Lait (2006) 401–405
© INRA, EDP Sciences, 2006
DOI: 10.1051/lait:2006015

Note

Growth inhibition of *Enterococcus mundtii* in Kefir by in situ production of bacteriocin ST8KF

Jillian E. POWELL[a], Svetoslav D. TODOROV[b], Carol A. VAN REENEN[b], Leon M.T. DICKS[b], R. Corli WITTHUHN[a]*

[a] Department of Food Science, Stellenbosch University, Private Bag X1, 7602 Matieland, South Africa
[b] Department of Microbiology, Stellenbosch University, Private Bag X1, 7602 Matieland, South Africa

Received 14 June 2006 – Accepted 16 August 2006

Abstract – *Lactobacillus plantarum* ST8KF, isolated from Kefir grains, produces a 3.5 kDa bacteriocin (bacST8KF) active against *Enterococcus mundtii* ST. Kefir produced with grains containing *L. plantarum* ST8KF prevented the growth of *E. mundtii* ST in situ. No inhibition of *E. mundtii* ST was recorded when Kefir was produced from grains containing a plasmid-free and bacteriocin-negative variant (ST8KF⁻). Cells of *E. mundtii* ST were detected by fluorescent in situ hybridization (FISH). This is the first report on the incorporation of a bacteriocin-producing starter culture in Kefir grains and in situ control of microbial growth.

Kefir / mass-cultured grains / bacteriocin ST8KF / *Lactobacillus plantarum*

摘要 – 开菲尔原位产生的细菌素 ST8KF 对蒙特肠球菌的生长抑制作用。从开菲尔粒中分离出来的植物乳杆菌 (*Lactobacillus plantarum*) ST8KF 可以产生 3.5 kDa 的细菌素 ST8KF，该细菌素对 *Enterococcus mundtii* ST （蒙特肠球菌）具有生长抑制作用。由含有植物乳杆菌 ST8KF (*L. plantarum* ST8KF) 的开菲尔粒发酵产生的开菲尔对 *E. mundtii* ST 的生长有抑制作用，而无质粒和细菌素阴性变异体的开菲尔粒发酵产生的开菲尔对 *E. mundtii* ST 的生长没有抑制作用。采用荧光素原位杂交法 (FISH) 对 *Enterococcus mundtii* ST 细胞进行了原位观察。本文首次报道了通过原位控制微生物的生长，高密度培养富含植物乳杆菌 ST8KF 高产细菌素 ST8KF 的开菲尔粒。

关键词 开菲尔 / 开菲尔粒的高密度培养 / 细菌素 ST8KF / 植物乳杆菌

Résumé – Inhibition de la croissance d'*Enterococcus mundtii* dans le kéfir par la production in situ de la bactériocine ST8KF. *Lactobacillus plantarum* ST8KF, isolée de grains de kéfir, produit une bactériocine (bacST8KF) de 3,5 kDa. Dans cette étude, du kéfir a été produit avec *L. plantarum* ST8KF dans le but de suivre la production de la bactériocine in situ. La croissance de *E. mundtii* ST était inhibée dans le kéfir ainsi obtenu. Aucune inhibition n'a été observée dans les kéfirs obtenus à partir de grains contenant le variant sans plasmide et le variant bactériocine-négative (ST8KF⁻). Les cellules de *E. mundtii* ST ont été détectées par hybridation de fluorescence in situ (FISH).

kéfir / grain cultivé en masse / bactériocine ST8KF / *Lactobacillus plantarum*

* Corresponding author (通讯作者): rcwit@sun.ac.za

Article published by EDP Sciences and available at http://www.edpsciences.org/lait or http://dx.doi.org/10.1051/lait:2006015

1. INTRODUCTION

Kefir is a fermented milk product with a slightly acidic taste, yeasty flavor and creamy consistency [12]. The starter cultures, which consist mainly of lactic acid bacteria, propionibacteria and yeasts, are entrapped in Kefir grains held together by polysaccharides [6, 12]. 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* [12, 18]. The reason for growth inhibition is not known, but may be due to the presence of lactic acid, volatile acids, hydrogen peroxide [4, 13], carbon dioxide, diacetaldehyde, acetaldehyde and bacteriocins [3]. The possibility of organic acids being responsible for antimicrobial activity was ruled out by growth inhibition obtained with pH-neutralized Kefir [7]. In at least one paper [1], antimicrobial activity was associated with antimicrobial peptides. Filter-sterilized Kefir treated with trypsin lost all activity [1], suggesting that growth inhibition could be caused by bacteriocins.

Bacteriocins are ribosomal synthesized proteins or peptides with activity against genetically closely related species [5, 14]. They are grouped into four classes, based on structure, mode of action, genetic origin, biochemical properties and spectrum of antimicrobial activity. The genetic determinants of most bacteriocins are located on plasmids, with only a few exceptions being chromosomally encoded. Little is known about the regulation of these peptides and in situ production [8].

The aim of this study was to incorporate Kefir grains with a bacteriocin-producing strain of *Lactobacillus plantarum* (ST8KF) and study the production of the bacteriocin (bacST8KF) in situ. *Enterococcus mundtii* ST served as a target organism. The results were compared with Kefir produced with a plasmid-free and bacteriocin-negative strain (ST8KF$^-$).

2. MATERIALS AND METHODS

2.1. Growth conditions

Lactobacillus plantarum ST8KF [10] isolated from Kefir grains, and *E. mundtii* ST from the culture collection of the Department of Microbiology, University of Stellenbosch, were cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa). The strains were stored at –80 °C in MRS broth (Biolab), supplemented with 15% (v/v) glycerol.

2.2. Isolation of plasmid DNA from *L. plantarum* ST8KF

Plasmid DNA was isolated from 24 h-old cells using the Qiagen Plasmid Midi Kit (Southern Cross Biotechnology, Cape Town, South Africa). Excess proteins were removed by an additional phenol treatment. Plasmid DNA was visualized by separation on 1% (m/v) agarose [17].

2.3. Plasmid curing

Plasmid curing was performed as described by Ruiz-Barba et al. [11]. Strain ST8KF was grown in MRS broth (Biolab), supplemented with 5 $\mu L \cdot mL^{-1}$ to 320 $\mu L \cdot mL^{-1}$ Novobiocin (Sigma, St Louis, USA) and 0.1 $mg \cdot mL^{-1}$ to 1.0 $mg \cdot mL^{-1}$ SDS, respectively, and inoculated with 0.3% (v/v) *L. plantarum* ST8KF. After 48 h at 30 °C, cultures resistant to the highest concentration of Novobiocin and SDS were selected, streaked onto MRS agar (Biolab) and incubated at 30 °C for 48 h. Colonies were randomly selected and tested for bacteriocin activity according to the triple-agar-layer method [15]. Isolates without zones of growth inhibition were selected and plasmid DNA isolated as described before.

2.4. Carbohydrate fermentation reactions

Carbohydrate fermentation reactions of *L. plantarum* ST8KF and ST8KF$^-$ were determined using the API 50 CHL test kit according to the instructions of the

manufacturer (bioMérieux® S.A., Marcy L'Étoile, France).

2.5. Mass cultivation and enrichment of Kefir grains

For the mass cultivation of Kefir grains full cream milk (400 mL), supplemented with 20 $g \cdot L^{-1}$ yeast extract (Biolab) and 5.0 $g \cdot L^{-1}$ urea (Biolab), was pasteurized (90 min at 80 °C) and inoculated with Kefir grains (40 g) from a stock culture. Fermentation was at 25 °C in a shaking water bath (120 rpm). Every 24 h the grains were sieved and replenished with pasteurized milk of the same composition.

Lactobacillus plantarum ST8KF and ST8KF$^-$ were cultured, separately, in 50 mL MRS broth (Biolab) to 1×10^8 $cfu \cdot mL^{-1}$, and the cells were harvested (6000× *g*, 10 min, 4 °C) and re-suspended in 1 mL sterile physiological salt. This was used to inoculate 20 g mass-cultured grains suspended in 200 mL pasteurized milk, supplemented with yeast extract and urea. Fermentation was at 25 °C at 120 rpm. Grains were sieved every 24 h and replenished with pasteurized milk of the same composition. Inoculation with *L. plantarum* ST8KF and ST8KF$^-$ was every 48 h. Enrichment was for 40 days at 25 °C at 120 rpm.

In a separate experiment, 5 g mass-cultured grains, and 5 g grains enriched with *L. plantarum* ST8KF and ST8KF$^-$, respectively, were added to 50 mL pasteurized full cream milk inoculated with 0.2% (v/v) of an 18 h-old culture of *E. mundtii* ST. Fermentation was for 24 h at 22 °C.

2.6. Fluorescent in situ hybridization (FISH)

Grains were sieved from Kefir containing *E. mundtii* ST and the cells hybridized in situ (FISH) with a DNA probe obtained from amplification with primers STF (TGAGAGAAGGT) and STR (TCCACTGAAAT). PCR was performed according to the method described by [19]. The probe was 5'-labeled with fluorescein isothiocyanate (Invitrogen, Karlsruhe, Germany). One µL (250 $ng \cdot mL^{-1}$) of the probe was added to a Kefir sample. Optimal stringency required the addition of 35% (v/v) hybridization buffer (180 µL 5 $mol \cdot L^{-1}$ NaCl, 20 µL 1 $mol \cdot L^{-1}$ Tris, 450 µL MilliQ water, 350 µL formamide and 1 µL 10%, m/v, SDS). Hybridizations were performed at 46 °C for 90 to 150 min. Fluorescence was detected with a Nikon eclipse E400 microscope (Innovative Met Products (IMP), Somerset-West, South Africa) equipped with a Nikon super high-pressure mercury lamp. Ten optical fields were counted for each sample, in triplicate.

3. RESULTS AND DISCUSSION

3.1. Plasmid isolation and curing

Lactobacillus plantarum ST8KF harbors at least 6 plasmids (Fig. 1). Growth in the presence of 80 $\mu L \cdot mL^{-1}$ Novobiocin resulted in the loss of a 3.9 kb plasmid and the ability to produce bacST8KF (Fig. 1). Growth in the presence of SDS did not result in plasmid loss or changes in bacST8KF activity. Based on these results, the genes encoding bacST8KF production are located on the plasmid. This is in agreement with most other reports for bacteriocins of *L. plantarum*. The genes coding for plantaricin 423 and plantaricin C11 are located on bigger plasmids of approximately 9 kb [9, 17]. Only a limited number of plantaricins are encoded by genes located on the genome, e.g. plantaricin UG1 [2] and plantaricin ST31 [16].

3.2. Carbohydrate fermentation reactions

Different fermentation reactions were recorded for *L. plantarum* ST8KF and ST8KF$^-$. Strain ST8KF$^-$ lost the ability to metabolize 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. The genes encoding the metabolism of these sugars may be located on plasmid pST8KF.

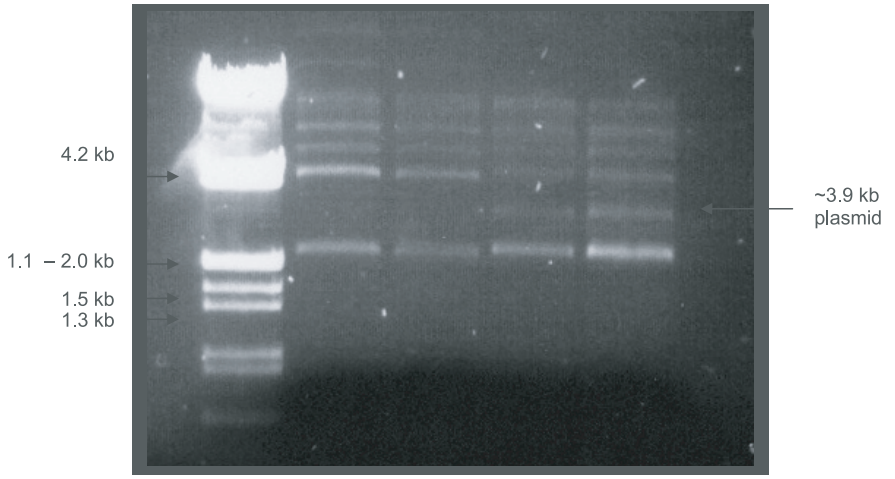

Figure 1. Agarose gel electrophoresis showing no plasmid DNA present in the cured strain, *L. plantarum* ST8KF^{-} (lanes 2 and 3) and the presence of a 3.9 kb plasmid in the wild-type strain, *L. plantarum* ST8KF (lanes 4 and 5). Lane 1: Lambda marker, digested with *Eco*RI and *Hin*dIII (Roche, Indianopolis, IN, USA).

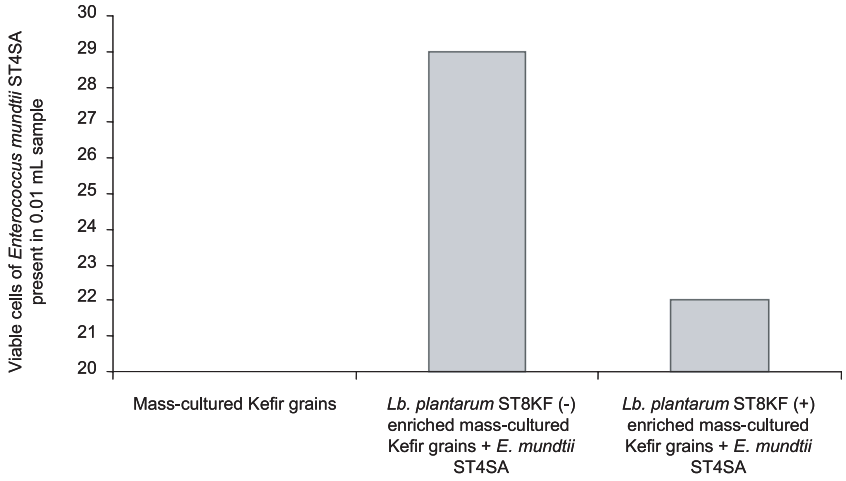

Figure 2. Survival of *E. mundtii* ST in Kefir produced from grains enriched with *L. plantarum* strains ST8KF and ST8KF^{-}, respectively. *Enterococcus mundtii* ST were recorded by FISH.

3.3. Survival of *E. mundtii* ST in Kefir

Low cell numbers of *E. mundtii* were recorded in Kefir produced with *L. plantarum* ST8KF (Fig. 2). However, high cell numbers of *E. mundtii* (28 cells per 10 µL sample) were recorded in Kefir produced with *L. plantarum* ST8KF^{-} (Fig. 2). Concluding from these results, the Kefir grains were successfully enriched with *L. plantarum* ST8KF, with the level of bacST8KF production high enough to inhibit the growth of *E. mundtii* ST. *Lactobacillus*

plantarum ST8KF could be used as a starter culture in Kefir production.

Acknowledgements: The National Foundation for Research (NRF), South Africa is thanked for their financial support.

REFERENCES

[1] Balasubramanyam B.V., Varadaraj M.C., Dahi as a potential source of lactic acid bacteria active against foodborne pathogenic and spoilage bacteria, J. Food Sci. Technol. 31 (1994) 241–243.

[2] Enan G., El-Essawy A.A., Uyttendaele M., Debevere J., Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1, Int. J. Food Microbiol. 30 (1996) 189–215.

[3] Helander I.M., von Wright A., Mattila-Sandholm T.M., Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria, Trends Food Sci. Technol. 8 (1997) 146–150.

[4] Juven B.J., Schved F., Linder P., Antagonistic compounds produced by a chicken intestinal strain of *Lactobacillus acidophilus*, J. Food Prot. 55 (1992) 157–161.

[5] Klaenhammer T.R., Bacteriocins of lactic acid bacteria, Biochimie 70 (1988) 337–349.

[6] Kwak H.S., Park S.K., Kim D.S., Biostabilization of kefir with a non-lactose-fermenting yeast, J. Dairy Sci. 79 (1996) 937–942.

[7] Morgan S.M., Hickey R., Ross R.P., Hill C., Efficient method for the detection of microbiologically-produced antibacterial substances from food systems, J. Appl. Microbiol. 89 (2000) 56–62.

[8] Naidu A.S., Bidlack W.R., Clemens R.A., Probiotic spectra of lactic acid bacteria (LAB), Critical Rev. Food Sci. Nutr. 38 (1999) 13–126.

[9] Olasupo N.A., Bacteriocins of *Lactobacillus plantarum* strains from fermented foods, Food Microbiol. 41 (1996) 130–136.

[10] Powell J.E., Witthuhn R.C., Todorov S.D., Dicks L.M.T., Characterization of bacteriocin ST8KF produced by a Kefir isolate *Lactobacillus plantarum* ST8KF, Int. Dairy J. (2006) doi: 10.1016/j.dairyj.2006.02.012.

[11] Ruiz-Barba J.L., Piard J.C., Jiménez-Diaz R., Plasmid profiles and curing plasmids of *Lactobacillus plantarum* strains isolated from green olive fermentations, J. Appl. Bacteriol. 71 (1991) 417–421.

[12] Saloff-Coste C.J., Kefir. Nutritional and Health Benefits of yoghurt and fermented milks, Danone World Newsletter 11 (1996) 1–7.

[13] Shahani K.M., Chandan R.C., Nutritional and healthful aspects of cultured and culture-containing dairy foods, J. Dairy Sci. 62 (1979) 1685–1694.

[14] Tagg J.R., Dajani A.S., Wannamaker L.W., Bacteriocins of Gram-positive bacteria, Bacteriol. Rev. 40 (1976) 722–756.

[15] Todorov S.D., Dicks L.M.T., *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria, Enzyme Microbiol. Technol. 36 (2005) 318–326.

[16] Todorov S., Onno B., Sorokine O., Chobert J.M., Ivanova I., Dousset X., Detection and characterization of a novel antibacterial substance produced by *Lactobacillus plantarum* ST 31 isolated from sourdough, Int. J. Food Microbiol. 48 (1999) 167–177.

[17] Van Reenen C.A., Chikindas M.L., van Zyl W.H., Dicks L.M.T., Characterisation and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*, Int. J. Food Microbiol. 81 (2003) 29–40.

[18] Van Wyk J., Britz T.J., Myburgh A.S., Arguments supporting Kefir marketing to the low-income urban African population in South Africa, Agrekon 41 (2002) 43–62.

[19] Zendo T., Eungruttanagorn N., Fujioka S., Tashiro Y., Nomura K., Sera Y., Kobayashi G., Nakayama J., Ishizaki A., Sonomoto K., Identification and production of a bacteriocin from *Enterococcus mundtii* QU2 isolated from soya, J. Appl. Microbiol. 99 (2005) 1181–1190.