kept for 8 – 10 days at 4°C (Koroleva, 1988b). Problems associated with traditional Kefir have led to the development of new techniques for its production.

To produce a Kefir of more consistent quality, some producers in Poland use concentrated lyophilized cultures made from the grains (Libudzisz & Piatkiewicz, 1990). The mother cultures obtained from the lyophilized cultures are used as bulk starters and are directly inoculated into milk. The mother culture is obtained by adding the whole contents of the package (1 g of grains) to 3 litres of milk (Libudzisz & Piatkiewicz, 1990). This mixture is then used to prepare the Kefir beverage, which is far more controlled and contains fewer steps than traditional methods, and as a result, a product of more consistent quality is produced (Saloff-Coste, 1996).

Kefir may also be produced from pure, defined cultures (Duitschaever *et al.*, 1987; Duitschaever *et al.*, 1988). Milk can either be inoculated simultaneously with LAB and yeasts, or the milk can undergo two separate fermentations. The first fermentation with LAB, and the second fermentation with yeasts (Saloff-Coste, 1996). Both methods allow for a better control of microorganisms involved, consistent quality and ease of production.

"Kefir tablets" are also produced and used in Poland for the domestic production of Kefir. One or two tablets are dissolved in a glass of milk and once dissolved, the milk is incubated at 25° – 30°C for 18 – 26 h, until curd is formed. This curd is then used as a starter to produce Kefir. Four to five tablespoons of the starter are used to inoculate one litre of milk. After inoculation, the milk is stored at 20° – 22°C until a curd is formed, which usually takes 14 – 18 h. Once formed, the milk is stored at room temperature for 4 h, cooled and refrigerated until consumed (Libudzisz & Piatkiewicz, 1990).

G. MASS CULTIVATION OF KEFIR GRAINS

Modern Kefir production is based on the continuous cultivation of Kefir grains in milk, resulting in a biomass increases of 5 – 7% (m/m) per day (Libudzisz &

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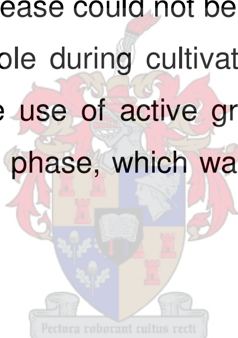
Piatkiewicz, 1990). As soon as the fermentation is complete, the grains are removed from the milk by sieving and are directly inoculated into fresh milk. Five hundred grams wet Kefir grains can double in weight in 7 – 10 days if transferred to 500 ml fresh milk six times a week (La Riviére *et al.*, 1967). This increase of grain biomass is greatly retarded if the grains are rinsed with water after each sieving. Libudzisz and Piatkiewicz (1990) recommend that the grains only be rinsed once a week with sterile, cooled water.

Schoevers and Britz (2003) investigated the factors influencing grain biomass increase and concluded that an incubation temperature of 25ºC was the optimum temperature for mass increase, as it resulted in a 145% (m/m) increase over a 10 day period as compared to a 123% (m/m) increase at 22°C. They also showed that the addition of either tryptose or yeast extract during the production (with agitation) greatly enhanced the increase in grain mass. The influence of milk fat content on the biomass increase could not be defined, and it appeared that milk fat played a more important role during cultivation without agitation, than during cultivation with agitation. The use of active grains during mass cultivation was also essential to prevent a lag phase, which was observed during cultivation with inactive grains.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H. NUTRITIONAL VALUE

The nutrient composition of Kefir is similar to that of milk, with Kefir containing more vitamins B_1, B_2 and folic acid (Libudzisz & Piatkiewicz, 1990). Kefir is also rich in vitamin K and amino acids and is an excellent source of biotin, a B vitamin that aids in the assimilation of the other B vitamins. The numerous benefits of maintaining adequate B vitamin intake range from regulation of the kidneys and liver, to helping regulate skin disorders, boost energy and promote longevity (Otes & Cagindi, 2003). Propionibacteria may be added to the Kefir grains to increase the vitamin B_{12} concentration of the beverage (Černà & Hrabovà, 1977). This is beneficial as the presence of vitamin B_{12} in milk decreases during the fermentation process (Kneifel *et al.*, 1989). Vitamin B_{12} may even decrease as much as 95% (m/v) (Liu & Moon, 1983; Kneifel *et al.*, 1989) during lactic acid fermentation. The

microorganisms in Kefir do not synthesise vitamin B_{12}, but they stimulate its production in mixed culture in the presence of propionic acid bacteria (Roczniakowa *et al.*, 1974). Van Wyk (2002) reported increases of vitamins B_{12} in Kefir enriched with *Propionibacterium freudenreichii* subsp. *shermanii*.

In addition to beneficial bacteria and yeasts, Kefir contains minerals and essential amino acids that promote healing and contribute to the maintenance functions of the body. The proteins in Kefir are partially digested and are, therefore, more easily utilised. Tryptophan, one of the essential amino acids abundant in Kefir, is well known for its relaxing effect on the nervous system. Kefir also offers an abundance of calcium and magnesium, which are important minerals for a healthy nervous system. The high phosphorous content contributes to the utilisation of carbohydrates, fats, and proteins by the body for cell growth, maintenance and energy (Otes & Cagindi, 2003).

I. ANTIMICROBIAL ACTIVITY OF KEFIR

Kefir possesses antimicrobial activity *in vitro* against a wide variety of Gram-positive and Gram-negative bacteria, as well as some fungi (Saloff-Coste, 1996; Garrote *et al.*, 2000). Some coliforms are actively inhibited by Kefir microorganisms, and pathogenic bacteria such as *Shigella* and *Salmonella* do not grow when they are introduced to Kefir (Koroleva, 1988a). *Lactobacillus acidophilus* isolated from Kefir, shows inhibitory activity against several Gram-positive and Gram-negative microorganisms (Gilliland & Speck, 1977; Apella *et al.*, 1992; Gupta *et al.*, 1996). Of all the Kefir starter microbial components, the microphilic homofermentative lactococci and acetic acid bacteria are the most active against coliforms. Van Wyk (2001) showed that Kefir possesses an inhibitory activity against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium tyrobutyricum* and *Listeria monocytogenes*. Studies have also indicated that yeasts such as *Torulaspora*, when separated from Kefir, possess pronounced antimicrobial activity against coliforms (Koroleva, 1988a; Naidu *et al.*, 1999).

The exact cause of the inhibition is not known, but may be due to the antagonistic action of various species of LAB (Gibson *et al.*, 1997; Naidu *et al.*, 1999). Lactic acid bacteria are also capable of preventing the adherence,

establishment, replication, and/or pathogenic action of certain enteropathogens (Saavedra, 1995). The precise mechanism of this antagonistic activity is not clear, but may include the activity of lactic acid or volatile acids, hydrogen peroxide (Shahani & Chandan, 1979, Juven *et al*., 1992), carbon dioxide, acetaldehyde and diacetyl, or bacteriocin and bacteriocin-like products (Helander *et al*., 1997).

Lactic acid and volatile acids

A decrease in the pH of the Kefir beverage is caused by the accumulation of organic acids, primarily lactic acid and acetic acid, produced as major end-products of carbohydrate metabolism by LAB. Accumulation of lactic acid and a subsequent decrease in pH results in a broad-spectrum inhibitory activity against Gram-positive and Gram-negative bacteria (Naidu *et al*., 1999).

The undissociated forms of lactic and acetic acid penetrates the microbial cell membrane. This results in acidification of the cytoplasm and the formation of inhibitions, especially against enzymes, by salt excesses (Piard & Desmazeaud, 1991). At a higher intracellular pH these acids dissociate to produce hydrogen ions, which interfere with important metabolic functions such as oxidative phosphorylation and substrate translocation (Baird-Parker, 1980; Naidu *et al*., 1999). The antimicrobial effect of lactic or acetic acid depends on the pK_a value of the acid, as well as the pH of the external environment (Adams & Hall, 1988; Piard & Desmazeaud, 1991). These acids are known to inhibit *E. coli* (Garrotte *et al*., 2000) and *B. cereus* (Rossland *et al*., 2005). At a pH 5.0 acetic acid inhibits the growth of *Salmonellae typhimurium* (Goepfert & Hicks, 1969). A synergism between lactic and acetic acid has been reported for the inhibition of *E. coli* and *Salmonella* spp. (Adams & Hall, 1988; Garrotte *et al*., 2000). Lactic acid (pK_a 3.86) is a stronger acid than acetic acid (pK_a 4.75) (Piard & Desmazeaud, 1991) and in well-buffered foods with a pH of 4 – 6, acetate has a stronger antimicrobial effect as a greater portion of the acid is undissociated (Adams & Hall, 1988).

Hydrogen peroxide

The production of hydrogen peroxide (H_2O_2) by LAB depends on the strain and the availability of oxygen (Helander *et al*., 1997). In the presence of oxygen, H_2O_2 is produced by LAB through electron transport via flavin enzymes. In the presence of H_2O_2, superoxide anions form destructive hydroxy radicals ($^{\cdot}OH$), leading to

increased membrane permeability (Kong & Davison, 1980) and to the peroxidation of membrane lipids (Piard & Desmazeaud, 1991). Bactericidal oxygen metabolites cause the destruction of nucleic acids and cell proteins, and have a strong oxidising effect on the bacterial cell (Dahl *et al*., 1989; Piard & Desmazeaud, 1991; Naidu *et al*., 1999). Hydrogen peroxide accumulates in the growth media and inhibits *Pseudomonas* spp. (Price & Lee, 1970) and *S. aureus* (Dahiya & Speck, 1968). Inhibitory compounds can also be formed from H_2O_2, such as in raw milk where it reacts with endogenous thiocyanate, catalysed by lactoperoxidase (Daeschel, 1989; Piard & Desmazeaud, 1991).

Carbon dioxide

Carbon dioxide (CO_2) is produced in substantial volumes by heterofermentative LAB as an end-product of hexose fermentation. Carbon dioxide may be produced by LAB from malate and citrate (Fleming *et al*., 1986; Naidu *et al*., 1999), by metabolising arginine via the arginine deaminase pathway (Poolman, 1993) or through the decarboxylation of the amino acids histidine and tyrosine (Naidu *et al*., 1999). Carbon dioxide contributes to the antimicrobial activity of LAB by replacing the existing molecular oxygen, creating an anaerobic environment (Ekland, 1984).

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Acetaldehyde and diacetyl

Acetaldehyde is responsible for the typical aroma in yoghurt. It is formed during carbohydrate metabolism by LAB and is then reduced to ethanol by re-oxidation of pyridine nucleotides. The reaction is catalysed by an NAD-dependent alcohol dehydrogenase (Naidu *et al*., 1999). Acetaldehyde, at concentrations of 10 to 100 ppm has antimicrobial activity against *Staph. aureus*, *E. coli* and *S. typhimurium* (Kulshrestha & Marth, 1974a; b; c).

Diacetyl (2,3-butanedione) is an end-product of pyruvate metabolism (Condon, 1987) of citrate-fermenting LAB (Hugenholtz, 1993; Naidu *et al*., 1999) that elicits antimicrobial activity against various spoilage microorganisms and food-borne pathogens (Jay, 1982; Helander *et al*., 1997). Diacetyl is effective against yeasts, moulds and Gram-negative bacteria. Archer *et al*. (1996) reported the inhibition of *S. typhimurium* by sublethal concentrations of diacetyl. The compound reacts with arginine-binding proteins of Gram-negative bacteria and interferes with arginine

utilisation (Jay, 1996; Kang & Fung, 1999; Naidu *et al.*, 1999). High concentrations of diacetyl are required for an antimicrobial effect. Dose-dependent inhibition experiments established that 0.2 $mg.ml^{-1}$ is required for the antimicrobial activity against Gram-negative bacteria and yeasts, while 0.3 $mg.ml^{-1}$ is required for the inhibition of non-lactic Gram-positive bacteria (Jay, 1982; Piard & Desmazeaud, 1991).

Bacteriocin and bacteriocin-like products

Bacteriocins are bacterial proteins or peptides with bactericidal or bacteriostatic activity against genetically closely related species (Tagg *et al.*, 1976). Bacteriocins generally vary with regards to their mode of action, molecular weight, genetic origin, biochemical properties and spectrum of activity. They can be produced spontaneously or induced and the genetic determinants of most bacteriocins are located on plasmids, with only a few exceptions being chromosomal encoded (Naidu *et al.*, 1999).

The release of bacteriocins from producer cells requires the expression and activity of bacteriocin-release proteins, and the presence of detergent resistant phospholipase A in the bacterial outer membrane (Naidu *et al.*, 1999). The bacteriocins that are released are species specific. The majority of bacteriocins produced by LAB have been characterised according to their activity as a proteinaceous inhibitor, on the estimation of their molecular mass, and on the determination of their spectrum of inhibition. In addition, recent developments in the biochemical and molecular characterisation of many of these compounds has led to the description of their genetic organisation, structure, and mode of activity (Klaenhammer, 1993). Bacteriocins produced by LAB display a high degree of heterogeneity and are divided into distinct classes.

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Class I – Lantibiotics

Lantibiotics are small, post-translationally modified (Hansen, 1993; Jack *et al.*, 1995), membrane-active peptides of less than 5 kDa (Klaenhammer, 1993). They typically have from 19 to more than 50 amino acids and are characterised by unusual amino acids, such as β-methyl-lanthionine, lanthionine and the dehydrated residues of dehydrobutyrine and dehydroalanine (Klaenhammer, 1993). The lantibiotics are ribosomally synthesised as pre-peptides containing an

unusual and structurally homologous leader sequence that may be involved in post-translational modification (Buchman *et al*., 1988; Schnell *et al*., 1988). Examples of these bacteriocins include nisin A and Z (Gross & Morell, 1971; Mulders *et al*., 1991; De Vos *et al*., 1993), subtilin (Gross & Kiltz, 1973), lactocin S (Mortveldt *et al*., 1991), sublancin 168 (Paik *et al*., 1998), carnocin U149 (Stoffels *et al*., 1992; 1994), and the two-component lantibiotics cytolysin produced by *Enterococcus faecalis* (Gilmore *et al*., 1990), lacticin 3147 produced by *L. lactis* (Ryan *et al*., 1999), and staphylococcin C55 produced by *Staph. aureus* (Navaratna *et al*., 1998; Sablon *et al*., 2000).

The class can be divided into type-A or type-B lantibiotics, based on the intra-chain positioning of the polycyclic structures and function. Type A lantibiotics, which include nisin, subtilin and epidermin consist of elongated, cationic peptides containing up to 34 residues and that show similarities in the arrangement of their lanthionine bridges (Hansen, 1993). These peptides primarily act by disrupting the membrane integrity of target bacterial species through formation of pores in the bacterial membrane (Altena *et al*., 2000). This group can further be divided into Class AI and Class AII based on the similarities in the net charge, sequence of the leaders and size (De Vos *et al*., 1995). Type-B lantibiotics are globular peptides and include mersacidin and actaragardine (Hansen, 1993). These peptides contain up to 19 residues (Jung, 1991; Hansen, 1993), have no net negative charge (Altena *et al*., 2000), and act through the disruption of enzyme function (Jung, 1991).

Class II

Class II bacteriocins are small, membrane-active, heat-stable, hydrophobic (Nes *et al*., 1996), non-lanthionine containing peptides that are less than 10 kDa in size and that undergo minimal post-translational modifications. They are characterised by the presence of a Gly-Gly$^{-1/-2}$ Xaa processing site. This site is present in the bacteriocin precursor and is not restricted to the Class II bacteriocins, as it is found in some lantibiotics (Havarstein *et al*., 1994; Sablon *et al*., 2000). The mature bacteriocins in this group form amphiphilic helices with varying p-sheet structures, hydrophobocity, and moderate (100°C) to high (121°C) heat stability. Class II bacteriocins are further subdivided into three subgroups, Classes IIa, IIb and IIc (Sablon *et al*., 2000).

Class IIa is the largest and most extensively studied subgroup of the Class II bacteriocins and are strong inhibitors of *L. monocytogenes*. Because of this anti-listerial effectiveness, Class IIa bacteriocins have significant potential as biopreservatives in food (Ennahar *et al*., 2000). Examples include leucocin A, mesentericin Y105, mundticin, sakacin A and P, enterocin A and P and pediocin AcH (Sablon *et al*., 2000).

The N-terminus of these peptides contains a consensus sequence of Tyr-Gly-Asn-Gly-Val, and two cysteines form a S-S bridge in the N-terminal half of the peptide (Cleveland *et al*., 2001). These bacteriocins contain between 37 (as in leucocin A) and 48 residues (as in carnobacteriocin B2) and they share considerable sequence similarity (Fleury *et al*., 1996). The Class IIa pediocins PA-1/AcH and JD elicit their bactericidal activity at the cytoplasmic membrane and cause a collapse in proton motive force and pH gradient (Bhunia *et al*., 1991; Christensen & Hutkins, 1992). Furthermore, they result in the leakage of UV-adsorbing materials and K^+ and in some cases cell lysis (Bhunia *et al*., 1991).

Pediocin PA-1 is a heat-stable (100°C, 60 min) protein, active against many LAB and *L. monocytogenes* (Klaenhammer, 1993). Production of the bacteriocin correlates with the presence of a 9.3 kb plasmid, pSRQ11 (Gonzalez & Kunka, 1987). Pediocin AcH production is linked to a 9.4 kb plasmid and the non-specific binding of the peptide requires lipoteichoic acid (Tagg *et al*., 1976; Bhunia *et al*., 1991).

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Class IIb are poration complexes formed by oligomers of two different proteinaceous peptides necessary for complete activity (Nes *et al*., 1996). Examples of this class include lactococcin G (Nissen-Meyer *et al*., 1992) and lacticin F (Muriana & Klaenhammer 1991a; 1991b). The cytoplasmic membrane is the site of activity for the Class IIb bacteriocin lactacin F, and treatment of *E. faecalis* cells with lactacin F results in the induction of membrane permeability (Klaenhammer, 1993). The Class IIb bacteriocin is to date the most hydrophobic peptide to be characterised (Klaenhammer, 1993). It is heat-stable (121°C, 15 min), proteinaceous and inhibitory against *E. faecalis* and other lactobacilli. The purified bacteriocin is 2.5 kDa, however, amino acid composition analysis indicates that the peptide may be as large as 6.2 kDa. The bacteriocin contains 54 – 57 amino acids and is produced by *Lactobacillus acidophilus* 11088 (NCK88) (Muriana & Klaenhammer, 1991a).

Class IIc are small, heat-stable, non-modified bacteriocins that require reduced cysteine residues for activity (Klaenhammer, 1993). In comparison to the other Class II bacteriocins, which require a secretion and maturation system to produce the extracellular, mature bacteriocin, Class IIc peptides utilise existing signal sequence-dependent general pathways present in the host (Worobo *et al*., 1995), such as the *sec* pathway in *E. coli*. Examples of this class include acidocin B (Leer *et al*., 1995) and divergicin A (Worobo *et al*., 1995).

Class III

Class III bacteriocins are large, heat-labile, hydrophilic (Nes *et al*., 1996) peptides less than 30 kDa in size and are relatively uncommon amongst the antibacterial compounds of LAB. Examples include acidophilin A, lacticin A and B and helveticin J and V1829 (Metha *et al*., 1983; Joerger & Klaenhammer, 1986; 1990; Toba *et al*., 1991; Vaughan *et al*., 1992; Sablon *et al*., 2000).

The exact mechanism of the Class III bacteriocins is still to be elucidated (Klaenhammer, 1993). The class III bacteriocins divergen A (Worobo *et al*., 1995) and acidocin B (Leer *et al*., 1995) are small, hydrophobic, thermostable bacteriocins produced by *Carnobacterium divergens* and *Lb. acidophilus*, respectively (Cintas *et al*., 1997). Helvetican J defines the Class III bacteriocins and is produced by *Lactobacillus helveticus* 481, and is a 37 kDa, heat-labile bacteriocin (Klaenhammer, 1993).

Class IV

A fourth class of bacteriocins was suggested by Klaenhammer (1993) and comprises the complex bacteriocins, which are composed of a protein and one or more chemical moieties such as a carbohydrate or lipid. Examples in this class include pediocin SJ-1, leucocin S, plantaricin S and lactocin 27 (Upreti & Hinsdill, 1973; Lewus *et al*., 1992; Jiménez-Diaz *et al*., 1993; Schved *et al*., 1993; Sablon *et al*., 2000). The existence of this class is based on the destruction of bacteriocin activity by lipolytic and glycolytic enzymes (Jiménez-Diaz *et al*., 1993). The inactivation of the bacteriocins by these enzymes has not been adequately characterised at a biochemical level and no purified bacteriocins have yet been shown to belong to this class (Nes *et al*., 1996). Experimental evidence further

suggests that antagonistic effects observed, form from interactions between constituents from the cells and the growth medium (Cleveland *et al.*, 2001).

The class IV bacteriocin lactacin 27 is membrane active and causes an efflux of K^+ ions through the membranes of sensitive cells (Upreti & Hinsdill, 1975). Leucocin S on the other hand has been reported to elicit a bacteriostatic mode of action, and dissipates the proton motive force of *Lactobacillus sake* (Klaenhammer, 1993). Lactacin 27, a class IV complex bacteriocin is a heat-stable glycoprotein produced by *Lb. helveticus*. It is 12.4 kDa and contains unusually high concentrations of glycine (15.1%) and alanine (18.1%) residues. Leucocin S, in contrast to other *Leuconostoc* bacteriocins, is not heat stable and loses 50% of its activity after heating at 60°C for 30 min (Lewus *et al.*, 1992).

Bacteriocin mode of activity

Lactic acid bacteria mostly produce bacteriocins that display a bactericidal effect on the sensitive cells (Davey, 1981; Zajdel *et al.*, 1985; Bhunia *et al.*, 1991). Bacteriocins such as leucocin A (Hastings & Stiles, 1991), lactocin 27 (Upreti & Hinsdill, 1975) and leucocin S (Lewus *et al.*, 1992) have been reported to act bacteriostatically.

A common mode of activity of LAB bacteriocins is by pore formation in the cytoplasmic membrane, and it has been suggested that this occurs through the 'barrel-stave' mechanism (Ennahar *et al.*, 1999). This model suggests that the peptide initially accumulates at the surface of the membrane, through ionic interactions with the phospholipid groups. The presence of these peptides results in the significant thinning of the membrane in these regions, caused by the localised displacement of the phospholipids. On application of a membrane potential, the molecules adopt a transmembrane orientation, thus forming a membrane pore (McAuliffe *et al.*, 2001). Some bacteriocins like lactacin F and lactococcin A, B and G require a specific receptor molecule for adsorption, whereas others like nisin exert a receptor-independent action. Bacteriocins with a narrow host-range usually require a specific receptor molecule, while those exhibiting a wide host-range do not (Sablon *et al.*, 2000).

Nisin elicits antimicrobial activity by forming an ion-permeable channel in the cytoplasmic membrane of target cells. This results in an increase in membrane permeability, resulting in a disturbance of the membrane potential and

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causing an efflux of ATP, essential ions and amino acids. Biosynthesis of macromolecules and energy production is thus inhibited, ultimately resulting in cell death. Nisin does not require a membrane receptor for activity (Sahl, 1991; Sablon *et al*., 2000) and acts in a voltage-dependent manner on energised cells, liposomes and membrane vesicles and its effect is enhanced by a change in pH (Gao *et al*., 1991). This characteristic is beneficial for fermentative bacteria that produce nisin and rapidly acidify the growth medium (Klaenhammer, 1993).

Characteristics of lactobacilli bacteriocins

The lactobacilli are a diverse group of homofermentative and heterofermentative species that are the most often cited for the production of bacteriocins (Klaenhammer *et al*., 1992). Bacteriocins produced by *Lactobacillus* are generally active against closely related species, which occupy similar ecological niches. For example, lacticins A and B produced by *Lactobacillus delbreuckii* target other related subspecies associated with fermented dairy products (Toba *et al*., 1991), while plantaricin A is bactericidal to numerous LAB normally associated with fermenting vegetables (Daeschel *et al*., 1990; Klaenhammer, 1993).

Lactobacillus bacteriocins are found within each of the four major classes of antimicrobial proteins produced by LAB and the lactobacilli produce many different bacteriocins of similar activity (Alpay Karaoglu *et al*., 2003) (Table 2). Among the lactobacilli, there has been great interest in *Lactobacillus plantarum*, due to the potential application of the microorganism as a starter bacterium for a variety of fermented foods (MacKay & Baldwin, 1990). The bacteriocins produced from *Lb. plantarum* have been found to be inhibitory towards closely related LAB, particularly the mesophilic and thermophilic lactobacilli (Suma *et al*., 1998).

J. CONCLUSION

Since Kefir has a pH of 4.2 – 4.6 (Odet, 1995) after fermentation and maturation, it may be that the inhibitory activity of the beverage and grains is due to the production of acids by LAB. However, a number of investigators have reported that acid is not the only contributor to the antimicrobial activity of Kefir and Kefir grains. Gulmez and Guven (2003) showed that when two Kefir samples, with no significant difference in acidity and pH were tested for antimicrobial activity, the

population of pathogenic microorganisms in the two samples showed varying degrees of inhibition. This suggests the potential effect of inhibitory substances other than organic acids and pH on the pathogenic microorganisms. Morgan *et al.* (2000) also showed that the inhibitory activity of Kefir may be ascribed to something other than acid inhibition

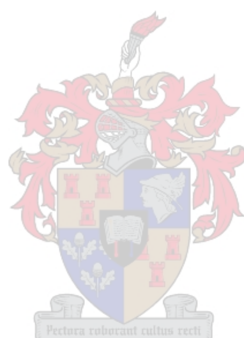

Table 2 *Lactobacillus plantarum* bacteriocins and their characteristics

Bacteriocin	Producer	Active spectrum	Characteristics	Reference
Plantaricin A (α and β)	*Lb. plantarum* C-11	LAB *Pediococcus* *Enterococcus faecalis*	α – 2.7 kDa β – 2.8 kDa; stable at 100°C for 30 min; active over pH 4-6.5; Class IIb	Daeschel *et al.*, 1990; Nissen-Meyer *et al.*, 1992
Plantaricin S	*Lb. plantarum* LPCO-10	LAB *Propionibacterium* *Clostridium tyrobutyricum* *Enterococcus* *Pediococcus* *Micrococcus*	2.5 kDa; sensitive to proteases, α-amylases and lipase; heat stable at 100°C for 60 min; active from pH 3-7; Class IV	Jimenez-Diaz *et al.*, 1990; 1993; 1995
Plantaricin T	*Lb. plantarum* LPCO10	LAB *Propionibacterium* *Cl. tyrobutyricum* *Micrococcus*	2 kDa; proteinaceous	Jimenez-Diaz *et al.*, 1990; 1993
Plantaricin C	*Lb. plantarum* LL441	LAB *Propionibacterium* *Streptococcus thermophilus* *E. faecalis* *Bacillus subtilis*	3.5 kDa	Gonzalez *et al.*, 1994
Pediocin AcH (Plantaricin AcH)	*Lb. plantarum* WHE 92	LAB *E. faecalis* *B. subtilis* *Micrococcus sedentarius* *Staphylococcus xylosus* *Listeria monocytogenes* *Listeria innocua* *Listeria seeligeri*	4.6 kDa ; Class IIa	Ennahar *et al.*, 2000
Plantaricin LC74	*Lb. plantarum* LC74	LAB *Bacillus stearothermophilus*	5 kDa	Rekhif *et al.*, 1994

Table 2 Cont.

Bacteriocin	Producer	Activity spectrum	Characteristics	Reference
		Enterococcus hirae		
Plantaricin B	*Lb. plantarum* NDCO 1193	LAB *Pediococcus damnosus*	Proteinaceous	West & Warner, 1988
Plantaricin EF and JK	*Lb. plantarum* C11	LAB *Pediococcus*	Class IIb	Anderssen *et al*., 1998
Plantaricin KW30	*Lb. plantarum* KW30	*Lactobacillus brevis* *Lb. plantarum* *Lactobacillus delbrueckii* subsp. *lactis*		Kelly *et al*., 1996
Plantaricin BN	*Lb. plantarum* BN	LAB *Listeria* sp. *Clostridium* sp.	>10 kDa	Lewus *et al*., 1991; 1992 Okereke & Montville, 1991
Plantaricin C19	*Lb. plantarum* C19	LAB *E. faecalis* *L. monocytogenes* *L. innocua* *Listeria grayi* *Staphylococcus*	3.5 kDa , Class IIa	Atrih *et al*., 1993
Plantaricin 1.25	*Lb. plantarum* TMW 1.25	LAB *B. subtilis* *Staphylococcus aureus*	6 kDa	Ehrmann *et al*., 2000
Plantaricin UG1	*Lb. plantarum* UG1	LAB *Bacillus cereus* *L. monocytogenes* *L. seeligery* *Clostridium sporogenes* *Clostridum perfringens*	3 – 10 kDa; Class IIa	Enan *et al*., 1996
Plantaricin F	*Lb. plantarum* BF001	LAB *L. monocytogenes* *S. aureus* *Pseudomonas*	0.5 – 3.5 kDa; Class IIa	Paynter *et al*., 1997

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Table 2 Cont.

Bacteriocin	**Producer**	**Activity spectrum**	**Characteristics**	**Reference**
		a*eruginosa* *Salmonella* spp.		
Plantaricin W (α and β)	*Lb.* p*lantarum*	Wide range Gram-positive organisms	Class AII lantibiotic	Holo *et al*., 2001
Plantaricin LP84	*Lb. plantarum* NCIM 2084	*Lb. amylovorus* DSM 20531 *B. cereus* F 4810 *Bacillus licheniformis* CFR 1621 *B. subtilus* CFR 1604 *P. aeruginosa* CFR 1704 *Escherichia coli* D 21	1-5 kDa; heat stable at 121°C for 20 min; stable to catalase; sensitive to trypsin and chymotrypsin	Suma *et al*., 1998
Plantaricin 35d	*Lb. plantarum* 35d	LAB *S. aureus* *L. monocytogenes* *Aeromonas hydrophila* *E. faecalis* *Enterococcus faecium* *B. subtilus* *Bacillus pumilus*	4.5 kDa; heat-stable at 80°C for 20 min; and storage at 4°C for 6 months; Class IIa	Messi *et al*., 2001
Plantaricin 423	*Lb. plantarum* 423	*Listeria* spp. *Staphylococcus* spp. *Pediococcus* spp. *Lactobacillus* spp.	Heat stable for up to 30 min at 100˚C and 60 min at 80˚C; Class IIa	Verellen *et al*., 1998; Van Reenen *et al.,* 2003

alone, and reported that neutralised Kefir fermentates were still capable of causing antimicrobial inhibition. Balasubramanyam and Varadaraj (1994) on the other hand reported that sterilised filtrates of LAB isolates lost all inhibitory activity after they were treated with the proteolytic enzyme trypsin. Trypsin is an enzyme capable of denaturing proteins, thereby indicating the proteinaceous nature of the antimicrobial populations, and suggesting that the inhibition could be attributed to bacteriocin activity.

Kefir is a complex microbial system that has been found to not only be nutritionally beneficial, but has also proven to inhibit a number of food-borne pathogens and spoilage microorganisms. Due to the high presence of LAB in the Kefir grains and Kefir beverage this inhibition could be due, in part, to the production of bacteriocins.

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CHAPTER 3

CHARACTERIZATION OF BACTERIOCIN ST8KF PRODUCED BY A KEFIR ISOLATE *LACTOBACILLUS PLANTARUM* ST8KF

Abstract

Lactobacillus plantarum ST8KF, isolated from Kefir, produced a 3.5 kDa bacteriocin (bacST8KF) active against *Lactobacillus casei*, *Lactobacillus salivarius*, *Lactobacillus curvatus* and *Listeria innocua*. BacST8KF was sensitive to proteolytic enzymes but stable between pH 2.0 and 10.0, and heat resistant (20 min at 121°C). BacST8KF did not adsorb to the surface of the producer cell. Maximum activity (25,600 $AU.ml^{-1}$) was recorded in modified MRS broth with glucose, in modified MRS broth with glucose replaced by sucrose, and in modified MRS broth with glucose, supplemented with KH_2PO_4 after 24 h at 30°C. Tri-ammonium citrate and glycerol in excess of 5.0 $g.l^{-1}$ repressed bacST8KF production. Production of bacST8KF increased from 800 $AU.ml^{-1}$ after 3 h of fermentation in complete MRS broth at 30°C to 12 800 $AU.ml^{-1}$ after 9 h and to 51 200 $AU.ml^{-1}$ after 27 h. These results suggest that bacST8KF may be a secondary metabolite and shows that its mode of activity is bacteriostatic.

Introduction

Kefir is a refreshing, naturally carbonated fermented milk beverage with a slightly acidic taste (Tamine & Marshall, 1997; Gulmez & Guven, 2003), yeasty flavour and creamy consistency (Wickerham, 1951; Duitschaever, 1989). When agitated, the beverage foams and fizzes (Obermann, 1985; Duitschaever, 1989), a characteristic that led to Kefir being named “the champagne of cultured dairy products” (Merin & Rosenthal, 1986).

Fermentation is initiated by the addition of Kefir grains to fresh milk (Garrote, Abraham & DeAntoni, 2000). The grains are insoluble in water and irregular in shape and size, varying from 0.3 – 3.5 cm in diameter (Garrote, Abraham & DeAntoni, 1997, Kosikowski & Mistry, 1997). When suspended in

milk, the grains swell to form a gelatinous-like product, kefiran (Kosikowski & Mistry, 1997), which contains lactic acid bacteria (LAB), yeast and acetic acid bacteria (Saloff-Coste, 1996). The majority of bacteria (as much as 80%) belong to the genus *Lactobacillus* (Witthuhn, Schoeman & Britz, 2004). The microbial composition of Kefir is determined by the source of the grains (Ottogalli, Galli, Resmini & Volonterio, 1973; Witthuhn *et al.*, 2004), the fermentation process (Molska, Moniuszco, Komorows & Merilanen, 1983) and storage conditions (Zourari & Anfantakis, 1988). Filamentous fungi have been described, but are only present in low numbers (Saloff-Coste, 1996; Garrote *et al.*, 1997).

Many health benefits have been attributed to Kefir, including its antimicrobial activity against a range of Gram-positive and Gram-negative bacteria, and fungi (Saloff-Coste, 1996; Garrote *et al.*, 2000). In *in vitro* tests with cell-free extracts of Kefir, the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium tyrobutyricum* and *Listeria monocytogenes* was inhibited (Van Wyk, 2001). In general, the antimicrobial activity of Kefir is ascribed to lactic acid, volatile acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and/or bacteriocins produced by LAB (Havenaar, Brink & Huis in't Veld, 1992; Sanders, 1993; Helander, Von Wright & Mattila-Sandholm, 1997).

In an *in vitro* study conducted by Rodrigues, Caputo, Carvalho, Evangelista & Schneedorf (2005), Kefir inhibited the growth of *Streptococcus pyogenes* and *Candida albicans*. In another study, strains of *Lactococcus cremoris*, *Lactococcus lactis*, *Streptococcus thermophilus* and *Streptococcus durans*, isolated from Kefir inhibited the growth of *S. aureus* (Yüksekdag, Beyatli & Aslim, 2004). In the same study, two strains of *Lac. lactis* and a strain of *Lac. cremoris* inhibited the growth of *E. coli* and *P. aeruginosa* (Yüksekdag *et al.,* 2004). These authors also described a strain of *Str. thermophilus* active against *P. aeruginosa*. Atanassova, Dousset, Montcheva, Ivanova & Haertle (1999) described a bacteriocin produced by a strain classified as *Lactobacillus* spp. with activity against *L. innocua* F. A number of *Lactobacillus* spp. isolated from Kefir displayed antimicrobial activity against enteropathogenic bacteria and affected the adhesion of *Salmonella typhimurium* to Caco-2 cells (Santos, San Mauro, Sanchez, Torres & Marquina, 2003).

The objective of this study was to characterize bacteriocin ST8KF, produced by *Lb. plantarum* strain ST8KF isolated from Kefir, with the aim of using the strain as a co-starter culture in Kefir fermentations.

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Materials and methods

Isolation of lactic acid bacteria and screening for bacteriocin activity

A combination of Kefir (50 ml) and Kefir grains (5 g), obtained from the Department of Food Science, Stellenbosch University, was macerated in a stomacher (BagMixer, Interscience, Weymouth, USA) for 10 min at 25℃. Serial dilutions of the sample were made with sterile saline (0.85%, w/v NaCl), plated onto complete MRS agar (Biolab, Biolab Diagnostics, Midrand, SA) and incubated at 30℃ for 24 h.

Screening for bacteriocin-producing isolates was carried out according to the triple-agar-layer method described by Todorov & Dicks (2005b). The second layer of agar (1.7%, w/v) was supplemented with 50.0 $mg.l^{-1}$ Natamycin (Delvocid, Gist-brocades, B.V., Delft, The Netherlands) to prevent fungal growth. All plates were incubated at 30°C for 24 h. Colonies were then overlaid with a third layer of 1% (w/v) Brain Heart Infusion (BHI) agar (Biolab), seeded with 10^6 $cfu.ml^{-1}$ Lactobacillus casei LHS, Escherichia coli ATCC 11775 and Staphylococcus aureus ATCC 12600, respectively. The plates were incubated at 37°C for 24 h. Colonies with the largest zones of growth inhibition were isolated, inoculated into complete MRS broth (Biolab) and incubated for 24 h at 30°C. Pure cultures were obtained by streaking onto complete MRS agar (Biolab).

Antimicrobial activity was confirmed by using the agar-spot-test method (Van Reenen, Dicks & Chikindas, 1998). Activity was expressed as arbitrary units (AU) per ml, with one AU defined as the highest dilution showing a clear zone of inhibition (Van Reenen et al., 1998; Todorov & Dicks, 2005b). Lactobacillus casei LHS was used as a sensitive test strain.

Identification of strain ST8KF

The morphology of strain ST8KF was determined by using a scanning electron microscope (Leo® 1430VP, Carl Zeiss Jena GmbH, Jena, Germany). Cells cultured for 24 h in complete MRS broth (Biolab) were harvested (8 000 x *g*, 10 min, 4℃), washed with sterile distilled water, and resuspended in 1 ml sterile

distilled water. Prior to imaging, samples were sputter-coated with either gold or carbon, depending on the application. Samples were identified with backscattered electron (BSE) and/or Secondary electron images, and phase compositions quantified by energy dispersive spectrometer (EDS) analysis using an Oxford Instruments®, location 133KeV detector and Oxford INCA software (Oxford Instruments, Witney, Oxon, UK). Beam conditions during the quantitative analyses were 20 KV and approximately 1.5 nA, with a working distance of 13 mm and a specimen beam current of -3.92 nA. Despite the relatively low energy of the beam, X-ray counts with the set-up used were typically ~ 5000 counts per second (cps). The counting time was 50 seconds live-time. Natural mineral standards of Co, Ti and Fe were used for standardization and verification of the analyses. Pure Co, as well as Ti and Fe in ilmenite were used periodically to correct for detector drift. Beam conditions during semi-quantitative analyses, when used in case of unpolished samples, were as described above without controlling the specimen beam current and the results were normalised to 100 % weight.

Further identification was by gram-reaction, and physiological and biochemical tests, as described by Schillinger & Lücke (1987), Stiles & Holzapfel (1997) and Collins, Phillips, & Zanoni (1989). Carbohydrate fermentation reactions were recorded by using the API 50 CHL test kit (BioMérieux® S.A., Marcy l`Etiole, France).

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Identification to species level was by PCR with primers specific for *Lactobacillus plantarum* (PlanF and REV), *Lactobacillus pentosus* (PentF and REV), and *Lactobacillus paraplantarum* (ParaF and REV), as described by Torriani, Felis & Dellaglio (2001). Confirmation of identification was obtained by amplifying the genomic DNA with primers F8 and R1512, as described by Felske, Rheims, Wolterink, Stackebrandt & Akkermans (1997). The amplified fragments were cleaned using SigmaSpin Post-Reaction Clean-Up Columns (Sigma, St Louis, MO, USA), sequenced, and compared to sequences in GenBank using BLAST, Basic Local Alignment Search Tool (Altschul, Madden, Schaffer, Zhang, Miller & Lipman, 1997).

Isolation of bacteriocin ST8KF

Strain ST8KF was cultured in complete MRS broth (Biolab) for 24 h at 30°C. The cells were harvested (8000 x *g*, 10 min, 4°C), the cell-free supernatant

was adjusted to pH 5.0 with 1 M NaOH, heat-treated (80°C for 10 min) and the bacteriocin (bacST8KF) precipitated with 80% saturated ammonium sulphate (Sambrook, Fritsch & Maniatis, 1989). The precipitate was resuspended in 20 ml of 25 mM ammonium-acetate (pH 6.5) and the amount of antimicrobial activity determined by testing against *Lb. casei* LHS, as described above.

The molecular size of bacST8KF was determined by tricine-SDS PAGE (Schägger & Von Jagow, 1987). A low molecular weight marker with sizes ranging from 2.5 to 45.0 kDa (Amersham Bioscience Europe GmbH, Freiburg, Germany) was used. One half of the gel was covered with *Lb. casei* LHS (10^6 $cfu.ml^{-1}$) imbedded in 1% (w/v) BHI agar (Biolab) and incubated at 37°C for 24 h. The other half was stained with Coomassie Brilliant Blue R (ICN Biomedicals Inc., 1263 South Chillicothe Rd, Aurora, Ohio 44202, USA).

Effect of enzymes, pH, detergents and temperature on bacST8KF

One ml of a cell-free supernatant, prepared as described before, was added to 1 $mg.ml^{-1}$ α-amylase (Sigma Diagnostics, St. Louis, MO, USA), 1 $mg.ml^{-1}$ Proteinase K (Roche, Indianopolis, IN, USA) and 1 $mg.ml^{-1}$ pronase (Roche), respectively. Samples were incubated at 30°C for 30 min and then heated at 95 – 97°C for 5 min. In a separate experiment the pH of 10 ml of cell-free supernatants was adjusted to 2.0, 4.0, 6.0, 8.0 or 10.0 with 1 M HCl or 1 M NaOH and incubated at 30°C for 1 h. Another batch of cell-free supernatants received 10 $mg.ml^{-1}$ of Triton X100 (BDH, BDH Chemicals Ltd, Poole, England), Triton X114 (Sigma), Tween 20 (Merck, Darmstadt, Germany), Tween 80 (Merck), SDS (Sigma), urea (Merck) or EDTA (Merck), respectively, and incubated for 30 min at 30°C. The effect of temperature on bacST8KF was determined by incubating cell-free supernatants at 30, 45, 60 and 100°C for 30 min and 2 h, respectively, and at 121°C for 20 min. The pH of all samples was adjusted to 5.0 and bacST8KF activity determined with *Lb. casei* LHS as sensitive strain, as described above.

Production of bacST8KF

Two ml of a 24 h culture was inoculated into 100 ml complete MRS broth (Biolab) and incubated at 30°C. Changes in optical density (600 nm) and pH were

determined hourly for 30 h. BacST8KF activity was determined every three hours, as described above.

Mode of bacST8KF activity

Complete MRS broth (Biolab) was inoculated with 1% (v/v) *Lb. casei* LHS and incubated for 3 h at 37°C. Twenty ml filter-sterilized cell-free supernatant was added to the culture and changes in optical density (at 600 nm) recorded every hour for 12 h.

Adsorption of bacST8KF to producer cells

Adsorption of bacST8KF to producer cells was studied by the method of Yang, Johnson & Ray (1992). An 18-h-old culture was adjusted to pH 5.0 with 1M NaOH, 10 ml of the cells harvested (8000 x *g*, 15 min, 4°C) and washed with an equal volume of sterile 0.1 M phosphate buffer (pH 6.5). The pellet was re-suspended in 10 ml 100 mM NaCl, pre-adjusted to pH 2.0 with 1 M HCl, and stirred for 1 h at 4°C. Cells were harvested (3000 x *g*, 30 min, 4°C) and the cell-free supernatant adjusted to pH 7.0 with sterile 1 M NaOH. BacST8KF activity was tested as described above.

Effect of medium components of bacST8KF production

Strain ST8KF (100 µl of a 24-h-old culture) was inoculated into 10 ml modified MRS broth (De Man, Rogosa & Sharpe, 1960), modified as indicated in Table 3. In the first set of experiments, glucose was replaced with either 20.0 $g.l^{-1}$ fructose, 20.0 $g.l^{-1}$ lactose, 20.0 $g.l^{-1}$ mannose, 20.0 $g.l^{-1}$ maltose, or 20.0 $g.l^{-1}$ saccharose. In the next set of experiments, the meat extract and yeast extract in modified MRS broth (De Man, Rogosa & Sharpe, 1960) were replaced with either 20.0 $g.l^{-1}$ tryptone (Oxoid), 20.0 $g.l^{-1}$ meat extract (Biolab), 20.0 $g.l^{-1}$ yeast extract (Biolab), 12.5 $g.l^{-1}$ tryptone (Oxoid) plus 7.5 $g.l^{-1}$ meat extract (Biolab), 12.5 $g.l^{-1}$ tryptone (Oxoid) plus 7.5 $g.l^{-1}$ yeast extract (Biolab), or 10 $g.l^{-1}$ meat extract (Biolab) plus 10 $g.l^{-1}$ yeast extract (Biolab). In a third set of experiments, strain ST8KF (100 µl of a 24-h-old culture) was also inoculated into 10 ml modified MRS broth (De Man *et al.*, 1960), modified by excluding magnesium sulphate, manganese sulphate and tri-ammonium citrate, respectively. In a fourth set of

experiments, modified MRS broth (De Man *et al.*, 1960) was supplemented with the following: glycerol at 1.0, 2.0, 5.0 or 10.0 $g.l^{-1}$, respectively; KH_2PO_4 at 5.0, 10.0 and 20.0 $g.l^{-1}$, respectively; K_2HPO_4 at 5.0, 10.0 and 20.0 $g.l^{-1}$, respectively; cyanocobalamin (vitamin B_{12}) (2.0 $mg.l^{-1}$); thiamine (vitamin B_1) (2.0 $mg.l^{-1}$); L–ascorbic acid (vitamin C) (2.0 $mg.l^{-1}$); DL–6,8–thioctic acid (2.0 $mg.l^{-1}$); and tri-ammonium citrate (5.0 and 10.0 $g.l^{-1}$, respectively). Complete MRS broth (Biolab) was adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, respectively; with 1 M NaOH or 1 M HCl . All cultures were incubated at 30°C for 24 h. BacST8KF activity was tested against *Lb.casei* LHS as before.

Results and discussion

Spectrum of antimicrobial activity

Thirty-five of the 48 isolates from Kefir and Kefir grains inhibited the growth of Lb. casei LHS. From these, the isolate with the strongest antimicrobial activity (isolate ST8KF) was screened against a panel of sensitive strains (Table 1). Cell-free supernatant, adjusted to pH 6.0, inhibited the growth of Enterococcus mundtii, Lactobacillus curvatus, Lactobacillus salivarius and Listeria innocua, but none of the other strains included in the test panel (Table 1). This narrow-spectrum of activity is unique for a bacteriocin produced by Lb. plantarum. Most of the bacteriocins described for Lb. plantarum are active against a much broader range of genera and species (De Vuyst & Vandamme, 1994).

Identification of isolate ST8KF

Isolate ST8KF is rod-shaped and based on sugar fermentation reactions (not shown), 99.9% related to *Lb. plantarum*. Amplification of genomic DNA with species-specific primers produced a 350 bp fragment, which corresponded in size to that of *Lb. plantarum* ATCC 14917^T (Fig. 1). The same DNA amplified with primers specific for *Lb. pentosus* and *Lb. paraplantarum*, and DNA from *Lb. paraplantarum* ATCC 700211^T and *Lb. pentosus* ATCC 8041^T amplified with primers specific for *Lb. plantarum* yielded no fragments (Fig. 1). The 16S rDNA amplified from isolate ST8KF revealed 99% homology to the 16S rDNA sequence

Table 1 Spectrum of antimicrobial activity of bacST8KF

Target strain	Growth temperature (in ^{o}C)	BacST8KF activity
Enterococcus faecalis 21, BFE 1071, FA2	37	-
Enterococcus faecalis FAIRE 77, FAIRE 88, FAIRE 90, FAIRE 92	37	-
Enterococcus mundtii ST4SA	30	+
Escherichia coli 40, RPEC 1	37	-
Klebsiella pneumoniae 30, 31, 39	37	-
Lactobacillus casei LHS	30	+
Lactobacillus curvatus DF38	30	+
Lactobacillus paracasei subsp. *Paracasei* ST11BR	30	-
Lactobacillus plantarum 423, AMA-K, ST8SH	30	-
Lactobacillus sakei DSM20017	30	-
Lactobacillus salivarius 241	30	+
Lactococcus lactis susbp. *lactis* HV219	30	-
Listeria innocua F, LMG 13568	37	+
Staphylococcus aureus 36, RPSA1	37	-
Streptococcus agalactiae RPSAG 39, RPSAG 48	37	-
Streptococcus sp. TL1	30	-
Streptococcus caprinus ATCC 700065, ATCC 700066	30	-
Streptococcus pneumoniae 29	37	-
Pseudomonas sp. 25	37	-

DSM = Deutsche Sammlung von Cellkulturen und Mikroorganismen, ATCC = American Type Culture Collection.

Lactic acid bacteria were cultured in complete MRS (Biolab) medium and all other bacteria in BHI (Biolab) medium.

+ = inhibition zone of at least 5mm in diameter, - = no inhibition zone recorded.

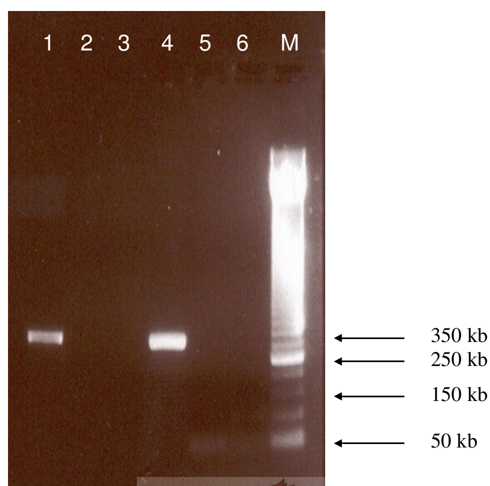

Figure 1 Agarose gel (1%, w/v) showing DNA fragments obtained after PCR amplification with primers specific for *Lactobacillus plantarum*. (1) Strain ST8KF, (2) *Lactobacillus pentosus* ATCC 8041^T, (3) *Lactobacillus paraplantarum* ATCC 700211^T, (4) *Lb. plantarum* ATCC 14917^T, (5) and (6) no DNA, (M) 50 kb molecular weight marker from Amersham (Bioscience, UK Limited, Budung-hamshire, UK).

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of *Lb. plantarum* (GenBank accession number AY383631.1). Isolate ST8KF is thus regarded a strain of *Lb. plantarum*.

Isolation of bacteriocin ST8KF

According to tricine-SDS PAGE, bacST8KF is approximately 3.5 kDa in size (Fig. 2). This is within the size range of most bacteriocins reported for the genus *Lactobacillus* (De Vuyst & Vandamme, 1994) and bacteriocins ST26MS and ST28MS described for *Lb. plantarum* (Todorov & Dicks, 2005b). BacST8KF is, however, much smaller than bacteriocin ST13BR (10.0 kDa) and bacteriocin ST194BZ (14.0 kDa) produced by *Lb. plantarum* ST13BR and ST194BZ, respectively (Todorov, Van Reenen & Dicks, 2004; Todorov & Dicks, 2005a).

Effect of enzymes, pH, detergents and temperature on bacST8KF

The activity of bacST8KF was destroyed after treatment with Proteinase K and pronase, but not when treated with α-amylase (Table 2). This suggested that the activity of bacST8KF is not dependent on glycosylation. Similar results have been reported for other bacteriocins of *Lb. plantarum* (DeVuyst & Vandamme, 1994; Kelly, Asmundson & Huang, 1996; Todorov *et al.*, 2004b). Leuconocin S, produced by *Leuconostoc paramesenteroides* (Lewus, Sun & Montville, 1992) and carnocin 54, produced by *Leuconostoc carnosum* (Keppler, Geisen & Holzapfel, 1994) are examples of amylase-sensitive bacteriocins.

BacST8KF remained stable after incubation (30°C) at pH 2.0, 4.0, 6.0, 8.0 and 10.0 (Table 2). No bacST8KF activity was recorded after treatment with Triton X100 and Triton X114, respectively (Table 2). However, treatment with Tween 20, Tween 80, SDS, urea and EDTA had no effect on the activity of bacST8KF (Table 2). Similar results were reported for bacteriocin J46 produced by *Lactococcus lactis* subsp. *cremoris* (Hout, Maghrous & Barena-Gonzalez, 1996). However, plantaricin C19 produced by *Lb. plantarum* C19 lost its activity after treatment with SDS or Triton X-100 (Atrih, Rekhif, Moir, Lebrihi & Lefebvre, 2001). Treatment of enterocin EJ97 produced by *Enterococcus faecalis* EJ97 (Gálvez, Valdivia & Abriouel, 1998), bozacin B14 produced by *Lacotcoccus lactis* subsp. *lactis* B14 (Ivanova, Kabadjova, Pantev, Danova & Dousset, 2000) and pediocin ST18

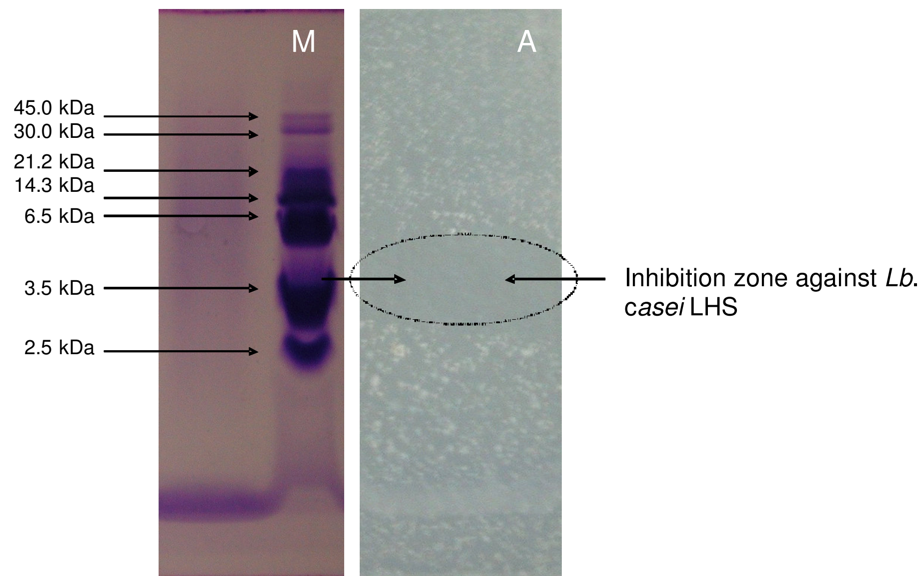

Figure 2 Separation of bacST8KF by SDS-PAGE. Inhibition of *Lactobacillus casei* LHS is indicated by the arrow. M = low molecular-weight rainbow marker (Amersham Bioscience Europe GmbH).

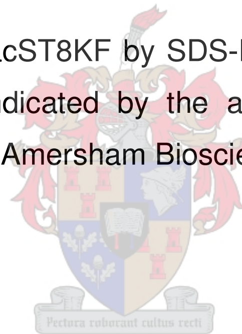

Table 2 Effect of enzymes, pH, detergents and temperature on bacST8KF

Component		Bacteriocin activity
Enzyme	α–amylase	+
	Proteinase K	-
	Pronase	-
pH	2.0 – 10.0	+
Temperature	30 ℃	+
	45 ℃	+
	60 ℃	+
	100 ℃	+
	121 ℃	+
Detergents	Triton X100, Triton X114	-
	Tween 20, Tween 80	+
	SDS, Urea	+
Protease inhibitor	EDTA	+

+ = inhibition zone of at least 5mm in diameter, - = no inhibition zone record

produced by *Pediococcus pentosaceus* ST18 (Todorov & Dicks, 2005c) with SDS did not result in any activity loss.

BacST8KF (pH 5.5) remained active after 20 min at 121°C. Similar results were recorded for a number of bacteriocins produced by *Lactobacillus* and *Lactococcus* spp. (Klaenhammer, 1988; Van Reenen *et al.*, 1998; Ko & Ahn, 2000; Todorov & Dicks, 2004b; Todorov & Dicks, 2005a, b, c). Moreover, lactocin NK24, produced by *Lc. lactis* NK24, lost 87.5% of its activity after 30 min at 100°C and was completely inactivated after 15 min at 121°C (Lee and Paik, 2001). In the case of lactocin MMFII produced by *Lc. lactis* MMFII, only 8.3% activity was recorded after 30 min at 110°C and 25% after 30 min at 80°C and 90°C (Ferchichi, Frere, Mabrouk & Manai, 2001). Nisin, produced by *Lc. lactis* subsp. *lactis* WNC20, was inactivated after 15 min at 121°C when incubated at pH 7.0, but not when incubated at pH 3.0 (Noonpakdee, Santivarangkna, Jumriangrit, Sonomoto & Panyim, 2003). Bozacin B14, produced by *Lc. lactis* subsp. *lactis* B14, was inactivated after 10 min at 90°C (Ivanova *et al.*, 2000).

Production of bacST8KF

The cell density of *Lb. plantarum* ST8KF increased from 0.3 to 10.0 (OD_{600}) during 30 h of growth at 30°C (Fig. 3). The pH decreased from 6.10 to 3.60 over the same period (Fig. 3). Production of bacST8KF increased from 800 $AU.ml^{-1}$ after 3 h of growth to 12 800 $AUml^{-1}$ during the following 6 h (Fig. 3). Production remained at 12 800 $AU.ml^{-1}$ for at least 12 h and increased to 51 200 $AU.ml^{-1}$ in

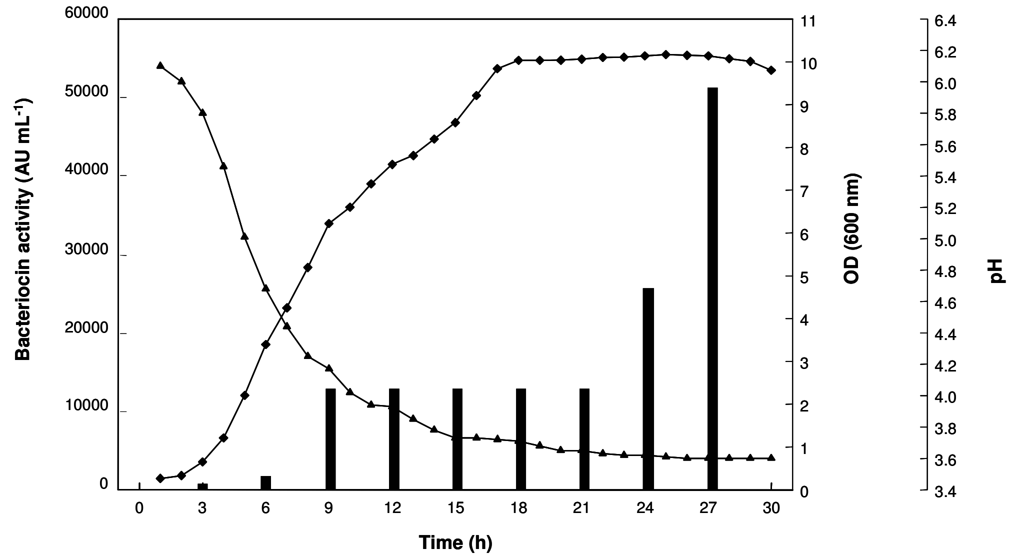

Figure 3 Growth of *Lactobacillus plantarum* ST8KF and bacST8KF production in complete MRS broth (Biolab). Symbols: ( ) = growth, (▲) = change in pH, ( ) = bacST8KF production. Incubation was at 30°C.

the next 6 h (Fig. 3). Production was still at 51 200 AU.ml^{-1} after 27 h. Optimal production of bacST8KF was recorded during stationary growth, which may suggest that the peptide is a secondary metabolite. Similar results were reported for plantaricin ST31 (Todorov *et al.*, 1999), bacteriocin ST26MS and bacteriocin ST28MS (Todorov & Dicks, 2005b). This is contrary to other bacteriocins thus far described for *Lb. plantarum* (Todorov *et al.*, 2004; Todorov & Dicks, 2004b; Van Reenen *et al.*, 1998). The pH of the culture decreased from 6.2 to approximately 4.1 during the first 9 h of fermentation (Fig. 3). The pH decreased to approximately 3.7 during the following 12 h and to 3.60 during the period of maximum production of bacST8KF (Fig. 3). These results show that bacST8KF is stable at pH 3.6, as determined with pH stability tests (Table 2). The sudden increase in activity from 12 800 AU.ml^{-1} to 51 200 AU.ml^{-1} occurred at pH 3.7 and 3.6 (Fig. 3) and cannot be ascribed to a change in culture pH. It is unlikely that such a small change in pH could trigger a sudden release of bacST8KF from the surface of the producer cell, as reported by Yang, Johnson & Ray (1992) and Van Reenen et al. (1998). The increase in activity could be due to the metabolism of remaining nutrients or medium component(s) not required for cell growth.

Effect of medium components on bacST8KF production

BacST8KF was produced at 25 600 AU.ml^{-1} when strain ST8KF was grown in modified MRS broth supplemented with 20 g.l^{-1} glucose (Fig. 3, Table 3). The same level of activity was recorded when glucose was replaced by 20 g.l^{-1} saccharose (Table 3). Fructose as sole carbon source, on the other hand, yielded only 200 AU.ml^{-1}, suggesting that the glucose moiety of sucrose is favoured for production (Table 3). Low levels of activity were recorded when the cells were grown in the presence of the same concentration of mannose (200 AU.ml^{-1}) and lactose (6 400 AU.ml^{-1}). No activity was recorded when the cells were grown in the presence of 20 g.l^{-1} maltose, suggesting that it may have a regulatory function.

Our results show that bacST8KF production is stimulated by glucose and sucrose. Similar results have been reported for plantaricin UG1 (Enan, Essawy, Uyttendaele & Debevere, 1996), plantaricin KW30 (Kelly *et al.*, 1996) and plantaricin ST31 (Todorov, Gotcheva, Dousset, Onno & Ivanova, 2000).

Table 3 Effect of carbohydrates, nitrogen, potassium, glycerol, vitamins and tri-ammonium sulphate on bacST8KF production

Component	Concentration ($g.l^{-1}$)	BacST8KF activity ($AU.ml^{-1}$)
Glucose	20.0	25,600
Fructose	20.0	200
Lactose	20.0	6,400
Mannose	20.0	200
Maltose	20.0	0
Saccharose	20.0	25,600
Tryptone	20.0	3,200
Meat Extract	20.0	12,800
Yeast Extract	20.0	1,600
Tryptone and Meat Extract	12.5 and 7.5	6,400
Tryptone and Yeast Extract	12.5 and 7.5	1,600
Meat Extract and Yeast Extract	10 and 10	6,400
Glycerol	1.0	12,800
	2.0	12,800
	5.0	6,400
	10.0	3,200
	20.0	0
KH_2PO_4	5.0	6,400
	10.0	25,600
	20.0	1,600
K_2HPO_4	5.0	3,200
	10.0	800
	20.0	3,200
Cyanocobalamin (B12)	0.002	12,800
Thiamine (B1)	0.002	12,800
L-ascorbic acid (C)	0.002	3,200
DL-6,8-thioctic acid	0.002	6,400
Tri-ammonium citrate	0	25,600
	5.0	12,800
	10.0	6,400

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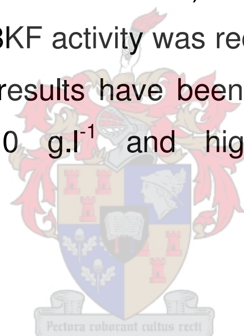
Of all nitrogen sources tested (Table 3), meat extract yielded the highest activity (12 800 AU.ml^{-1}). A combination of meat extract and tryptone (7.5:12.5 g.l^{-1}) , or meat extract and yeast extract (10.0:10.0 g.l^{-1}) yielded 6 400 AU.ml^{-1} (Table 3), suggesting that meat extract in excess of 10 g.l^{-1} is required for optimal bacteriocin production. BacST8KF production was not stimulated by yeast extract (Table 3). These results are contradictory to those reported for plantaricin 423 produced by *Lb. plantarum* 423. Highest production of plantaricin 423 was obtained in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract (Verellen, Bruggeman, Van Reenen, Dicks & Vandamme, 1998). Stimulation of bacteriocin production by meat extract has been reported for pediocin AcH (Bhunia, Kim, Johnson & Ray, 1998) and helveticin J (Joerger and Klaenhammer, 1986)

Modified MRS medium, supplemented with 1.0 g.l^{-1} and 2.0 g.l^{-1} glycerol yielded higher levels of bacST8KF (12 800 AU.ml^{-1}) compared to 5.0 g.l^{-1} and 10.0 g.l^{-1} glycerol (Table 3). No bacST8KF activity was recorded in the presence of 20.0 g.l^{-1} glycerol (Table 3). Similar results have been reported for plantaricin ST31, in which case glycerol at 2.0 g.l^{-1} and higher resulted in lower activity



(Todorov *et al.*, 2000). Glycerol is not used as a carbon source and the decrease in bacteriocin production may be due to changes in osmotic stress (Todorov & Dicks, 2005a). This merits further research.

Little is known about the influence of potassium ions on the production of bacteriocins (Todorov & Dicks, 2004a). Levels of 10.0 g.l^{-1} KH_2PO_4 yielded optimal levels of bacST8KF (25 600 AU.ml^{-1}), while the same level of K_2HPO_4 yielded only 800 AU.ml^{-1} (Table 3). The increase in activity cannot be due to pH changes caused by higher potassium levels, since all media were adjusted to pH 6.5 before inoculation. In the case of plantaricin UG1, 7.0 g.l^{-1} K_2HPO_4 resulted in increased activity (Enan *et al.*, 1996). The optimal level of K_2HPO_4 recorded for plantaricin ST31 was between 2.0 g.l^{-1} and 5 g.l^{-1} (Todorov *et al.*, 2000). On the other hand, no difference in antibacterial activity was recorded when *Lb. plantarum* ST194BZ was grown in the presence of 2.0 g.l^{-1} KH_2PO_4 and 2.0 g.l^{-1} K_2HPO_4.

BacST8KF production was stimulated by cyanobalamin and thiamine (12 800 AU.ml^{-1}), but not by L–ascorbic acid (3 200 AU.ml^{-1}) and DL–6,8–thioctic acid (6 400 AU.ml^{-1}) (Table 3). Similar results have been reported for bacteriocin ST13BR (Todorov *et al.*, 2004) and bacteriocin ST194BZ (Todorov & Dicks, 2005a).

BacST8KF production was lower in the presence of 5.0 g.l^{-1} and 10.0 g.l^{-1} tri-ammonium citrate (12 800 AU.ml^{-1} and 6 400 AU.mL^{l1}, repectively), compared to growth in its absence (25 600 AU.ml^{-1}) (Table 3). Bacteriocin production of 6 400 AU.ml^{-1} was observed in the absence of magnesium sulphate, while no bacteriocin production was observed in the absence of manganese sulphate (data not shown). Therefore, manganese sulphate is required for bacteriocin production, and magnesium sulphate has a stimulatory effect on bacST8KF.

BacST8KF was not produced in complete MRS broth with an initial pH of 4.5 while 3 200 AU.ml^{-1} were produced at a media pH of 5.0. Bacteriocin production of 25 600 AU.ml^{-1} was observed for all other pH media tested (pH 5.5, 6.0, 6.5). Similar results were reported for other bacteriocins produced by *Lb. plantarum* (Kelly *et al.*, 1996; Todorov *et al.*, 2000; Daeschel, McKeney & Mac Donald, 1990; Todorov & Dicks, 2005b). From these results and literature data (Kelly *et al.*, 1996; Todorov *et al.*, 2000; Daeschel *et al.*, 1990; Todorov & Dicks,

2005b) it can be concluded that an initial pH of 5.5 or greater is required for optimal growth of *Lb. plantarum* ST8KF and bacteriocin production.

Mode of activity

Lactobacillus casei LHS treated with bacST8KF (12 800 $AU.ml^{-1}$) increased from OD_{600} 0.3 to 1.0 over 9 h (Fig. 4). The control (not treated with bacST8KF) increased from OD_{600} 0.3 to 4.0 over the same period (Fig. 4). The slight increase in optical density of cells treated with bacST8KF suggests that the mode of activity is bacteriostatic. The cell density of treated cells remained more-or-less the same (OD 600 $_{nm}$ = 1.2) for the last 3 h of growth, which suggests that the cells do not recover from the treatment. Similar results were reported for bacteriocins ST11BR, ST151BR and ST34BR (Todorov & Dicks, 2004b). Bacteriocins ST28MS and ST26MS, on the other hand, repressed the growth of strain LHS for only 2 h (Todorov & Dicks, 2005b).

Adsorption of bacST8KF to producer cells

Low bacST8KF activity was detected after treatment of 18-h-old cells of strain ST8KF with 100 mM NaCl. The activity was, however lower than the activity recorded in the cell-free supernatant, suggesting that bacST8KF adsorbs to the surface of the producer cells in low concentrations. Similar results were reported for plantaricin C19, maximal adsorption to the producer cells was recorded between pH 5 and 7, with complete loss of adsorption at pH 1.5 and 2.0 (Atrih *et al*., 2001). In the case of plantaricin ST31 (Todorov *et al.*, 1999), pediocin ST18 (Todorov & Dicks, 2005c) and bozacin B14 (Ivanova *et al.*, 2000) no bacteriocin activity was recorded on the cell surface of the producer strains.

Conclusions

BacST8KF (3.5 kDa) has a narrow spectrum of activity, is heat resistant and stable between pH 2.0 and 10.0, adsorbs to the surface of the producer cell in low concentrations and is produced at 51 200 $AU.ml^{-1}$ after 27 h of fermentation in the presence of 2% (w/v) D-glucose. Low activity levels (6 400 $AU.ml^{-1}$) have been

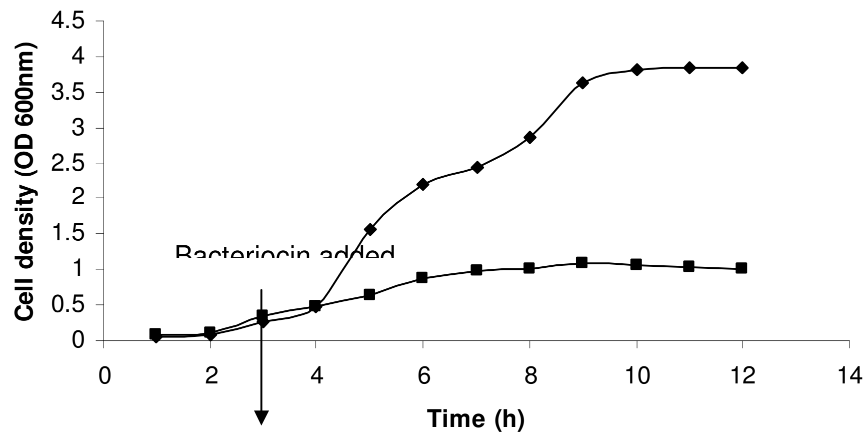


Figure 4 Effect of bacST8KF on the growth of *Lactobacillus casei* LHS over a period of 12 h (■). The control ( ) received no bacST8KF.

Pectora roborant cultus recti

recorded in the presence of lactose, and in the presence of citrate (tri-ammonium citrate), suggesting that the bacteriocin may not be produced at high levels during the initial phases of Kefir production. However, bacST8KF production may increase towards the end of the production process when the citrate has been consumed by the rest of the microflora. Strain ST8KF may be used in a mixed starter culture. Further research on the production of specific flavor compounds is in progress.

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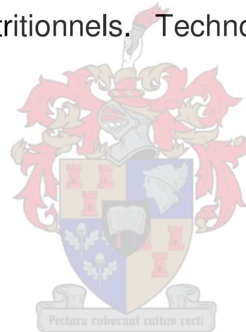


CHAPTER 4

PARAMETERS AFFECTING THE ADSORPTION OF BACTERIOCIN ST8KF PRODUCED BY *LACTOBACILLUS PLANTARUM* ST8KF ISOLATED FROM KEFIR

Abstract

Bacteriocin ST8KF (bacST8KF), produced by *Lactobacillus plantarum* ST8KF isolated from Kefir, inhibits the growth of *Enterococcus faecalis* E88, *Lactobacillus casei* LHS, *Lactobacillus curvatus* DF38, *Lactobacillus sakei* DSM 20017, *Lactobacillus salivarius* 241 and *Listeria innocua* LMG 13568. High levels of adsorption of bacST8KF (80%) to *Lb. casei* and *Lb. sakei* were recorded. Adsorption of the bacteriocin to *Lactobacillus paraplantarum* ATCC 700211^T and *Streptococcus caprinus* ATCC 700066, which are not sensitive to the bacteriocin, was also recorded. The best adsorption to *E. faecalis* E88 was recorded at 25°C and to *L. innocua* LMG 13568 at 4°C, 10°C and 25°C. Optimal adsorption was recorded at pH 6.0 for *L. innocua* LMG 13568 and at pH 2.0 for *E. faecalis* E88. Triton X-100 and Triton X-114 increased the adsorption of bacST8KF to *L. innocua* LMG 13568 and *E. faecalis* E88 by 40%. Potassium ions (K_2HPO_4, KH_2PO_4, KCl and KI), $MgCl_2$, Tris, NH_4-citrate, Na-acetate, Na_2CO_3, EDTA and SDS led to a decrease in adsorption. NaCl and β-mercaptoethanol also resulted in a decrease of adsorption to *E. faecalis* E88, but not to *L. innocua* LMG 13568, while methanol resulted in a decrease of adsorption to *L. innocua* LMG 13568 but not to *E. faecalis* E88. Neither 80% ethanol nor chloroform had an effect on bacteriocin adsorption. *Listeria innocua* LMG 13568 and *E. faecalis* E88 treated with bacST8KF secreted DNA and β-galactosidase. Growth of *Lb. plantarum* ST8KF and *L. innocua* LMG 13568 in mixed culture yielded an increase in bacST8KF production (from 12 800 AU.ml^{-1} to 25 600 AU.ml^{-1} in 32 h). The aim of this study was to determine the parameters affecting the adsorption of bacST8KF, regarding future application of the bacteriocin in various food systems.

Introduction

The ability of lactic acid bacteria (LAB) to inhibit the growth of undesirable bacteria is well known. Inhibition may be due to production of organic acids, hydrogen peroxide (Shahani & Chandan, 1979; Juven *et al*., 1992), carbon dioxide, acetaldehyde, diacetyl or bacteriocins (Helander *et al*., 1997). Bacteriocins are ribosomal synthesised polypeptides with activity against genetically closely related bacteria (Klaenhammer, 1988). These peptides are divided into three categories, i.e. (i) peptides with a narrow inhibitory spectrum, affecting only closely-related species, (ii) peptides inhibitory to Gram-positive bacteria, including food-borne pathogens, and (iii) peptides with an unusual spectrum of activity, including Gram-negative bacteria, viruses and yeast (Klaenhammer, 1988; Piard & Desmazeaud, 1992; Okkers *et al.,* 1999; Wachsman *et al*., 1999; Serkedjieva *et al*., 2000; Todorov & Dicks, 2005; Todorov *et al*., 2005). Bacteriocins active against Gram-negative bacteria include plantaricin 35d, active against *Aeromonas hydrophilas* strains (Messi *et al*., 2001); plantaricin LP84, active against *Escherichia coli* D21 and *Pseudomonas aeruginosa* CFR 1704 (Suma *et al*., 1998); and nisin, active against several *Salmonella* species and other selected Gram-negative bacteria (Stevens *et al*., 1991).

Lactobacillus plantarum ST8KF was isolated from Kefir and produces a bacteriocin (bacST8KF) which inhibits several food spoilage bacteria and foodborne pathogens, including *Enterococcus mundtii* ST4SA, *Lactobacillus casei* LHS, *Lactobacillus curvatus* DF38, *Lactobacillus salivarius* 241 and *Listeria innocua* F and LMG 13568 (Powell *et al*., 2006). BacST8KF is resistant to treatment at 100°C for 120 min and at 121°C for 20 min. The peptide remains active after incubation at pH 2.0 to 10.0 and is inactivated when treated with proteolytic enzymes such as Proteinase K and pronase. The mechanism of activity is bacteriostatic, as determined against *Lb*. *casei* LHS (Powell *et al*., 2006).

A limited number of papers have been published on the adsorption of bacteriocins to target (sensitive) cells. The adsorption of pediocin N5p, produced by *Pediococcus pentosaceus*, has been found to be selective to both Gram-positive and Gram-negative bacteria. The presence of Mg^{2+} and Mn^{2+} increased pediocin N5p binding to *P. pentosaceus* by 80% and 100%, respectively.

Treatment of the target strain with 1% SDS increased the adsorption of pediocin N5p by 25% (Manca de Nadra *et al.*, 1998). The adsorption of buchnericin LB, produced by *Lactobacillus buchneri*, is influenced by pH and not temperature or contact time. Anions and lipoteichoic acid reduced or inhibited the adsorption of buchnericin LB to the target cells. Treatment of the cells or their cell walls with detergents or organic solvents had no effect on the adsorption of buchnericin LB (Yildirim *et al.*, 2002).

In this study, the mode of activity of bacST8KF and parameters affecting its adsorption to target bacteria were determined.

Material and Methods

Growth conditions and preparation of bacST8KF

Lactobacillus plantarum ST8KF was grown in 10 ml complete MRS broth (Biolab, Biolab Diagnostics, Midrand, SA) and incubated at 30°C for 24 h. The other strains used in this study (Table 1) were grown in 10 ml complete MRS broth or Brain Heart Infusion (BHI) broth (Biolab), at temperatures indicated in the respective culture collection catalogues. All strains were stored at -80℃ in complete MRS or BHI broth supplemented with 15% (v/v) glycerol.

Lactobacillus plantarum ST8KF inoculated from culture stored at -80°C, was inoculated (2%, v/v) into 100 ml complete MRS broth and incubated at 30°C for 24 h. The cells were harvested (8 000 x *g*, 15 min, 4°C), the pH of the cell-free supernatant containing bacST8KF adjusted to 5.0 with sterile 1 M NaOH, heated for 10 min at 80°C, and then filter-sterilised (0.20 µm, Minisart®, Sartorius).

Bacteriocin bioassay

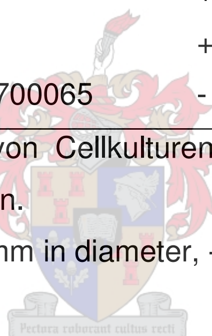
Bacteriocin activity tests were performed on cell-free supernatants by using the agar-spot test and the well-diffusion method (Todorov & Dicks, 2005). Cell-free supernatants were adjusted to pH 5.0 with sterile 1 M NaOH before testing. Target microorganisms (Table 1) were cultured in 10 ml complete MRS broth (*Enterococcus faecalis* DFE 1071 and E88, *Lactobacillus casei* LHS, *Lactobacillus curvatus* DF38, *Lactobacillus paraplantarum* ATCC 700211^{T}, *Lactobacillus*

Table 1 Spectrum of activity of bacteriocin ST8KF and its adsorption to the cells, expressed as a percentage value

Strains	**Sensitive to bacST8KF**	**Adsorption (%)**
Enterococcus faecalis BFE 1071	-	60
Enterococcus faecalis E88	+	60
Lactobacillus casei LHS	+	80
Lactobacillus curvatus DF38	+	60
Lactobacillus paraplantarum ATCC 700211^{T}	-	80
Lactobacillus pentosus NCFB 363	-	60
Lactobacillus plantarum 423	-	60
Lactobacillus plantarum LMG 13556	-	20
Lactobacillus sakei DSM 20017	+	80
Lactobacillus salivarius 241	+	60
Listeria innocua LMG 13568	+	60
Streptococcus caprinus ATCC 700065	-	80

DSM = Deutsche Sammlung von Cellkulturen und Mikroorganismen, ATCC = American Type Culture Collection.

+ = inhibition zone of at least 5 mm in diameter, - = no inhibition zone recorded.



pentosus NCFB 363, *Lactobacillus plantarum* 423 and LMG 13556, *Lactobacillus sakei* DSM 20017, *Lactobacillus salivarius* 241 and *Streptococcus caprinus* ATCC 700065), or in 10 ml BHI broth (*Listeria innocua* LMG 13568) at 30°C or 37°C. Antimicrobial activity was expressed as arbitrary units AU per ml, calculated as follows: a^b x 100, where “a” represents the dilution factor and “b” the last dilution that produces an inhibition zone of at least 5 mm in diameter. Activity was expressed per ml by multiplication by 100 (Todorov & Dicks, 2005).

Effect of bacST8KF on growth of Enterococcus faecalis *E88 and* Listeria innocua *LMG 13568*

Twenty ml (12 800 AU.ml^{-1}) of bacST8KF-containing cell-free supernatant (pH 5.0) was filter-sterilised (0.20 µm, Minisart®, Sartorius) and added to 100 ml 3-h-old cultures ($OD_{600\ nm}$ = 0.1 – 0.2) of *E. faecalis* E88 and *L. innocua* LMG 13568 grown in complete MRS and BHI, respectively. *Enterococcus faecalis* E88 and *L. innocua* LMG 13568 grown under the same conditions, in 100 ml complete MRS or BHI broth without the addition of bacST8KF were used as controls. Optical density readings were taken at 600 nm, hourly for 10 h.

Effect of bacST8KF on cell permeability

Cells of 10 ml cultures of *E. faecalis* E88 and *L. innocua* LMG 13568 (18-h-old) were harvested by centrifugation (10 000 x *g*, 15 min, 4°C) and washed twice with sterile 5 mM phosphate buffer (pH 6.5) and re-suspended in 10 ml of the same buffer. BacST8KF (25 600 AU.ml^{-1}) was then added to the washed cells of *E. faecalis* E88 and *L. innocua* LMG 13568 at a ratio of 0.1:1.0. After incubation at 37°C for 1 h, the cells were harvested (8 000 x *g*, 15 min, 4°C) and the supernatant filtered through a 0.20 µm membrane (Minisart®, Sartorius). DNA concentration was determined by measuring absorbancy of the cell-free supernatants at 260 nm. Controls were *E. faecalis* E88 and *L. innocua* LMG 13568 suspended in 5 mM phosphate buffer, but without bacteriocin, and in the same buffer containing bacST8KF, but without cells.

In a separate experiment, extracellular levels of β-galactosidase activity was monitored. Eleven-h-old cultures of *E. faecalis* E88 and *L. innocua* LMG

13568 (10 ml each) were harvested and the cells washed twice with 0.03 M sodium phosphate buffer (pH 6.5) and re-suspended in 2 ml of the same buffer. The cell suspensions were treated with 2 ml bacST8KF (25 600 $AU.ml^{-1}$) for 5 min at 25°C, followed by the addition of 0.2 ml 0.1 M ONPG (O-nitrophenyl-β-D-galactopyranoside) in 0.03 M sodium phosphate buffer (pH 6.8) and then incubated at 37°C for 10 min. The reaction of β-galactosidase was stopped by the addition of 2.0 ml 0.1 M sodium carbonate. The cells were harvested (8 000 x *g*, 15 min, 25°C) and absorbance readings of the supernatant recorded at 420 nm. The controls consisted of cells prepared the same way, but not treated with bacST8KF (Nagy *et al.*, 2001; Hsu *et al.*, 2005).

Adsorption of bacST8KF to target cells

Adsorption of bacST8KF to target cells was performed according to the method described by Yildirim *et al.* (2002). The target strains (Table 1) were grown overnight in 10 ml complete MRS (*Enterococcus faecalis* DFE 1071 and E88, *Lactobacillus casei* LHS, *Lactobacillus curvatus* DF38, *Lactobacillus paraplantarum* ATCC 700211^T, *Lactobacillus pentosus* NCFB 363, *Lactobacillus plantarum* 423 and LMG 13556, *Lactobacillus sakei* DSM 20017, *Lactobacillus salivarius* 241 and *Streptococcus caprinus* ATCC 700065), or in 10 ml BHI (*Listeria innocua* LMG 13568) broth at 30°C or 37°C and then centrifuged (8 000 x *g*, 15 min, 4°C). Cells were washed twice with sterile 5 mM phosphate buffer (pH 6.5) and re-suspended to the original volume in the same buffer. Each cell suspension (0.7 ml) was mixed with bacST8KF (25 600 $AU.ml^{-1}$) (0.7 ml) and incubated at 37°C for 1 h. After removal of cells by centrifugation (8 000 x *g*, 15 min, 25°C), the activity of unbound bacST8KF in the supernatant was determined as described in bacteriocin bioassay of this chapter. This experiment was performed twice and no difference in the data was observed.

The percentage adsorption of bacST8KF to the target cells was calculated according to the following formula:

$$\% \text{ adsorption} = 100 - \left(\frac{\text{bacteriocin activity after treatment}}{\text{original bacteriocin activity}} \times 100 \right)$$

Effect of pH and temperature on the adsorption of bacST8KF

Enterococcus faecalis E88 and *L. innocua* LMG 13568 were grown overnight in 10 ml complete MRS or BHI broth at 30°C or 37°C and then centrifuged (8 000 x *g*, 15 min, 4°C). For the experiment regarding pH, cells were washed twice with sterile distilled water and re-suspended to the original volume in sterile distilled water. In the experiment regarding temperature, cells were washed twice with sterile 5 mM phosphate buffer (pH 6.5) and re-suspended to the original volume in the same buffer. Each cell suspension was then mixed with bacST8KF (25 600 $AU.ml^{-1}$) at a ratio of 1:1 and incubated for 1 h at different temperatures (4°C, 10°C, 25°C, 30°C, 37°C, 45°C and 60°C) and at different pH (2.0, 4.0, 6.0, 8.0 and 10.0). The cells were harvested (8 000 x *g*, 15 min, 25°C) and the pH of the cell-free supernatant adjusted to 5.0 with sterile 1M NaOH. Bacteriocin activity in the supernatant was determined as described in bacteriocin bioassay of this chapter.

Effect of SDS, inorganic salts and organic compounds on the adsorption of bacST8KF to target cells

Eighteen-h-old cultures of *E. faecalis* E88 and *L. innocua* LMG 13568 were treated with 1% (m/v) NaCl (Saarchem Pty Ltd., Muldersdrift, SA), K_2HPO_4 (Merck, Darmstadt, Germany), KH_2PO_4 (UnivAR, Saarchem Pty Ltd., Muldersdrift, SA), $MgCl_2$, KCl (Saarchem), KI (Saarchem), Tris (Kimix, Johannesburg, SA), $(NH_4)_3C_6H_5O_7$ (Saarchem), CH_3COONa (Saarchem), Na_2CO_3 (Saarchem), EDTA (Merck), SDS (Sigma, St Louis, USA) and 1% (v/v) Triton X-100 (BDH, BDH Chemicals LTD, Poole, England), Triton X-114 (Sigma), β-mercaptoethanol (BioRad-Hercules, CA, SA), 80% ethanol (Saarchem), methanol (Saarchem) and chloroform (Saarchem), respectively. BacST8KF was added to the treated cells, as described in adsorption of bacST8KF to target cells of this chapter, and incubated for 1 h at 37°C. The cells were then harvested (8 000 x *g*, 15 min, 25°C) and the activity of bacST8KF in the cell-free supernatant determined as described in bacteriocin bioassay of this chapter.

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Cell growth and bacST8KF production in mixed culture.

Eighteen-h-old cultures of *Lb. plantarum* ST8KF and *L. innocua* LMG 13568 were inoculated into 100 ml complete MRS broth at concentrations of 2.0% (v/v) and 0.1% (v/v), respectively. The mixed culture was incubated at 30°C for 32 h. Two ml samples of the mixed culture were taken at 3-h time intervals in order to determine changes in pH. One ml samples were also taken at 3-h time intervals in order to determine the cell numbers of *L. innocua* LMG 13568 on *Listeria* Enrichment Broth (LEB) (Merck). Total cell counts were determined on complete MRS agar. The cell count of *Lb. plantarum* ST8KF was determined as the difference between the total cell count and the cell count recorded for *L. innocua* LMG 13568. Bacteriocin production was determined by using the agar-spot method as described in bacteriocin bioassay of this chapter.

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Results and Discussion

Spectrum of antimicrobial activity

Cell-free supernatant (25 600 AU.ml^{-1}) of *Lb. plantarum* ST8KF, adjusted to pH 5.0, inhibited the growth of *Enterococcus faecalis* E88, *Lactobacillus casei* LHS, *Lactobacillus curvatus* DF38, *Lactobacillus sakei* DSM 20017, *Lactobacillus salivarius* 241 and *L. innocua* LMG 13568, but none of the other strains included in the test panel (Table 1). This experiment was performed in duplicate but no difference in the results was observed.

Effect of bacST8KF on growth of Enterococcus faecalis E88 and Listeria innocua LMG 13568

Addition of the bacST8KF to early logarithmic-phase cells of *E. faecalis* E88 or *L. innocua* LMG13568 resulted in a decrease of cell growth, when compared to the controls (Fig. 1). BacST8KF was added after 3 h growth, which resulted in an increase from $OD_{600\ nm}$ 0.02 to 0.20 for *E. faecalis* E88 over 10 h, and an increase from $OD_{600\ nm}$ 0.01 to 0.51 for *L. innocua* LMG 13568 over 10 h. The control (not treated with bacST8KF) increased for $OD_{600\ nm}$ 0.05 to 1.55 and from $OD_{600\ nm}$ 0.10 to 2.05 for *E. faecalis* E88 and *L. innocua* LMG13568 respectively over the same time period. The slight increase in optical density of cells treated with bacST8KF

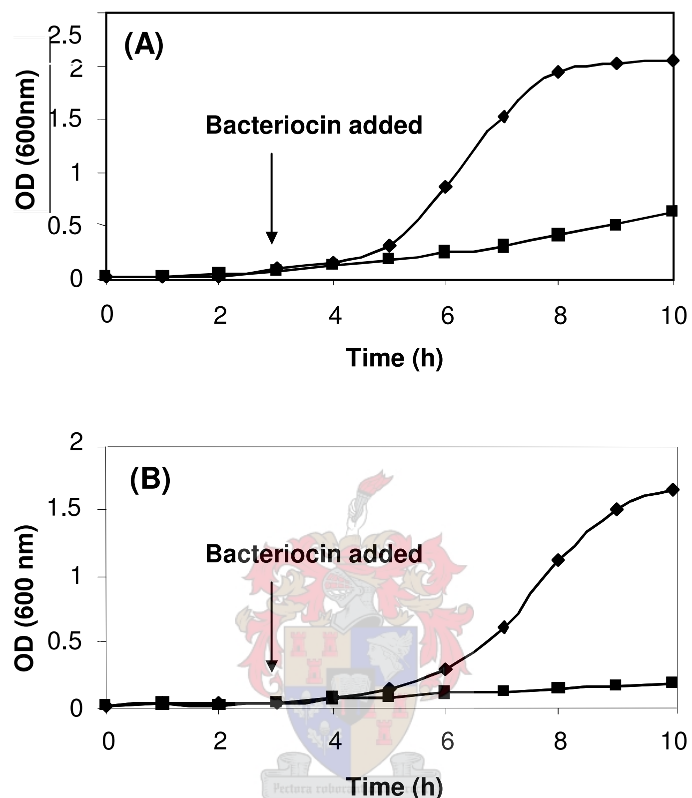

Figure 1 Effect of bacST8KF (12 800 AU.ml^{-1}) on exponential phase cells of (A) *Listeria innocua* LMG 13568 and (B) *Enterococcus faecalis* E88. Symbols: - ♦ - = in the absence of bacteriocin; - ■ - in the presence of bacST8KF (12 800 AU.ml^{-1}). Bacteriocin (12 800 AU.ml^{-1}) was added at 3 h from the beginning of fermentation.

suggests that the mode of activity is bacteriostatic. Similar results were reported for bacteriocins ST11BR, ST151BR and ST34BR (Todorov & Dicks, 2004). Bacteriocins ST28MS and ST26MS, on the other hand, repressed the growth of *Lb. casei* LHS for only 2 h (Todorov & Dicks, 2005).

Effect of bacST8KF on cell membrane permeability

Eighteen-h-old cultures of *Enterococcus faecalis* E88 and *L. innocua* LMG 13568 treated with bacST8KF resulted in the leakage of DNA and β-galactosidase (Table 2). Concluded from these results, the mode of action of bacST8KF is most probably destabilising the permeability of the cell membrane. Similar results have been reported for buchnericin LB (Yildirim *et al.*, 1999; 2002) and pediocin AcH (Bhunia *et al.*, 1991). The experiment was not done in duplicate and it is suggested that it be repeated in future work.

Adsorption of bacST8KF to target cells

BacST8KF adsorbed to sensitive and resistant cells of Gram-positive bacteria (Table 1). Adsorption ranged from 20% for *Lb. plantarum* LMG 13556 to 80% for *Lb. casei* LHS, *Lb. paraplantarum* ATCC 700211^T, *Lb. sakei* DSM 20017 and *Streptococcus caprinus* ATCC 700065. Both strains sensitive to bacST8KF as well as strains insensitive to the peptide, showed strong adsorption (80%) of the peptide. This suggests that the adsorption of bacST8KF to target strains does not confirm the activity of the peptide against the target strain. This warrants further research. This experiment was done in duplicate and no difference in the results was observed. Similar results have been reported by Yildirim *et al.* (2002). In the case of buhnericin LB, 100% adsorption to *Lb. plantarum*, *Pediococcus dextranicus*, *Oenococcus oeni* (previously *Leuconostoc oenos*) and *E. faecalis* has been reported (Yildirim *et al.*, 2002). The authors also reported 100% adsorption of the peptide to a strain of *Pediococcus cerevisiae* insensitive to buchnericin LB. Similar results have been recorded by Manca de Nadra *et al.* (1998). Higher adsorption of pediocin N5p was observed to sensitive strains. Adsorption of 100% was recorded for *O. oeni* X2L, 80% for *Lactobacillus hilgardii*,

Table 2 Extracellular levels of DNA and β-galactocidase recorded after treatment of *Listeria innocua* LMG 13568 and *Enterococcus faecalis* E88 with bacST8KF for 1 h

	Absorbance	
	DNA (260 nm)	ß-galactosidase (420 nm)
BacST8KF solution	0.090	0.015
Untreated cells of *L. innocua* LMG 13568	0.240	0.022
Untreated cells of *E. faecalis* E88	0.190	0.016
L. innocua LMG 13568 treated with bacST8KF solution	2.00	0.103
E. faecalis E88 treated with bacST8KF	1.90	0.112

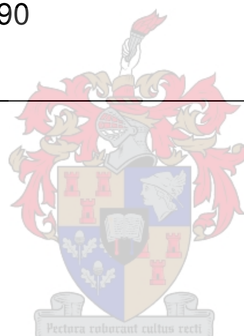

Table 3 Effect of pH, temperature, inorganic and organic salts on adsorption of bacST8KF (25 600 AU.ml^{-1}) to *Listeria innocua* LMG 13568 and *Enterococcus faecalis* E88

Treatment with 1%	Adsorption to *L. innocua* LMG 13568 (%)	Adsorption to *E. faecalis* E88 (%)
NaCl	60	20
K_2HPO_4	20	0
KH_2PO_4	0	20
$MgCl_2$	20	0
KCl	20	40
KI	0	0
Tris	0	0
NH_4-citrate	20	20
Na-acetate	20	40
Na_2CO_3	0	20
EDTA (-Na)	40	40
SDS	0	40
Triton X-100	100	100
Triton X-114	100	100
B-mercaptoethanol	60	40
80% ethanol	60	60
Methanol	40	60
Chloroform	60	60
Control	60	60
Effect of temperature (^{o}C)		
4	60	30
10	60	40
25	60	60
30	40	40
37	30	30
45	30	30
60	30	30
Effect of pH		
2.0	40	80
4.0	40	40
6.0	60	60
8.0	40	40
10.0	40	40

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O. oeni L10, and 70% for *Lb. hilgardii* 6D. Pediocin N5p adsorbed to resistant bacteria at levels below 20% (Manca de Nadra *et al.,* 1998).

Effect of pH, temperature, SDS, inorganic salts and organic compounds on the adsorption of bacST8KF to target cells

Optimal adsorption of bacST8KF (25 600 $AU.ml^{-1}$) to *E. faecalis* E88 was recorded at pH 2.0 (Table 3). In the case of *L. innocua* LMG 13568, optimal adsorption of bacST8KF was recorded at pH 6.0. The reason for these differences in adsorption rates is not known, but may be due to specific interaction between bacST8KF and the target strain. In the case of buchnericin LB, optimal levels of adsorption to *Lb. plantarum* were recorded at pH 5.0 to 8.0 (Yildirim *et al*., 2002). The adsorption at pH 6.0 served as a control for this experiment.

The best adsorption of bacST8KF (25 600 $AU.ml^{-1}$) to *E. faecalis* E88 was noted at 25°C, while higher adsorption (60%) to *L. innocua* LMG 13568 was recorded after treatment at 4°C, 10°C and 25°C (Table 3). In the case of buchnericin LB, identical adsorption to cells of *Lb. plantarum* was recorded after treatment at 0°C, 10°C, 25°C, 50°C and 80°C (Yildirim *et al*., 2002). Adsorption at 30°C served as a control for this experiment.

Reduction in adsorption of bacST8KF to *E. faecalis* E88 was observed when cells were treated with NaCl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris, NH_4-citrate, Na-acetate, Na_2CO_3, EDTA, SDS, β-mercapto-ethanol (Table 3). Treatment of the cells with 80% ethanol, methanol and chloroform yielded the same levels of adsorption compared to untreated cells of *E. faecalis* E88 (Table 3). An increase in the adsorption of bacST8KF to *E. faecalis* E88 was observed in the presence of Triton X-100 and Triton X-114 (Table 3).

Cells of *L. innocua* LMG 13568 treated with K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris, NH_4-citrate, Na-acetate, Na_2CO_3, EDTA, SDS and methanol led to a reduction in bacST8KF adsorption (Table 3). No change in adsorption was observed in the presence of NaCl, β-mercaptoethanol, 80% ethanol and chloroform whereas an increase in adsorption was observed in the presence of Triton X-100 and Triton X-114 (Table 3). These experiments were performed in order to determine whether the presence of specific detergents had an effect on

bacteriocin adsorption. The reason why the effects on adsorption occurred renders further work and could be considered for future research.

Adsorption of buchnericin LB to *Lb. plantarum* was reduced by NaCl, NH_4Cl, $MgCl_2$, KCl, KI and Tris. Treatment of cells with NH_4-citrate, Na-acetate, $NaCO_3$, EDTA**,** SDS, Triton X-100, 2-mercaptoethanol, 80% ethanol and 80% methanol had no effect on adsorption of buchnericin LB to *Lb. plantarum* (Yildirim *et al*., 2002). The adsorption of pediocin N5p to *P. pentosaceus* E5p increased in the presence of $MgCl_2$, $MgSO_4$, $MnCl_2$, $MnSO_4$, whereas NaCl, KCl, KI, NH_4Cl, $CaCl_2$, Na_3PO_4, Na_2SO_4, EDTA and ethanol had no affect on its adsorption. Organic salts and Na-acetate reduced pediocin N5p adsorption to the target cells. Adsorption of pediocin N5p increased with 25% in the presence of SDS (Manca de Nadra *et al*., 1998).

Cell growth and bacST8KF production in mixed culture

Production of bacST8KF by *Lb. plantarum* ST8KF increased to 25 600 AU.ml^{-1}, recorded at 23 h when the strain was cultured in the presence of viable cells of *L. innocua* LMG 13568 (Fig. 2). Production of bacST8KF in complete MRS (control) showed the same activity, but only at 27h (Powell *et al*., 2006). The bacteriocin activity recorded represents that of the cell-free peptides and does not represent peptide molecules that may still be bound to the producer cell. Nevertheless, it would seem that bacST8KF production is stimulated by the presence of the target organism, most probably by receptors in its cell wall.

High cell numbers of *Lb. plantarum* ST8KF and *L. innocua* LMG 13568 were recorded when co-cultured (Fig. 2). However, the cell numbers of *L. innocua* LMG 13568 remained constant, from 3.1 x 10^3 colony forming units (cfu) per ml to 3.0 x 10^3 cfu.ml^{-1} in 32 h (Fig. 2). The bacteriocin activity could be higher, as some active peptides remain bound to the cell surface of the target strain. The latter phenomenon has been shown by Yildirim *et al.* (2002). This experiment was performed once.

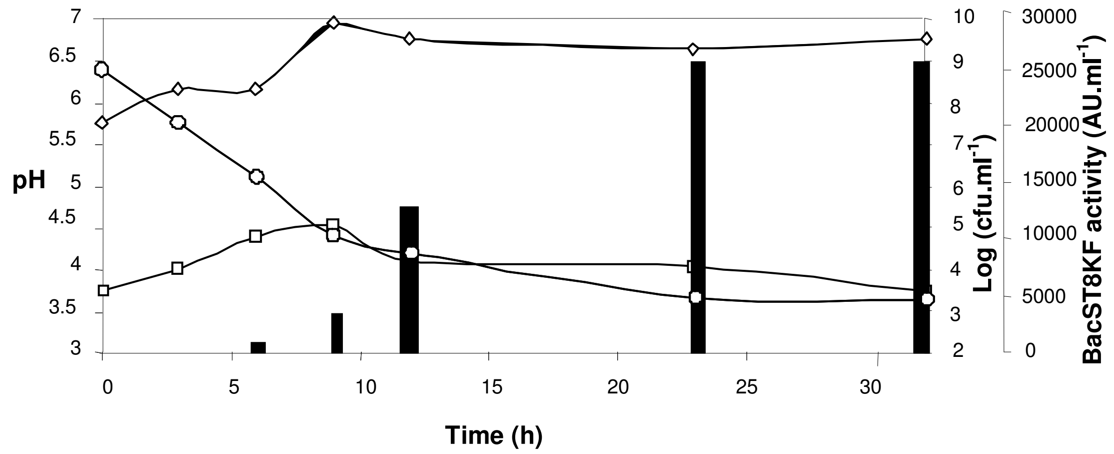
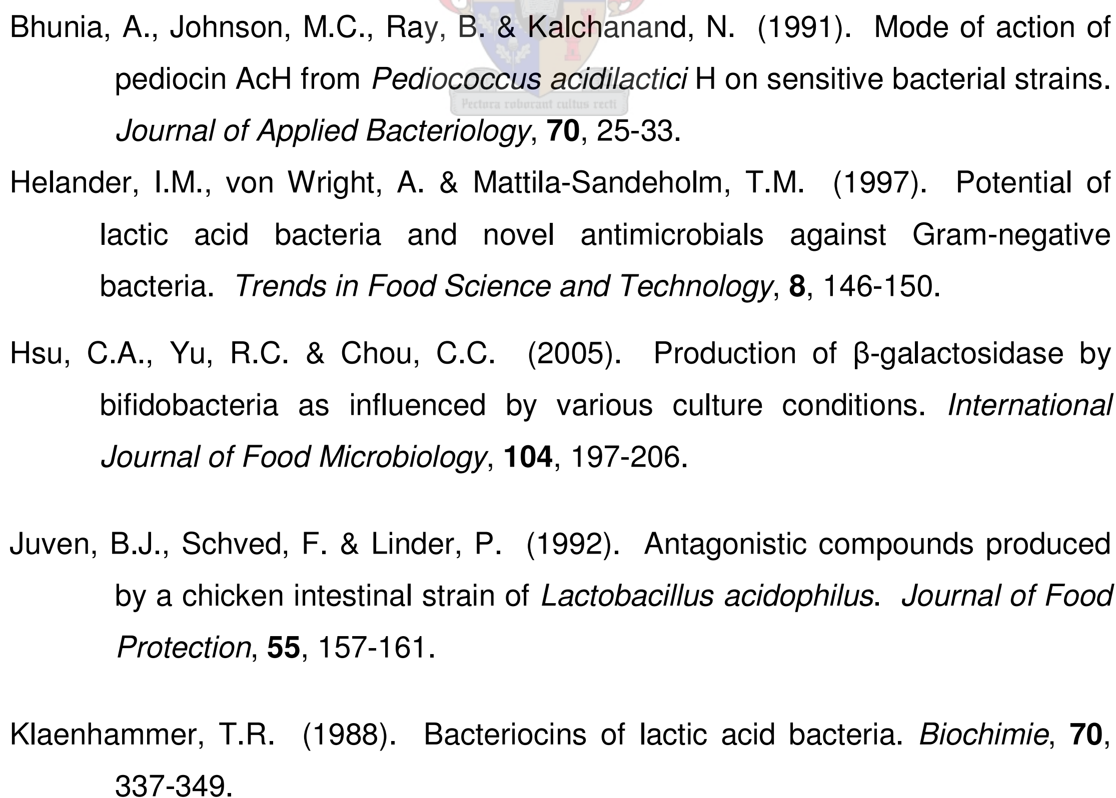


Figure 2 Growth (- ◊ -) of *Lactobacills plantarum* ST8KF, (- □ -) *Listeria innocua* LMG 13568, and (- ○ -) decrease in pH. (- ▌ -) production of bacteriocin ST8KF in mixed culture.

Conclusions

BacST8KF has a narrow spectrum of activity, and the presence of SDS, inorganic salts and organic compounds decreases the adsorption of bacST8KF to target cells. Optimal bacteriocin adsorption was observed at 25°C, and pH did not influence the adsorption of bacST8KF to target cells. The presence of different detergents had an effect on the adsorption of bacST8KF. Bacteriocin production increases when the producer *Lb. plantarum* ST8KF is grown in mixed culture. The mode of bacST8KF activity is bacteriostatic and the bacteriocin destabilises the permeability of the cell membrane of target cells. This suggests that the adsorption of bacST8KF is optimal in Kefir.

However, differences recorded for *E. faecalis* E88 and *L. innocua LMG* 13568 suggests that conditions required for adsorption is species-specific. From an industrial point of view, for instance the application of bacST8KF as a food preservative, these are important factors that will have to be taken into account.



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CHAPTER 5

INCORPORATION OF BACTERIOCIN PRODUCING *LACTOBACILLUS PLANTARUM* ST8KF(+) AND THE CURED ST8KF(-) INTO MASS-CULTURED KEFIR GRAINS, AND THE INFLUENCE OF THESE GRAINS ON A BACTERIOCIN SENSITIVE STRAIN DURING KEFIR PRODUCTION

Abstract

Lactobacillus plantarum ST8KF, isolated from Kefir produces bacteriocin ST8KF (bacST8KF). The genes encoding for bacST8KF are located on a plasmid approximately 3.9 kilo base pairs (kb) in size. The plasmid was cured from the host using 80 $\mu l.ml^{-1}$ novobiocin. The cured strain [ST8KF(-)] differed regarding antibiotic resistance and carbohydrate fermentation reactions. Strains ST8KF(+) and ST8KF(-) were incorporated into Kefir grains during mass-cultivation. *Enterococus mundtii* ST4SA was used as sensitive starin as the bacterium is sensitive to bacST8KF, and a probe could be designed from the specific gene sequence of the bacterium. The survival of this strain was monitored using fluorescent *in situ* hybridization (FISH) in Kefir produced from enriched mass-cultured grains. *Enterococcus mundtii* ST4SA was present in higher numbers (28 *E. mundtii* ST4SA cells per 10 µl Kefir sample) in the ST8KF(-) Kefir system when compared to the ST8KF(+) system (22 *E. mundtii* ST4SA cells per 10 µl Kefir sample). This is an indication that *Lb. plantarum* ST8KF(+) contributes to the antimicrobial activity of Kefir through the production of bacST8KF.

Introduction

Kefir is a refreshing, self-carbonated fermented milk with a slightly acidic taste (Saloff-Coste, 1996), yeasty flavour and creamy consistency. When agitated, the beverage foams and fizzes (Obermann, 1985; Duitschaever, 1989). It is a traditional fermented milk (Duitschaever, 1989) that is obtained from the fermentative activity of Kefir grains (Garrotte *et al.*, 2000). The Kefir grains are comprised of a mass of actively growing bacteria and yeasts (Farnworth, 2003), polysaccharides and other products of bacterial metabolism, together with milk protein (Hertzler & Clancy, 2003), and primarily include lactic acid bacteria (LAB), namely lactococci and lactobacilli, as well as lactose fermenting and non-lactose

fermenting yeasts (Kwak *et al*., 1996), acetic acid bacteria and filamentous fungi (Saloff-Coste, 1996).

The antimicrobial activity of Kefir has been well documented and the beverage is known to inhibit a number of spoilage microorganisms and foodborne pathogens, including *Bacillus cereus*, *Clostridium tyrobutyricum*, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* (Saloff-Coste, 1996; Garrotte *et al*., 2000; Van Wyk, 2001). The exact cause of this inhibition is not known, but may include the presence of lactic and volatile acids, hydrogen peroxide (Shahani & Chandan, 1979; Juven *et al*., 1992), carbon dioxide, diacetaldehyde and acetaldehyde, or bacteriocins (Helander *et al*., 1997). Although the inhibition could be due to a combination of these factors, Morgan *et al*. (2000) showed that neutralised Kefir fermentates were still capable of causing inhibition. Balasubramanyam & Varadaraj (1994) reported that the sterilised filtrates of LAB isolates lost all inhibitory activity after they were treated with the proteolytic enzyme trypsin. Trypsin is capable of denaturing proteins, thereby indicating the proteinateous nature of the antimicrobial compound.

Bacteriocins are bacterial proteins or peptides with bactericidal or bacteriostatic activity against genetically closely related species (Tagg *et al*., 1976; Klaenhammer, 1988). Bacteriocins vary with regards to their mode of action, molecular weight, genetic origin, biochemical properties and spectrum of activity. They are produced spontaneously or induced. The genetic determinants of most bacteriocins are located on plasmids, with only a few exceptions being chromosomally encoded (Naidu *et al*., 1999).

The aim of this study was to cure *Lactobacillus plantarum* ST8KF from the plasmid encoding bacteriocin ST8KF (bacST8KF) and to incorporate both strains ST8KF(+) and ST8KF(-) into Kefir grains during mass-cultivation. The second aim was to determine whether the enriched mass-cultured Kefir grains show antimicrobial activity against *Enterococcus mundtii* ST4SA, which is sensitive to bacST8KF, using fluorescent *in situ* hybridization (FISH).

Materials and methods

Growth condition of test strains

Lactobacillus plantarum ST8KF (Powell *et al*., 2006), isolated from Kefir grains, and *E. mundtii* ST4SA, obtained from the Department of Microbiology, Stellenbosch University were cultured in 10 ml complete MRS broth (Biolab, Biolab Diagnostics, Midrand, SA) at 30°C for 24 h. The strains were stored at -80°C in complete MRS broth supplemented with 15% (v/v) glycerol.

Plasmid isolation from Lactobacillus plantarum ST8KF

A 2% (v/v) inoculum of an 18-h-old culture of *Lb. plantarum* ST8KF grown in complete MRS broth was added to 10 ml complete MRS broth and incubated for 24 h at 30°C. Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit (Southern Cross Biotechnology, Cape Town). An additional phenol step was incorporated due to the high concentration of proteins present in the sample. The isolated plasmid was visualised on a 1% (m/v) agarose gel after electrophoresis (van Reenen *et al*., 1998).

Plasmid curing of Lactobacillus plantarum *ST8KF*

Plasmid curing was performed as described by Ruiz-Barba *et al*. (1991). Different concentrations of novobiocin (Sigma, St Louis, USA) or SDS (Sigma), ranging from 5 $\mu l.ml^{-1}$ to 320 $\mu l.ml^{-1}$ and from 0.1 $mg.ml^{-1}$ to 1.0 $mg.ml^{-1}$, respectively were added to complete MRS broth. This mixture was then inoculated with 0.3% (v/v) of an 18-h-old culture of *Lb. plantarum* ST8KF grown in complete MRS broth and incubated at 30°C for 48 h. After incubation, cells resistant to the highest concentration of the curing agent were selected. A streak plate was prepared on complete MRS agar and the plate incubated at 30°C for 48 h. Colonies were randomly selected and tested for bacteriocin activity according to the triple-agar-layer method described by Todorov and Dicks (2005). Colonies showing a loss in antimicrobial activity were then randomly selected for plasmid isolation. Plasmid DNA was again isolated using the Qiagen Plasmid Midi Kit (Southern Cross Biotechnology, Cape Town).

Antibiotic resistance of strains ST8KF(+) and ST8KF(-)

Complete MRS plates seeded with *Lb. plantarum* ST8KF(+) and ST8KF(-) (1 x 10^6 (cfu per ml) were prepared. Antibiotic discs (Oxoid, Ltd, Basingstoke, Hampshire, England) were aseptically placed on the plates, and these were

incubated at 30°C for 24 h. The various antibiotics tested are listed in Table 1. Zones of inhibition were measured and zones of at least 5 mm in diameter were considered a positive result.

Carbohydrate fermentation by Lb. plantarum *ST8KF(+) and ST8KF(-)*

Cell cultures for carbohydrate fermentation reactions were prepared by inoculating 10 ml complete MRS broth with 1% (v/v) *Lb. plantarum* ST8KF(+) and ST8KF(-), grown for 18-h in complete MRS broth. Samples were incubated for 24 h at 30°C. Carbohydrate fermentation reactions were recorded using an API 50 CHL test kit (bioMérieux® S.A., Marcy, l'Etiole, France).

Mass-cultivation and enrichment of Kefir grains with Lactobacillus plantarum *ST8KF(+) and ST8KF(-)*

The mass-cultivation of Kefir grains was done according to the method developed by Schoevers and Britz (2003). The milk mixture was prepared by the addition of 20 $g.l^{-1}$ yeast extract (Biolab) and 5.0 $g.l^{-1}$ urea (Biolab) to pasteurised full cream milk. The milk was heat-treated at 80°C for 90 min. Four hundred ml of the milk mixture and 40 g Kefir grains, obtained from the Department of Food Science, Stellenbosch University, were incubated at 25°C in a water bath and shaken constantly at 120 rpm. The Kefir grains were sieved and the milk mixture replaced every 24 h.

To enrich the mass-cultured Kefir grains with *Lb. plantarum*, 20 g of mass-cultured grains were placed in sterile 500 ml containers and filled with 200 ml of heat-treated milk mixture. The bottles were incubated at 25°C in a water bath, shaken constantly at 120 rpm. The grains were sieved and the milk mixture replaced every 24 hrs. Every 48 hrs, 50 ml of 1×10^8 $cfu.ml^{-1}$ *Lb. plantarum* ST8KF(+) or ST8KF(-) grown in complete MRS broth, were centrifuged (6 000 x *g*, 10 min, 4°C), re-suspended in 1 ml sterile physiological salt solution (0.85% (m/v) NaCl), and added to the appropriate container. The enrichment was carried out for 40 days.

Table 1 Antibiotic resistance of *Lactobacillus plantarum* strains ST8KF(+) and ST8KF(-)

Antibiotic	**Antibiotic resistance (mm)**	
	ST8KF(+)	**ST8KF(-)**
Metronidazole	-	-
Nalidixic acid	-	-
Sulphamethoxazole	-	-
Neomycin	9	17
Tobramycin	8	10.5
Nitrofurantion	30	15.5
Cirprofloxacin	-	-
Ceforoxime	19	-
Fusidic acid	12	17.5
Clindamycin	30	-
Furazolidone	19	-
Cefotaxime	22	-
Rifampicin	20	25
Tetracycline	23	24.5
Sulphonamides	-	-
Ofloxacin	10	10
Oxacillin	-	-
Cefepime	21	-
Amikacin	11	17.5
Cephazolin	11	-
Ceftazidime	19	-
Ceftriaxone	21	-
Streptomycin	-	10
Erythromycin	25	27.5
Chloramphenicol	25	27.5
Vancomycin	-	-
Sulphamethoxazole	26	-
Sulphafurazole	-	-
Trimethoprim	20	-

+ = inhibition zone of at least 5 mm in diameter, - = No zone of inhibition was observed.

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Fluorescent in situ *hybridisation (FISH) monitoring of the survival of* Enterococcus mundtii *ST4SA*

Mass-cultured Kefir grains (5 g) and 5 g *Lb. plantarum* ST8KF(+) or ST8KF(-) enriched mass-cultured Kefir grains were placed in 100 ml sterile containers and 50 ml heat-treated, full cream milk (DairyBelle) was added. An 18-h-old culture of *E. mundtii* ST4SA (0.2%, v/v) grown in complete MRS broth was added to the milk mixture and all the containers incubated at 22°C for 24 h. After incubation, the grains were sieved and the resulting Kefir beverage was evaluated for the survival of the sensitive strain, using FISH. An 18-h-old culture of *E. mundtii* ST4SA, Kefir prepared with mass-cultured Kefir grains, and milk inoculated with the 0.2% (v/v) of an 18-h-old culture of *E. mundtii* ST4SA incubated at 22°C for 24 h, served as controls.

FISH was performed as described by Manz *et al.* (1999) using primer FluoroST4 that hybridizes with the specific gene sequence of *E. mundtii* ST4SA. The probe was 5'-labelled with fluorescein isothiocyanate (Invitrogen, Karlsruhe, Germany) and 1 µl probe (250 $ng.ml^{-1}$) was added to each Kefir sample to be hybridized. Optimal hybridization stringency required the addition of 35% (v/v) hybridization buffer (180 µl 5 M NaCl, 20 µl 1 M Tris, 450 µl MilliQ water, 350µl formamide and 1µl 10% (m/v) SDS), and all hybridizations were performed at 46°C for 90 min to 150 min. Optical sections were viewed with a Nikon eclipse E400 microscope equipped with a Nikon super high-pressure mercury lamp. Ten optical sections were viewed for each hybridised Kefir sample, and the experiment was repeated twice.

Results and discussion

Plasmid isolation and curing

Lactobacillus plantarum ST8KF harbours more than one plasmid (Fig. 1). Curing of plasmid ST8KF was achieved by growth in the presence of 80 $\mu l.ml^{-1}$ novobiocin. All concentrations of SDS were ineffective in generating ST8KF(-) variants (data not shown). All cured strains lost a 3.9 kb plasmid (Fig. 1).

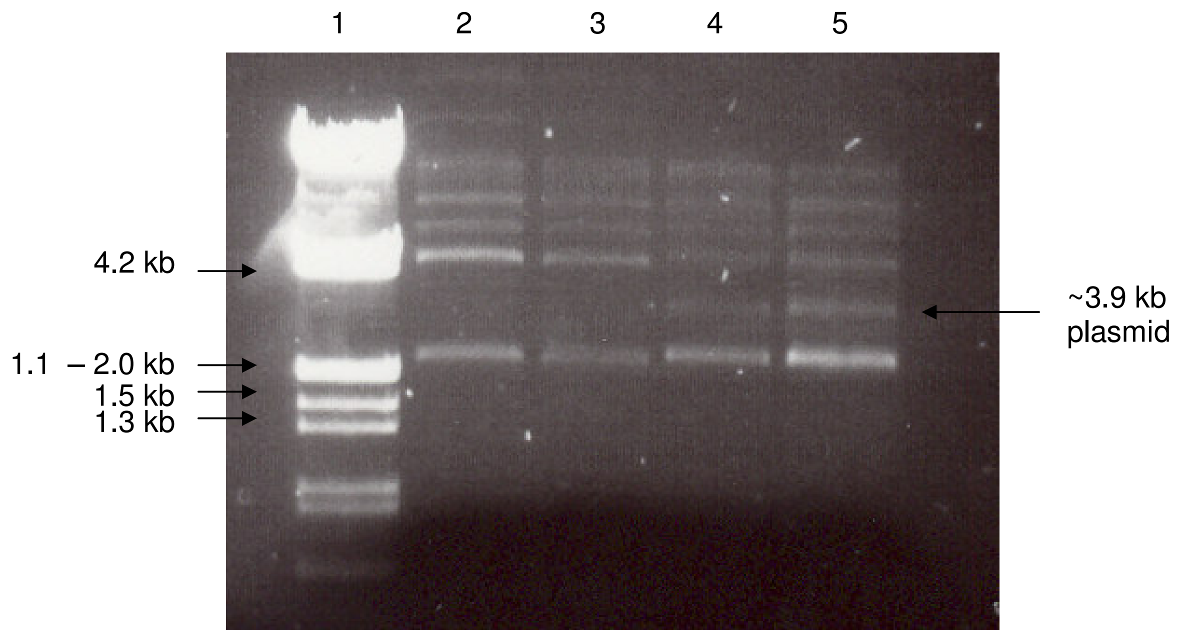

Figure 1 Agarose gel electrophoresis of plasmid DNA from *Lactobacillus plantarum* ST8KF(+) and the cured strain ST8KF(-). Lane 1 - Lambda marker, digested with *Eco*RI and *Hin*dIII (Roche, Indianopolis, IN, USA), Lane 2 and 3 – cured *Lactobacillus plantarum* ST8KF(-), Lane 4 and 5 – *Lactobacillus plantarum* ST8KF(+).

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Testing of *Lb. plantarum* ST8KF(+) and ST8KF(-) strains for antimicrobial activity (Todorov & Dicks, 2005) showed a loss in bacteriocin activity in the cured strain ST8KF(-) (data not shown). It would therefore appear that the genes responsible for bacST8KF production are located on the plasmid that is approximately 3.9 kb in size. It is suggested that the plasmid of approximate size 3.9 kb be isolated and sequenced. It is unknown whether this is a single plasmid, and sequencing would determine whether other plasmids are cryptic or not.

The genes encoding bacteriocin production for plantaricin 423, produced by *Lb. plantarum* 423 (Van Reenen *et al.*, 2003) and for plantaricin A, produced by *Lb. plantarum* C11 (Olasupo, 1996) are plasmid encoded. Plantaricin 423 is a small (approximately 3.5 kDa) plasmid-encoded protein and bacteriocin production is associated with the presence of an approximately 9 kb plasmid (Van Reenen *et al.*, 2003). The genes encoding plantaricin UG1, produced by *Lb. plantarum* UG1 (Enan *et al.*, 1996), plantaricin S and T, produced by *Lb. plantarum* LPCO10 (Jiménez-Diaz *et al.*, 1993) and plantaricin ST31 produced by *Lb. plantarum* ST31 (Todorov *et al.*, 1999) on the other hand, are chromosomal encoded.

Antibiotic resistance

Lactobacillus plantarum ST8KF(+) is sensitive to Neomycin, Tobramycin, Nitrofurantion, Ceforoxime, Fusidic Acid, Clindamcyin, Furazolidone, Cefotaxime, Rifampicin, Tetracycline, Ofloxacin, Cefepime, Amikacin, Cephazolin, Ceftazidime, Ceftriaxone, Erythromycin, Chloramphenicol, Sulphamethoxazole, Trimethoprim. After curing, *Lb. plantarum* ST8KF(-) became resistant to Ceforoxime, Clindamcyin, Furazolidone, Cefotaxime, Cefepime, Cephazolin, Ceftazidime, Ceftriaxone,

Sulphamethoxazole and Trimethoprim, and sensitive to Streptomycin (Table 1). These results show that the genes encoding antibiotic resistance for the affected antibiotics are located on the cured plasmid. To our knowledge, no information is available on the differences in antibiotic resistance between bacteriocin producing and bacteriocin mutant strains. These results should be further investigated in order to determine reproducibility, as the lost plasmid could harbour the genes encoding the specific mechanisms for the uptake of the antibiotics for which the test bacterium lost resistance.

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Carbohydrate fermentation reactions

Different fermentation reactions were recorded for *Lb. plantarum* ST8KF(+) and ST8KF(-). Strain ST8KF(-) lost the ability to utilise D-mannose, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, amygdalin, arbutin, salicin, D-cellobiose, D-lactose, D-saccharose, D-trehalose, D-melezitose and gentiobiose compared to the non-cured strain ST8KF(+). The genes encoding the metabolism of these sugars may be located on plasmid ST8KF cured from *Lb. plantarum* ST8KF.

Sugar fermentation reactions of the API system do not represent a reliable method for strain identification. This is due to the possibility that test strains could loose plasmids due to environmental stress, leading to inaccurate sugar fermentation reactions and identification. Bio-molecular methods should thus be used for strain identification as they supply more accurate results (Dicks & van Vuuren, 1987; Du Plessis & Dicks, 1995).

Fluorescent in situ *hybridization (FISH) monitoring of the survival of* Enterococcus mundtii *ST4SA*

The probe FluoroST4 was added to an 18-h-old culture of *E. mundtii* ST4SA and the sensitive strain viewed under fluorescent light with a Nikon Eclipse E400 microscope. The bacteria were found to fluoresce, indicating that the probe annealed to the specific gene sequence of the sensitive strain (data not shown). No cells of *E. mundtii* ST4SA were present in traditional Kefir produced by mass-cultured Kefir grains (Fig. 2). This result was expected as *E. mundtii* ST4SA is not an organism

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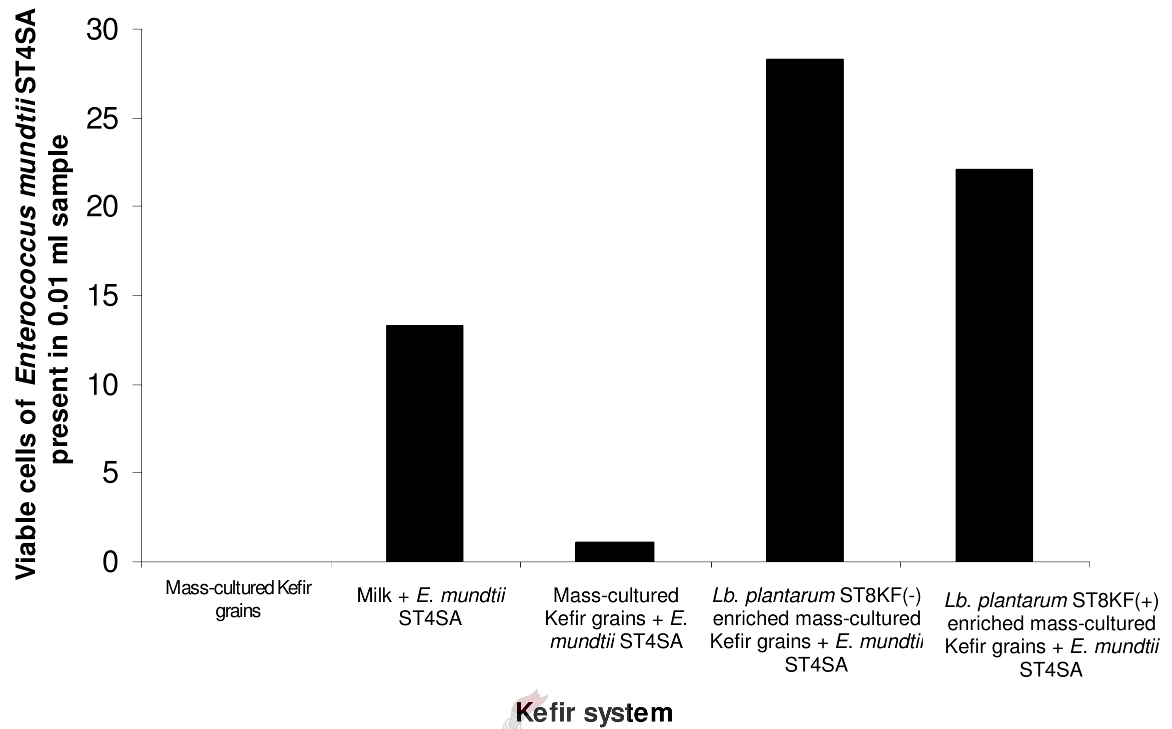

Figure 2 Survival of *Enterococcus mundtii* ST4SA in Kefir produced with *Lactobacillus plantarum* ST8KF(+) enriched mass-cultured Kefir grains, *Lactobacillus plantarum* ST8KF(-) enriched mass-cultured Kefir grains, mass-cultured Kefir grains, and milk. The data is an average of ten measurements.

naturally present in Kefir. Survival of *E. mundtii* ST4SA in milk indicates that the organism is not sensitive to the natural environmental stress conditions present in milk. When added to mass-cultured Kefir, *E. mundtii* ST4SA survived in low numbers (Fig. 2). This result was also expected as Kefir is a known inhibitor of a variety of spoilage and pathogenic bacteria (Garrotte e*t al*. 2000; Saloff-Coste, 1996; van Wyk, 2001). These three Kefir, milk systems were used as controls and from these tests it was concluded that the organism does not naturally occur in Kefir, that the sensitive strain has the ability to survive in milk, which indicates that a decrease in the sensitive strain in a Kefir system is not contributed to an inhibitory effect by milk, and that Kefir does have an inhibitory effect on the sensitive strain.

From these results it can be speculated that Kefir has an inhibitory effect on *E. mundtii* ST4SA, and by monitoring the survival of the sensitive strain in Kefir produced with mass-cultured Kefir grains enriched with *Lb. plantarum* ST8KF(+) and ST8KF(-) it can be determined whether this inhibition is due to the production of bacST8KF. *Enterococcus mundtii* ST4SA was found to be present in higher numbers (28 *E. mundtii* ST4SA cells per 10 µl Kefir sample) in the ST8KF(-) Kefir system when compared to the ST8KF(+) system (22 *E. mundtii* ST4SA cells per 10 µl Kefir sample) (Fig. 2). This result suggests that bacST8KF has an effect on the growth and survival of *E. mundtii* ST4SA in Kefir. From this result it can be speculated that *Lb. plantarum* ST8KF contributes to the antimicrobial activity of Kefir through the production of bacST8KF. *Enterococcus mundtii* ST4SA was used as a model spoilage microorganism, and from the result obtained for the sensitive strain is can also be speculated that *Lb. plantarum* ST8KF(+) is an active bacteriocin producer in Kefir. *Lactobacillus plantarum* ST8KF(+) has potential as an ideal starter culture, as bacST8KF is active against *Enterococcus faecalis* E88, *Lactobacillus casei* LHS, *Lactobacillus curvatus* DF38, *Lactobacillus sakei* DSM 20017, *Lactobacillus salivarius* 241 and *Listeria innocua* LMG 13568 and F. This experiment should be done in triplicate in order to determine the significance of this difference, and to confirm reproducibility.

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Conclusions

A plasmid of approximately 3.9 kb in size was successfully isolated from *Lb. plantarum* ST8KF and was found to encode bacteriocin production. Differences in sugar fermentation reactions and antibiotic resistance were shown between the bacteriocin producer and negative mutant *Lb. plantarum* ST8KF. This indicates the unreliability of the API system in the identification of microorganism to species level, and highlights the importance of the application of biomolecular techniques in species identification. The successful re-incorporation of *Lb. plantarum* ST8KF(+) and incorporation of *Lb. plantarum* ST8KF(-) into mass-cultured Kefir grains was done to determine the effect of the produced bacST8KF against food spoilage microorganisms. The reduction of *E. mundtii* ST4SA, which was used as a model strain, was shown in the presence of the bacteriocin producer *Lb. plantarum* ST8KF(+), which was incorporated into mass-cultured Kefir grains. From these results it can be concluded that the antimicrobial activity of Kefir and Kefir grains may be due to the presence of bacteriocins.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The antimicrobial activity of Kefir is well documented, and the beverage is known to have broad antitumor, antibacterial and antifungal properties (Saloff-Coste, 1996). Kefir is known to inhibit the growth of spoilage and pathogenic bacteria, including *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Clostridium tyrobutyricum* and *Listeria monocytogenes* (Van Wyk, 2001). Although the cause of this inhibition could be due to the production of organic acids, hydrogen peroxide (Shahani & Chandan, 1979; Juven *et al*., 1992), acetaldehyde, diacetyl or carbon dioxide produced by lactic acid bacteria (LAB) present in the Kefir grains (Helander *et al*., 1997), it has been reported that bacteriocins produced by LAB may play a role in the inhibitory activity of the Kefir beverage (Balasubramanyam *et al*., 1994; Morgan *et al*., 2000; Gulmez & Guven, 2003). The objective of this study was to determine whether the inhibitory activity of Kefir can in part be ascribed to the production of bacteriocins by LAB present in the Kefir grains.

The first aim was to isolate a bacteriocin producing strain from Kefir and Kefir grains, and to characterise the isolated bacteriocin. *Lactobacillus plantarum* ST8KF was isolated from Kefir and Kefir grains and was found to produce a bacteriocin (bacST8KF) 3.5 kDa in size. The bacteriocin is heat resistant, stable between pH 2.0 and 10.0 and adsorbs to the surface of the producer cell in low concentrations. BacST8KF also showed high levels of activity after 27 h of fermentation and was produced at lower levels during the initial stages of fermentation, indicating that bacST8KF may be a secondary metabolite. High levels of bacST8KF production towards the end of fermentation indicates that bacteriocin production within the Kefir system is optimal and suggests that fermentation should be initiated for at least 24 h to ensure maximum bacteriocin production. From these results it can be concluded that bacST8KF has potential for application in a variety of food processes as it remains stable at various temperatures and pH values.

The second aim of this study was to determine the parameters affecting the adsorption of the bacteriocin (bacST8KF). Optimal adsorption of bacST8KF was recorded at 25°C and pH 2.0 for *Enterococcus faecalis* E88 and at 4°C, 10°C and

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25°C and pH 6.0 for *Listeria innocua* LMG13568. Adsorption decreased in the presence of SDS, inorganic salts and organic compounds, and the growth of *Lb. plantarum* ST8KF and *L. innocua* LMG 13568 in mixed culture led to an increase in bacST8KF production. The wide tolerance range of bacST8KF may allow it future application in various fermentation processes, and bacST8KF could be used in a number of food products to prevent the growth of spoilage microorganisms.

The third aim was to determine whether bacST8KF contributes to the inhibitory activity of Kefir and Kefir grains. The genes encoding bacST8KF were found to be located on a plasmid approximately 3.9 kilo bases (kb) in size. *Lactobacillus plantarum* strains cured of this plasmid showed a loss in antimicrobial activity, indicating that the genes encoding bacteriocin production are plasmid encoded. Monitoring *Enterococcus mundtii* ST4SA, which is sensitive to bacST8KF, in Kefir produced with mass-cultured Kefir grains enriched with *Lb. plantarum* ST8KF(+) and the cured *Lb. plantarum* ST8KF(-) using fluorescent *in situ* hybridization, showed a decrease in the growth of the sensitive strain in the presence of the bacteriocin. It therefore may be that the inhibitory activity of Kefir and Kefir grains can be attributed to the production of bacST8KF by *Lb. plantarum* ST8KF.

The recent demand by consumers for natural preservatives has led to an increased interest in the use of natural antimicrobials in food products. The results obtained for bacST8KF indicates the potential use of the bacteriocin in the preservation of food products, and in the inhibition of food and spoilage microorganisms. It also indicates the potential use of *Lb. plantarum* ST8KF as a starter culture.

Future research could investigate the determination of the amino acid sequence of bacST8KF. Application of the bacteriocin producer strain in a model food system needs to be investigated in order to determine the bacteriocin effectivity. Interaction between the bacteriocin producer strain *Lb. plantarum* ST8KF and natural Kefir microflora as well as spoilage microflora needs to be investigated in detail. Effect of Kefir components, such as kefiran on bacteriocin activity must also be determined.

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