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ANALYSIS OF NATURAL ISOLATES OF LACTOBACILLI RESISTANT TO BACTERIOCIN NISIN

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The collection of lactic acid bacteria (LAB) was made by isolation of microorganisms from fermented products traditionally manufactured in different geographical regions (high mountains, river valleys, seaside, etc). Among collected LAB, 51 isolates were identified as *Lactobacillus* sp. Results showed that all isolated lactobacilli were mesophilic strains, since they grew at 15°C and 30°C but not at 45°C. Testing the ability of isolated lactobacilli to grow in the presence of nisin revealed that Lactobacillus sp. isolates designed BGCGK4, BGHN40, BGBUK2-8, BGBUK2-7 and BGBUK2-16 were resistant to nisin. Determination of the minimal inhibitory concentrations (MIC) for nisin revealed that the most resistant isolate was *Lactobacillus* sp. BGCGK4. Isolate BGBUK2-16, determined as *Lactobacillus paracasei* subsp. *paracasei*, produces bacteriocin, named Bac217 and showed a resistance to 8000 IU/ml of nisin. Plasmid curing of BGBUK2-16 resulted in derivatives BGBUK2-

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16/K2 and BGBUK2-16/K4. Derivative BGBUK2-16/K2 retained resistance to Bac217 and nisin, but lost the ability to synthesise Bac217. Derivative BGBUK2-16/K4 lost concomitantly the resistance to both Bac217 and nisin.

Key words: *Lactobacillus*, natural isolates, antimicrobial activity, nisin, resistance

INTRODUCTION

Many factors contribute to a successful natural fermentation of carbohydrate-rich food and feed products. Metabolic activities of lactic acid bacteria (LAB) play a leading role in this process. Besides product of lactic acid, their other specific effects are difficult to quantitate (hydrogen peroxide, diacetyl, etc). Nisin is the best known bacteriocin of LAB. It belongs to the class I bacteriocins, which are known as lantibiotics (NES, *et al*., 1996). It has been shown that chromosomally located conjugative transposons in *L. lactis* were identified to be involved in the transmission of the structural gene of nisin among lactococci (HOM *et al*.,1991; RAUCH and DE VOS, 1992). The production of bacteriocin nisin by LAB and their resistance to nisin are sometimes difficult to evaluate as well (HARRIS *et al*., 1992).

It is known that nisin as well as other bactericins may play a role in selecting the microflora which initiates the fermentation. Bacteriocins are believed to be important in the ability of LAB to compete in non-fermentative ecosystems. Moreover, interest has arisen in the use of other antagonistic activities of LAB to extend the shelf-life of protein rich products (DAESCHEL *et al.*, 1991).

There are several reasons for studying production and resistance to bacteriocins in lactobacilli. Firstly, the genetic determination for bacteriocin production and resistance to it has great potential as genetic markers in recombinant DNA technology (COLLINS-THOMPSON *et al.*,1985). Secondly, using of bacteriocin producing starter cultures may result in a more reliable fermentation process preventing growth of spoilage bacteria. Moreover, nisin resistant (Nis^r) strains of lactobacilli could be possibly used as either as a component of existing starter cultures or solely in combination with food-grade additive nisin for controlled lactic acid fermentation (DE REYTER *et al.*,1996).

The purpose of this study was to analyse the resistance to nisin of lactobacilli isolated from different traditionally manufactured fermented products.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions. The bacterial strains used in this study are listed in Table 1. Preliminary identification of isolated LAB was done on the basis of their biochemical properties. The determination of some isolates was also done according to its fermentation ability by using API 50CHL and API 50 Strep, (Api System S.A., Bio-Merieux, Montelieu–Vercieu, France) and other classical microbiological techniques. *Lactobacillus* strains were cultured in MRS broth (Merck GmbH Darmstadt, Germany). *Lactococcus* strain used in this

study was grown in M17 medium (Merck GmbH Darmstadt, Germany) supplemented with glucose (0.5%, w/v) (GM17 broth) and incubated at 30°C for 24 h. To each medium agar (2%, w/v) (Difco, Detroit, Mich.) was added when used as a solid medium.

Table 1. The bacterial strains used in this study

Strains	Description	Source or reference
Lactobacillus paracasei subsp. *paracasei*		
BGBUK2-16	Natural isolate, $Bac217^+$, 217^r, Prt^+	(LOZO *et al*., 2004)
BGBUK2-16/K2	Derivative of BGBUK2-16, $Bac217^-$, 217^r, Prt^+	(LOZO *et al*., 2004)
BGBUK2-16/K4	Derivative of BGBUK2-16, $Bac217^-$, 217^s, Prt^+	(LOZO *et al*., 2004)
Lactobacillus sp.		
BGBUK2-8	Natural isolate, Bac^+, Prt^+	Laboratory collection
BGBUK2-7	Natural isolate, Bac^+, Prt^+	Laboratory collection
BGCGK4	Natural isolate	Laboratory collection
BGHN40	Natural isolate	Laboratory collection
BGLI15	Natural isolate	Laboratory collection
Lactococcus lactis subsp. *lactis*		
NP 45	Nisin producer, Nis^+, Nis^r	Laboratory collection

Bac^+ - bacteriocin producer; Bac^- - bacteriocin non-producer; Bac^r – resistance to bacteriocin; Bac^s – bacteriocin sensitive; Prt^+ - proteinase producer; Nis^+ - nisin producer; Nis^r – resistance to nisin

Plasmid curing. Plasmid curing was achieved by growing the cells in the presence of novobiocin at sublethal temperature (GAJIĆ *et al.,* 1999). Prewarmed MRS broth (43°C) containing novobiocin (8 μg/ml) was inoculated with 10^3 cells per ml. After incubation for 2 h cells were collected by centrifugation and resuspended in the same volume of prewarmed MRS broth containing novobiocin to avoid a bacteriocin-killing-effect towards cured (Bac^-, Bac^s) cells. This procedure was repeated five times, and then aliquots (0.1 ml) were plated onto MRS agar plates that were incubated at 30°C for 48 h. To detect Bac^-, Bac^s derivatives, master plates were made in duplicate. One of the plates was overlaid with MRS soft agar (0.7%) containing indicator strain *Lactobacillus* sp. BGLI15 and incubated overnight at 30°C. Colonies that did not inhibit the indicator strain were taken from the original master plate and retested for their Bac^-, Bac^s phenotype by using them as indicator strains.

The influence of bacteriocin nisin on the growth of natural isolates. To determine the influence of food-grade bacteriocin nisin on the growth of natural isolates of lactobacilli an agar-well diffusion assay was performed (TAGG and

MCCGIVEN, 1971). Aliquots (100 μl) of prepared dilutions of commercially used nisin (Applin and Barrett, Trowbridge, England) in rising concentrations (100, 500, 1000, 5000, 10000 and 25000 IU/ml) were poured into the wells. *Lactococcus lactis* subsp. *lactis* NP45, the nisin producer was used as a control.

Minimal inhibitory concentration (MIC) determination. Determination of the MIC was conducted as described previously (ARSENIJEVIĆ and TOPISIROVIĆ, 2000). The MRS medium (5 ml) containing increasing concentrations of nisin was inoculated with 1000 times diluted overnight culture of lactobacilli grown in the absence of nisin. The tubes were incubated at appropriate temperature. The final cells number (CFU/ml) were determined at the moment of inoculation, after 24 h and 48 h of incubation. The MIC value represents the concentration of nisin at which no growth of inoculated bacteria was observed after 48 h of incubation. *Lactococcus lactis* subsp. *lactis* NP45, the nisin producer was used as a control.

RESULTS

Screening for nisin resistant isolates. The collection of LAB was intentionally made by isolating the strains from traditionally manufactured fermented products from geographically different regions (high mountains, river valleys, seaside, etc). Among collected LAB, 51 isolates were identified as *Lactobacillus* sp. by using their carbohydrate fermentation abilities.

Analysis of isolated lactobacilli showed that all of them were mesophilic strains having the ability to grow in MRS broth at 15°C and 30°C but not at 45°C. All 51 isolates were tested for the resistance to nisin by using an agar-well diffusion assay. Antimicrobial activity of nisin was scored by clear zone appearance around the well in the lawn of indicator nisin-sensitive strains, which is the indication of the growth inhibition (data not shown). Results showed that isolates, *Lactobacillus* sp. BGCGK4, BGHN40, BGBUK2-8, BGBUK2-7 and BGBUK2-16 were resistant to nisin i.e. there is no a clear zone of growth inhibition around the well containing the rising concentration of pure nisin. Isolate BGBUK2-16 was identified as *Lactobacillus paracasei* subsp. *paracasei* by molecular determination (data not shown). Plasmid curing of BGBUK2-16 resulted in obtaining derivatives, designated BGBUK2-16/K2 and BGBUK2-16/K4. The analysis of these derivatives revealed that derivative BGBUK2-16/K2 retained Nis^r phenotype whereas BGBUK2-16/K4 lost it. Interestingly, isolate BGBUK2-16 is a producer of bacteriocin, named Bac217. The derivative BGBUK2-16/K2 lost the ability to produce Bac217 but retained the resistance to it. In contrast, derivative BGBUK2-16/K4 lost both Bac217 production as well as resistance to it.

Determination of the minimal inhibitory concentration (MIC). To determine the MIC values, the five selected *Lactobacillus* isolates (BGCGK4, BGHN40, BGBUK2-8, BGBUK2-7 and BGBUK2-16) and two derivatives of BGBUK2-16 (BGBUK2-16/K2 and BGBUK2-16/K4) were grown on the rising concentrations of nisin. Concentrations of nisin used for MIC evaluation were selected by previously determined the resistance to nisin of selected lactobacilli by

agar-well diffusion assay. Therefore, BGCGK4 was grown in the MRS broth containing nisin in the range of 0 to 20000 IU/ml (Fig. 1A), whereas isolates BGHN40, BGBUK2-7 and BGBUK2-8 were grown in the range of nisin from 0 to 10000 IU/ml (Fig. 1B, C, D).

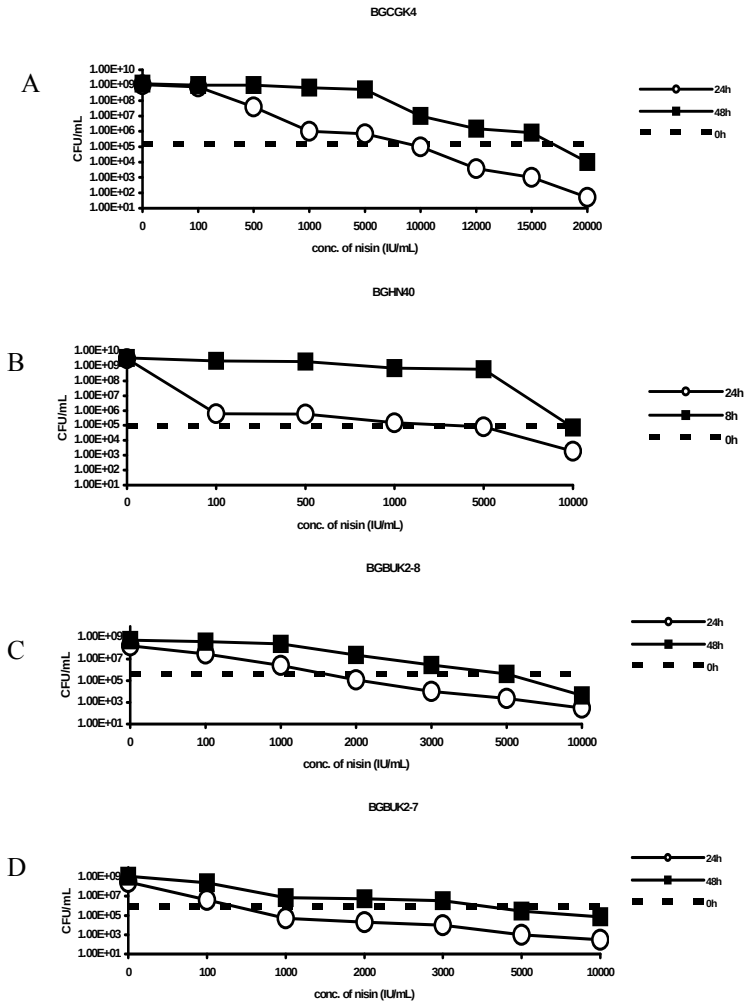

Fig. 1. Growth of the strains BGCGK4 (A), BGHN40 (B), BGBUK2-8 (C) and BGBUK2-7 (D) in the rising concentrations of nisin

The strain *Lactobacillus paracasei* subsp. *paracasei* BGBUK2-16 and its derivative BGBUK2-16/K2 were grown in MRS broth containing nisin in the range of 0 to 10000 IU/ml (Fig. 2A, B). Derivative BGBUK2-16/K4 was not able to grow in the presence of nisin (100 IU/ml) (Fig. 2C). After incubation of 48 h at

30°C the MIC values for nisin were determined. Results showed that the most resistant isolate was BGCGK4 (Table 2).

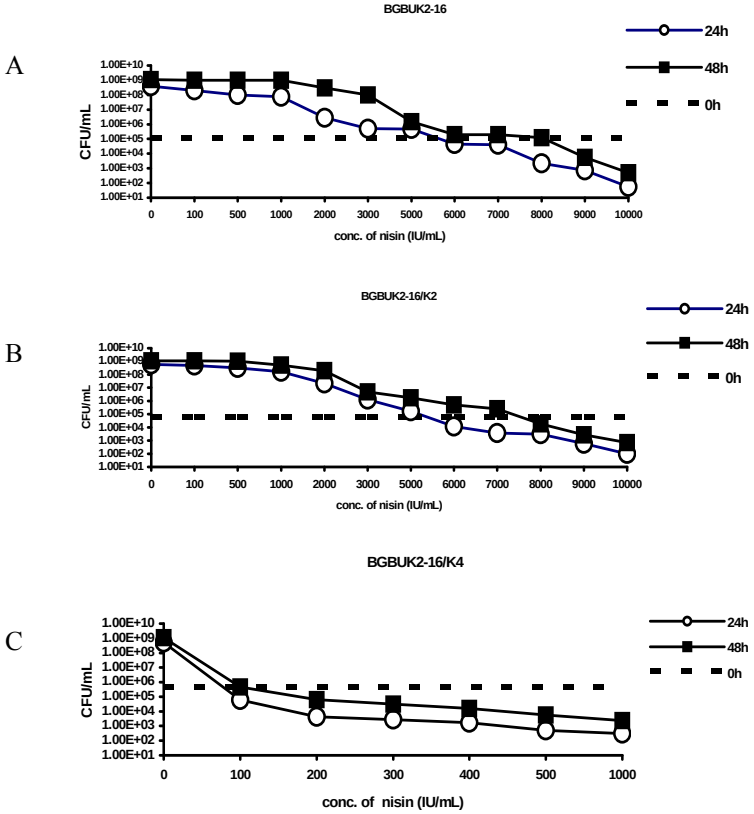

Fig. 2. Growth of the strain BGBUK2-16 (A), and its derivatives BGBUK2-16/K2 (B) and BGBUK2-16/K4 (C) in the rising concentrations of nisin

Table 2. Determined MIC values for selected strains

Strain	MIC (IU/ml)
BGCGK4	15000
BGHN40	10000
BGBUK2-8	5000
BGBUK2-7	5000
BGBUK2-16	8000
BGBUK2-16/K2	8000
BGBUK2-16/K4	100

DISCUSSION

Lactic acid bacteria (LAB) have an important role in the production of fermented foods, considering their ability to produce agents capable of inhibiting

the growth of a wide variety of food spoilage organisms. The most widely applied natural preservative in food industry is a nisin (JUNG, 1991; SAHL and BIERBAUM, 1998). Limitation of the application of nisin as a natural preservative is the high sensitivity of the starter microorganisms to nisin. Recently, increasing attention has been focused on the development of new starter cultures. The isolation and characterisation of the LAB from the home-made dairy product could be useful since these strains potentially harbour the ability to produce new flavours and other relevant characteristics. The main goal of this work was to identify nisin resistant lactobacilli from natural ecosystems.

Recently analysed *Lactobacillus* strains were sensitive to the low nisin concentrations as well as the most Gram positive bacteria (CHOI and PARK, 2000). The preliminary results obtained by testing isolates of lactobacilli from our laboratory collection indicated that five of 51 were naturally resistant to nisin. The minimal inhibitory concentrations to nisin were determined for strains BGCGK4, BGHN40, BGBUK2-8, BGBUK2-7, BGBUK2-16, and its derivatives BGBUK2-16/K2 and BGBUK2-16/K4. The obtained results clearly showed that the strain BGCGK4 was resistant to the highest nisin concentration (15000 IU/ml). Moreover, the strains BGHN40, BGBUK2-8, BGBUK2-7 and BGBUK2-16 had also very high MIC values. Considering the obtained data it could be speculated that the strains BGCGK4, BGHN40, BGBUK2-8, BGBUK2-7 and BGBUK2-16 carrying genetic information for the nisin resistance. This suggests the possible use of these strains as starter cultures for dairy products with the nisin as a natural preservative.

One of the BGBUK2-16 derivatives obtained after plasmid curing, BGBUK2-16/K2, kept the resistance to its own bacteriocin Bac217 and retained the same level of the resistance to nisin as the parental strain BGBUK2-16. On the other hand, plasmid-cured derivative BGBUK2-16/K4 lost both resistance to Bac217 and nisin. These results suggested that there is possible correlation between the resistance to Bac217 and nisin. Further research will give an answer to this proposition.

CONCLUSION

In this paper we presented the natural isolates of lactobacilli, resistant to the high nisin concentration that could be possibly used as a component of existing starter cultures or solely in combination with food-grade additive nisin for controlled lactic acid fermentation.

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PRIRODNI IZOLATI LAKTOBACILA REZISTENTNI NA BAKTERIOCIN NIZIN

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I z v o d

Kolekcija bakterija mlečne kiseline (BMK) je napravljena od mikroorganizama izolovanih iz fermentisanih mlečno-kiselinskih proizvoda dobijenih na tradicionalan način. Iz kolekcije 51 izolat je identifikovan kao *Lactobacillus* sp. Svi izolovani laktobacili pripadaju grupi mezofilnih sojeva koji dobro rastu na temperaturama od 15°C i 30°C, a ne rastu na temperaturi od 45°C. Testiranje sposobnosti rasta u prisustvu nizina pokazalo je da su izolati BGCGK4, BGHN40, BGBUK2-8, BGBUK2-7 i BGBUK2-16 rezistentni na bakteriocin nizin. U eksperimentu odredjivanja minimalne inhibitorne koncentracije (MIK) za nizin pokazano je da je najrezistentniji izolat *Lactobacillus* sp. BGCGK4. Izolat BGBUK2-16, determinisan kao *Lactobacillus paracasei* subsp. *paracasei,* produkuje bakteriocin označen kao Bac217 i pokazuje rezistenciju na 8000 IU/ml. Čišćenjem plazmida iz soja BGBUK2-16 dobijena su 2 derivata označena kao BGBUK2-16/K2 i BGBUK2-16/K4. Derivat BGBUK2-16/K2 zadržao je rezistenciju na Bac217 i nizin, ali je izgubio sposobnost sinteze Bac217, dok je derivat BGBUK2-16/K4 pored gubitka sposobnosti sinteze Bac217 postao senzitivan na Bac217 i nizin. Prirodno rezistentni laktobacili se mogu iskoristiti za pripremanje starter kultura u kombinaciji sa nizinom kao konzervansom u cilju kontrolisane mlečno-kiselinske fermentacije.

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