Bacteriocin production and resistance to drugs are advantageous features for *Lactobacillus acidophilus* La-14, a potential probiotic strain

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SUMMARY

L. acidophilus La-14 produces bacteriocin active against *L. monocytogenes* ScottA (1600 AU/ml) in MRS broth at 30°C or 37°C. The bacteriocin proved inhibitory to different serological types of *Listeria* spp. Antimicrobial activity was completely lost after treatment of the cell-free supernatant with proteolytic enzymes. Addition of bacteriocin produced by *L. acidophilus* La-14 to a 3 h-old culture of *L. monocytogenes* ScottA repressed cell growth in the following 8h. Treatment of stationary phase cells of *L. monocytogenes* ScottA (10^7-10^8 CFU/ml) by the bacteriocin resulted in growth inhibition.
Growth of *L. acidophilus* La-14 was not inhibited by commercial drugs from different generic groups, including non-steroidal anti-inflammatory drugs (NSAID) containing diclofenac potassium or ibuprofen arginine. Only one non-antibiotic drug tested, Atlansil (an antiarrhythmic agent), had an inhibitory effect on *L. acidophilus* La-14 with MIC of 2.5 mg/ml. *L. acidophilus* La-14 was not affected by drugs containing sodium or potassium diclofenac. *L. acidophilus* La-14 shows a good resistance to several drugs and may be applied in combination for therapeutic use.

KEY WORDS: Lactobacillus acidophilus, Probiotic, Bacteriocin, Medicaments

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INTRODUCTION

Bacteriocins are ribosomally synthesized antibacterial peptides and are usually active against genetically related species. They have been grouped into 4 classes based on their structure and mode of action (Heng *et al.*, 2007). In the last two decades several reports focused on the production of bacteriocins from lactic acid bacteria isolated from different fermented products, vegetables, fruits, meat, fish, human and animal gastrointestinal tract (GIT) (Todorov, 2009).
Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2001). The best known examples of probiotic foods are fermented milks and yogurts, which are generally consumed within days or weeks of manufacture (Nagpal *et al.*, 2007), as well as other dairy products, including cheeses (Cruz *et al.*, 2009b) and ice-creams (Cruz *et al.*, 2009a). Besides better growth and survival during food manufacturing and storage and in the GIT, protection against acid, bile, and gastrointestinal enzymes, and adhesion to intestinal epithelium, antimicrobial properties and antibiotic resistance could be considered factors that might be important in maintaining probiotic efficacy (Ranadheera *et al.*, 2010).

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Probiotic *Lactobacillus* species have been implicated in a variety of beneficial roles for the human body, including maintenance of the normal intestinal microbiota, pathogen interference, exclusion and antagonism, immunostimulation and immunomodulation, anticarcinogenic and antimutagenic activities, deconjugation of bile acids, and lactase release *in vivo* (Klaenhammer, 1988; Guarner and Malagelada, 2003; Shah, 2007; Tuohy *et al.*, 2003). Consequently, the potential health-promoting effect of dairy products that incorporate *Lactobacillus* species and other probiotic organisms has stimulated considerable research (Buriti *et al.*, 2005). *Lactobacillus acidophilus* La-14 (Danisco) is a commercially available potential probiotic strain of human origin and has been deposited in the American Type Culture Collection as SD5212 (Danisco).

In a double-blind, randomized, controlled trial with 83 healthy volunteers aged 18 up to 72 years who received two capsules per day of the test product containing 10 log CFU of bacteria in a maltodextrin carrier, *L. acidophilus* La-14 was administered to 9 of those volunteers. The serum IgG was reported to increase significantly in those volunteers in an early response compared with controls (P=0.01) 7 days after the second vaccine administration. Since IgG are involved in immune memory, *L. acidophilus* La-14 was suggested to possibly contribute to disease prevention in the long term (Paineau *et al.*, 2008).

Probiotic lactic acid bacteria may prevent the use of certain antibiotics in animal feeds (Park *et al.*, 2002) and if carefully selected, control the proliferation of pathogenic bacteria that may lead to diarrhoea and other clinical disorders, such as cancer and inflammatory bowel disease (Fooks *et al.*, 1999).

They may offer a safe and practical means of modulating the function and metabolic activity of the human intestinal microbiota, excluding pathogens and helping to keep the gut homeostasis by influencing the mucosal immune system (Morita *et al.*, 2006). Recent clinical and animal studies have supported the hypothesis that lactobacilli, particularly certain selected strains with immunomodulatory properties, can modify the responses of the host, thereby inducing beneficial effects (Ezendam and van Loveren, 2008; Shida and Nanno, 2008). Recently, there has been much interest in the use of probiotic bacteria for treating diseases and allergic disorders (Ezendam and van Loveren, 2008; Ghadimi *et al.*, 2008; He *et al.*, 2001; Shida and Nanno, 2008).

Apart from competition for binding sites, production of hydrogen peroxide and bacteriocins play a key role in competitive exclusion and probiotic properties (Boris and Barbes, 2000; Lepargneur and Rousseau, 2002; Reid and Burton, 2002; Galdeano *et al.*, 2007). Although the role of bacteriocins and their significance in controlling the proliferation of pathogenic bacteria in the intestinal tract is questionable (Brink *et al.*, 2006), several reports on bacteriocins active against Gram-negative bacteria (Ivanova *et al.*, 1998; Messi *et al.*, 2001; Caridi, 2002; Todorov and Dicsk, 2005a; Todorov and Dicks, 2005b; Todorov and Dicks, 2005c) aroused a renewed interest in these peptides and their interaction with intestinal pathogens. Only few papers reported bacteriocin production and potential probiotic properties of lactic acid bacteria isolated from different ecological niches (Van Reenen *et al.*, 1998; Todorov and Dicks, 2005a; Todorov and Dicks, 2005c; Todorov and Dicks, 2006; Todorov *et al.*, 2006; Powell *et al.*, 2007; Todorov *et al.*, 2007; Todorov *et al.*, 2008; Todorov and Dicks, 2008). Probably, bacteriocin production increases the chances for the probiotic strain to survive in the competing GIT environment. In fact, according to O'Flaherty and Klaenhammer (2010), there is strong evidence from *in vitro* studies that probiotic bacteria are able to make use of antimicrobial effects *in vivo*.

The survival of probiotic bacteria in the human or animal GIT is a complex process and involves the availability of nutrients, type of diet, interactions with autochthonous bacteria in the GIT, adhesion properties and auto-aggregation and co-aggregation characteristics of the probiotic cells. Survival of probiotics in the GIT of patients treated for the chronic illnesses that become dependent on permanent drug treatment may be less effective. Recent studies on potential probiotics have shown that these bacteria may be affected by non-antibiotic drugs (Boris and Barbes, 2000; Todorov *et al.*, 2007; Botes *et al.*, 2008; Todorov and Dicks, 2008; Carvalho *et al.*, 2009).

This article focuses on the investigation into bacteriocin production by the potential probiotic strain of *L. acidophilus* La-14 and determination of some aspects of bacteriocin mode of action.

The effect of selected drugs from different generic groups on growth of *L. acidophilus* La-14 was also determined and discussed.

MATERIALS AND METHODS

Strains and media

L. acidophilus La-14 was provided by Danisco (Dangé, France). The strain was grown in MRS broth (Difco) at 37ºC for 24 h. The test microorganisms used in this study and their culturing condition are listed in Table 1. All strains were stored at -80°C in MRS broth supplemented with 80% (v/v) glycerol.

Test for bacteriocin production

L. acidophilus La-14 was tested for antimicrobial compounds production against *Listeria monocytogenes* ScottA, using the agar spot-test (Todorov, 2008). Activity was expressed as arbitrary units (AU)/ml. One AU was defined as the reciprocal of the highest serial twofold dilution showing a clear zone of growth inhibition of the indicator strain (Todorov, 2008). The antimicrobial effect of lactic acid was eliminated by adjusting the pH of the supernatants to 6.0 with sterile 1 N NaOH. To rule out the effect of proteolytic enzymes and H_2O_2, the cell-free supernatant was heated at 80ºC for 10 minutes.

Confirmation of the identity of *L. acidophilus* La-14

L. acidophilus La-14 was identified to genus-level according to its physiological and biochemical characteristics, as described by Stiles and Holzapfel (1997). Carbohydrate fermentation reactions were recorded by using API50CHL (Biomérieux, Marcy-l'Etiole, France). Results were compared to carbohydrate fermentation pattern listed in Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986).

Dynamics of bacteriocin production

MRS broth was inoculated with an 18h-old culture (2 %, v/v) of *L. acidophilus* La-14 and incubated at 37°C without agitation. Antimicrobial activity (AU/ml) of the bacteriocin, and changes in pH and optical density (at 600 nm) of the cultures, were determined at 3 h and 1 h intervals, respectively for 48 h. *L. monocytogenes* ScottA was used as sensitive strain. In addition, several Gram-positive and Gram-negative bacterial strains were used for determination of spectrum activity. These strains were cultured in MRS or BHI broth, as shown in Table 1, at 30°C or 37°C, respectively.

Effect of enzymes, pH, detergents and temperature on bacteriocin activity

Cell-free supernatants of *L. acidophilus* La-14, obtained by centrifugation (8.000 x *g*, 10 min, 4°C) of a 18 h culture in MRS broth at 37°C, were adjusted to pH 6.0 with 1 N NaOH. Samples of 2 ml were incubated for 2 h in the presence of 1.0 mg/ml (final concentration) Proteinase type XIV (Roche), Proteinase (Roche), α-chymotrypsin (Roche), catalase (Roche) and α-amylase (Roche), and then tested for antimicrobial activity using

TABLE 1 - *Spectrum of activity of the antibacterial compound produced by Lactobacillus acidophilus La-14.*

Test microorganisms	*Antibacterial compound produced by L. acidophilus La-14 (diameter of the inhibition zone)*
Listeria monocytogenes	
ATCC 7644 (BHI, 37°C)	0
ScottA	8
Serotype 4b	
101	8
211, 302, 620, 703	0
724	10
Serotype 1/2a	
103	5
104, 506, 709	0
106	7
409	7
Serotype 1/2b	
426	10
603, 607	0
Serotype 1/2c	
408, 637, 712	0
422	9
711	5
Listeria innocua ATCC 33090 (BHI, 37°C)	10
Listeria sakei ATCC 15521 (MRS, 37°C)	0
Staphylococcus aureus ATCC 6538 (BHI, 37°C)	0
Staphylococcus aureus ATCC 29213 (BHI, 37°C)	0
Bacillus cereus ATCC 11778 (BHI, 37°C)	0

the agar-spot test method. Samples of plain MRS added of the listed enzymes in same concentrations were used as controls. In a separate experiment, the effect of SDS, Tween 20, Tween 80, urea, Na-EDTA and NaCl (1%, m/v, v/v) on bacteriocin stability were determined as described by Todorov and Dicks (2006). The same chemicals were applied as controls in plain MRS and incubated in similar conditions. The effect of pH on the bacteriocin stability was determined by adjusting the cell-free supernatant to pH 2.0 up to 12.0 with sterile 1 N HCl or 1 N NaOH. After 2 h of incubation at 37°C, the samples were readjusted to pH 6.5 with sterile 1 N HCl or 1 N NaOH and the activity was determined as described before (Klaenhammer, 1998). The effect of temperature on the bacteriocin stability was tested by heating the cell-free supernatants to 30, 37, 45, 60 and 100°C. Residual bacteriocin activity was tested after 30, 60 and 120 min at each of these temperatures, as described before (Todorov and Dicks, 2006). As control, plain MRS broth was exposed to the same temperatures and pH and tested against *L. monocytogenes* ScottA

Growth of the test-microorganisms in the presence of bacteriocin produced by *L. acidophilus* La-14

A 20 ml aliquot of bacteriocin-containing filter-sterilized (0.20 µm, Minisart®, Sartorius) supernatant (pH 6.0) was added to a 100 ml culture of *L. monocytogenes* ScottA in early exponential phase (OD_{600} = 0.064) and incubated for 14 h. Optical density readings (at 600 nm) were recorded at 1 h intervals.

Determination of the reduction of viable cells of test microorganisms in presence of bacteriocin produced by *L. acidophilus La-14*

Cells of an early stationary phase (18h-old) culture of *L. monocytogenes* ScottA were harvested (5000 x g, 5 min, 4°C), washed twice with sterile saline water and re-suspended in 10 ml of sterile saline water. Equal volumes of the cell suspensions and filter-sterilized (0.20 m, Minisart®, Sartorius) cell-free supernatant of *L. acidophilus* La-14 containing bacteriocin were mixed. Viable cell numbers were determined before and after incubation for 1 h at 37°C by plating onto MRS agar. Cell suspension of *L. monocytogenes* ScottA without added bacteriocins served as controls.

Adsorption study of the bacteriocin to the producer cells

The ability of a bacteriocin to adsorb to producer cells was studied according to the method described by Yang *et al.* (1992). After 18 h of growth at 37°C, the culture pH was adjusted to pH 6.0, the cells harvested (10 000 x *g*, 15 min, 4°C) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The cells were re-suspended in 10 ml 100 mM NaCl (pH 2.0), stirred for 1 h at 4°C and then harvested (12 000 x *g*, 15 min, 4°C). The cell-free supernatant was neutralized to pH 7.0 with sterile 1 N NaOH and tested for activity as described elsewhere.

Susceptibility of *L. acidophilus La-14* to medicaments

L. acidophilus La-14 was tested for susceptibility to commercially available drugs [analgesic, combination of analgesics and vasoconstrictor, narcotic analgesic, antipyretic, anorexiant/sympathomimetic, antiarrhythmic, antibiotic, antiemetic, antifungal agents, antihistaminic, antihypertensive (Alpha blocker, Angiotensin Converting Enzyme (ACE) inhibitor), antitussives (central and peripheral mode of action), association of analgesic/antipyretic, antihistaminic and decongestant, contraceptive, diuretic, histamine H2-receptor antagonist that inhibits stomach acid production (Proton pump inhibitor), hypolipidemic, mucolytic agent, non-steroidal anti-inflammatory drug (NSAID), proton pump inhibitor, selective serotonin reuptake inhibitor (SSRI) antidepressant, thiazide diuretic] was determined (Table 3). Strains were inoculated separately into 10 ml MRS broth (Difco) and incubated at 37°C for 18 h and imbedded into MRS soft agar (1.0%, w/v, Difco) at 10^6 CFU/ml. Ten µl of each drug was spotted onto the surface of the agar. The plates were examined for the presence of inhibition zones after 24 h of incubation at 37°C. The drugs presenting the inhibition zones larger than 2 mm were subjected to the determination of the minimal inhibition concentration, using serial twofold dilutions of the medicaments. For the test, 10 µl of each dilution were spotted onto the surface of the agar, previously imbedded with *L. acidophilus* La-14. The plates were incubated for 24 h at 37°C and examined for inhibition zones. Those presenting inhibition zones above 2 mm in diameter were considered positive.

RESULTS

Identification of the *L. acidophilus* La-14 strain

Based on the biochemical test and API50CHL, the identity of the strain grown from the commercial available lyophilized product of Danisco (Dangé, France) was confirmed to be *L. acidophilus*.

Bacteriocin production

No significant differences in growth and production of bacteriocin were observed when the strain *L. acidophilus* La-14 was cultured for 24 h in MRS broth at 30°C or at 37°C. At this two incubation temperatures, activity against *L. monocytogenes* ScottA was 1600 AU/ml. All further experiments were conducted at 37°C, since strain *L. acidophilus* La-14 is a potential probiotic strain.

Production of bacteriocin by *L. acidophilus* La-14 was detected at maximum levels (1600 AU/ml) after 16 h and remained stable up to 24 h of fermentation in MRS broth.

After 24 h, the activity against *L. monocytogenes* ScottA decreased and was progressively reduced to 400 AU/ml at 48 h of incubation (Fig. 1). During this period, the medium pH of *L. acidophilus* La-14 culture decreased from 6.40 to 4.25 and the cell density increased from 0.022 to 7.35 (as detected at 39 h) and decreased slightly to 0.669 in the following 9 h (Fig. 1). Low levels of bacteriocin produced by *L. acidophilus* La-14 (approx. 400 AU/ml) were recorded after 3 h of growth in MRS broth at 37°C.

Spectrum of activity

The bacteriocin produced by *L. acidophilus* La-14 proved inhibitory to different serotypes of *L. innocua* and *L. monocytogenes* listed in Table 1. However, no activity was recorded against *Staphylococcus aureus*, *Lactobacillus sakei* and *Bacillus cereus*.

Effect of enzymes, pH, detergents and temperature on bacteriocin activity

Treatment with α-amylase and lipase did not change the antimicrobial activity (Table 2). Activity of the bacteriocin produced by *L. acidophilus* La-14 was not affected by 1% SDS, Tween 20, Tween 80, Urea, EDTA or NaCl (Table 2). Bacteriocin produced by *L. acidophilus* La-14 remained stable after incubation for 2 h at pH from 2.0 up to 12.0 (Table 2).

Stability of bacteriocin produced by *L. acidophilus* La-14 was recorded after 120 min at 25, 30, 45, 60 or 100°C (Table 2). Heating at 121°C for 20 min did not inactivate the bacteriocin, but caused a reduction of activity, as smaller inhibition zone against *L. monocytogenes* ScottA were observed (Table 2). Treatment of bacteriocin at pH 6.0 at 121°C for 20 min resulted in a decreased activity from 1600 AU/ml to 400 AU/ml.

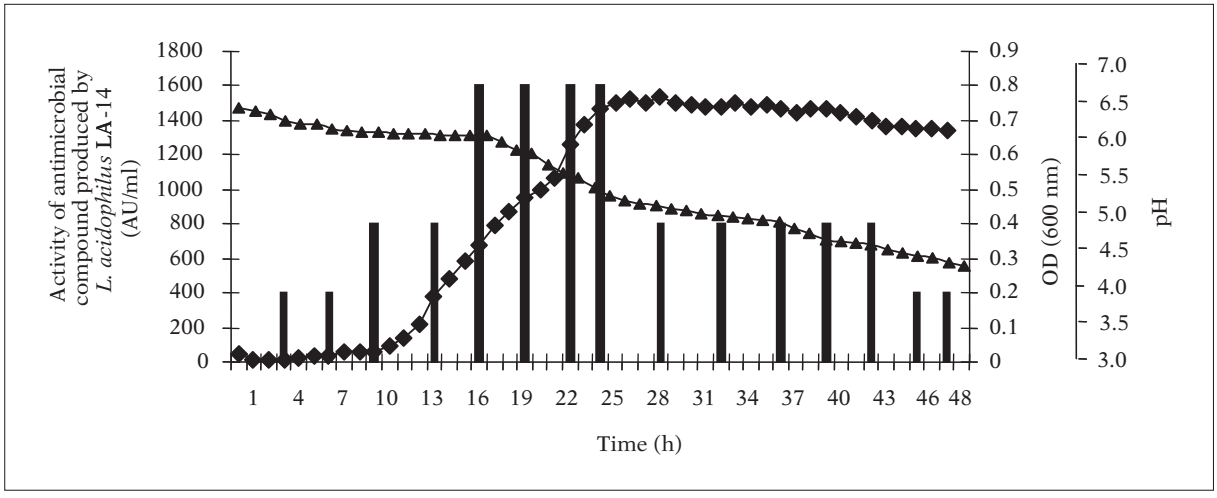

FIGURE 1 - *Production of bacteriocin by Lactobacillus acidophilus La-14 in MRS broth (pH 6.5, 37°C). Antimicrobial activity is presented as AU/ml (bars) against Listeria monocytogenes ScottA. Changes in optical density (-♦-) and pH (-▲-) are indicated. Standard deviation recorded from three repeats was less that 5% and is not indicated.*

TABLE 2 - *Effect of enzymes, detergents, NaCl, temperature and pH on the stability of the antibacterial compound produced by Lactobacillus acidophilus La-14.*

Treatment	*Test microorganism*	
	L. monocytogenes ScottA	*L. monocytogenes 724 serotype 4b*
α-amylase, catalase	+	+
Proteinase type XIV	-	-
Proteinase	-	-
α-chymotrypsin	-	-
Tween 20, Tween 80	+	+
Urea, SDS, EDTA, NaCl	+	+
25, 30, 45, 60, 100°C for 2h	+	+
121°C for 20 min	+	+
pH 2-10	+	+
pH 12	+	+
No treatment (control)	+	+

Activity was expressed as: + = presence of inhibition zone ≥2 mm diameter, - = no inhibition.

Growth of the test-microorganisms in the presence of bacteriocin produced by *L. acidophilus* La-14

Addition of bacteriocin produced by *L. acidophilus* La-14 obtained from a 24 h old culture, to a 3-h-old culture of *L. monocytogenes* ScottA ($OD_{600nm} \approx 0.044$) repressed cell growth in the following 8 h and slightly increased in the next 4 h (Fig. 2), but no viable cells were recorded in 6, 8, and 10 h. Levels of 10^2-10^3 CFU/ml for *L. monocytogenes* ScottA were recorded at 12 and 14 h, pointing the bacteriostatic mode of action of this bacteriocin against this test microorganism.

Reduction in CFU/ml of *L. monocytogenes* ScottA after exposure to bacteriocin produced by *L. acidophilus* La-14

Treatment of stationary phase cells of *L. monocytogenes* ScottA (10^7-10^8 CFU/ml) with the bacteriocin produced by *L. acidophilus* La-14 resulted in growth inhibition. After 1 h of contact, low levels (10^1-10^2 CFU/ml) of viable cells of *L. monocytogenes* ScottA were detected. No significant changes in cell numbers of *L. monocytogenes* ScottA were recorded in the untreated (control) sample.

Adsorption study of the bacteriocin to the producer cells

After treatment of the cell suspension of *L. acidophilus* La-14 with 100 mM NaCl (pH 2.0) for 1 h, no adsorption of the bacteriocin was recorded, showing that this bacteriocin probably does not adhere to the producer cell surface.

Sensitivity of *L. acidophilus* La-14 to drugs

Only two antibiotics (Amoxil and Urotrobel) and the non-antibiotic drug Atlansil (an antiarrhythmic agent) inhibited growth of *L. acidophilus* La-14 in a MIC of <0.5 mg/ml, 5.0 mg/ml and 2.5 mg/ml, respectively (Table 3). Growth of *L. acidophilus* La-14 was not inhibited by other medicaments belonging to different generic

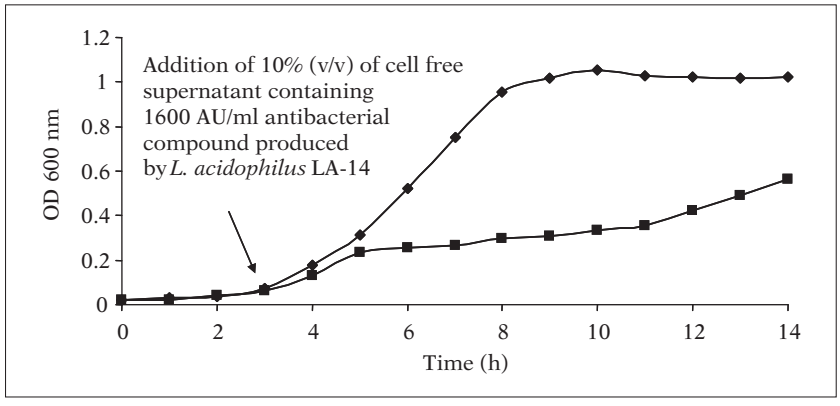

FIGURE 2 - *Effect of bacteriocin produced by Lactobacillus acidophilus La-14 on growth of Listeria monocytogenes ScottA. Arrow indicates the time of the addition of the bacteriocin.*

TABLE 3 - *Effect of commercial drugs on the growth of Lactobacillus acidophilus La-14.*

Medicament (commercial name)	*Applied concentration (mg/ml)*	*Active substance*	*Medication group*	*L. acidophilus La-14*	
				Inhibition (mm)	*MIC (mg/ml)*
AAS	20	Acetylsalicylic acid	Analgesic/Antipyretic	0	
Amoxil	100	Amoxicillin	Antibiotic/β-Lactam antibiotic (Penicilin)	36	<0.4
Antak	30	Ranitidine hydrochloride	Histamine H2-receptor antagonist that inhibits stomach acid production (Proton pump inhibitor)	0	
Arotin	4	Paroxetine	Selective serotonin reuptake inhibitor (SSRI) antidepressant	0	
Aspirina	100	Acetylsalicylic acid	Analgesic / Antipyretic	0	
Atlansil	40	Amiodarone	Antiarrhythmic	13	2.5
Cataflam	10	Diclofenac potassium	Non-steroidal anti-inflammatory drug (NSAID)	0	
Celebra	40	Celecoxib	Non-steroidal anti-inflammatory drug (NSAID)	0	
Clorana	5	Hydrochlorothiazide	Diuretic	0	
Coristina R		Acetylsalicylic acid, Pheniramine maleate, Phenylephrine hydrochloride, Cafein	Association of Analgesic/ Antipyretic, antihistaminic and decongestant	0	
Diclofenac potasico[1]	10	Diclofenac potassium	Non-steroidal anti-inflammatory drug (NSAID)	0	
Diclofenaco potasico[1]	10	Diclofenac potassium	Non-steroidal anti-inflammatory drug (NSAID)	0	
Dorflex		Orphenadrine citrate, Metamizole sodium, Cafein	Analgesic	0	
Doxuran	0.8	Doxazosin	Antihypertensive (Alpha blocker)/Treatment of benign prostatic hyperplasia	0	
Dramin	20	Dimenhydrinate	Antiemetic	0	
Fenergan	5	Promethazine hydrochloride	Antihistaminic	0	

continue

follow TABLE 3 - *Effect of commercial drugs on the growth of Lactobacillus acidophilus La-14.*

Medicament (commercial name)	*Applied concentration (mg/ml)*	*Active substance*	*Medication group*	*L. acidophilus La-14*	
				Inhibition (mm)	*MIC (mg/ml)*
Fluimucil	8	Acetylcysteine	Mucolitic agent	0	
Flutec	30	Fluconazole	Antifungal	0	
Higroton	10	Chlorthalidone	Thiazide diuretic	0	
Omeprazole	4	Omeprazole	Proton pump inhibitor	0	
Neosaldina	60	Metamizole sodium, isometheptene mucate, cafein	Analgesic (combination of analgesics and a vasoconstrictor)	0	
Nimesulida	20	Nimesulide	Non-steroidal anti-inflammatory drug (NSAID)	0	
Nisulid	20	Nimesulide (NSAID)	Non-steroidal anti-inflammatory drug	0	
Redulip	3	Sibutramine hydrochloride monohydrate	Anorexiant/ Sympathomimetic	0	
Seki	3.54	Cloperastine	Antitussives (central and periferic mode of action)	0	
Spidufen	120	Ibuprofen arginine	Non-steroidal anti-inflammatory drug (NSAID)	0	
Superhist	80	Acetylsalicylic acid, Pheniramine maleate, Phenylephrine hydrochloride	Association of Analgesic/ Antipyretic, antihistaminic and decongestant	0	
Tylenol	150	Paracetamol	Analgesic/Antipyretic	0	
Tylex	6	Paracetamol, Codein	Analgesic/Narcotic analgesic	10	5.0
Urotrobel	80	Norfloxacin	Antibiotic	0	
Yasmin	0.6	Ethinylestradiol, drospirenone	Contraceptive	0	
Zestril	4	Lisinopril	Antihypertensive (Angiotensin-converting enzyme (ACE) inhibitor)	0	
Zocor	2	Simvastatin	Hypolipidemic	0	
Zyrtec	2	Cetirizine hydrochloride	Antihistaminic	0	

groups, including non-steroidal anti-inflammatory drugs (NSAID) containing diclofenac potassium or ibuprofen arginine, and drugs containing sodium or potassium diclofenac (Table 3).

DISCUSSION

Similar levels of bacteriocin production were recorded for *L. acidophilus* La-14 when cultured for 24 h in MRS broth at 30°C or at 37°C. This is in agreement with the results recorded for other bacteriocins (Todorov and Dicks, 2006). Optimal levels of other bacteriocins were recorded in growth media that supported high biomass production, e.g. MRS and TGE (Biswas *et al.*, 1991; Ray *et al.*, 1992; Yang and Ray, 1994). However, during cultivation of *L. acidophilus* La-14 in MRS broth, the reduction in bacteriocin activity levels was recorded at pH values below 5.1, suggesting that production is blocked in these conditions. Only genetic studies on the expression of the genes encoding the bacteriocin production can confirm this hypothesis.

Similar results were observed for other bacteriocins (Todorov and Dicks, 2005b). The decreased activity by the end of the monitored period might be explained by degradation of the bacteriocin by extracellular proteolytic enzymes, as a previously similar decrease in activity was shown for bacteriocins produced by *Lactobacillus plantarum* ST414BZ (Todorov and Dicks, 2006), *Pediococcus acidilactici* NRRL B5627 (Anastasiadou *et al.*, 2008a) and *Pediococcus pentosaceus* (Anastasiadou *et al.*, 2008b). From a metabolic point of view, this trend is characteristic of a primary metabolite production, as observed for several bacteriocins produced by *Pediococcus* spp. (Bhunia *et al.*, 1988; Ray *et al.*, 1989; Bhunia *et al.*, 1991; Anastasiadou *et al.*, 2008a; Anastasiadou *et al.*, 2008b).

The bacteriocin produced by *L. acidophilus* La-14 showed the inhibitory spectrum summarised in Table 1. It is important to highlight the activity against *L. monocytogenes*, an important human and food pathogen. Based on strong activity against *L. monocytogenes*, the bacteriocin produced by *L. acidophilus* La-14 is probably a class IIa bacteriocin (Klaenhammer, 1988; Heng *et al.*, 2007), but this preliminary conclusion needs to be confirmed by determination of the amino-acid sequence of the antimicrobial molecule. Production of bacteriocins may be considered an advantage for the probiotic strains, since this antimicrobial compound will give them a benefit in the competition with the GIT pathogens, such as *L. monocytogenes*. Previous reports have shown that several probiotic and potential probiotic strains are bacteriocin producers (Todorov and Dicks, 2005a; Todorov and Dicks, 2005c; Todorov *et al.*, 2005; Todorov and Dicks, 2006; Todorov *et al.*, 2006; Powell *et al.*, 2007; Todorov *et al.*, 2007; Botes *et al.*, 2008a; Botes *et al.*, 2008b; Todorov and Dicks, 2008; Todorov *et al.*, 2008; Todorov and Dicks, 2009).

Activity against pathogens is one of the important properties a probiotic strain ought to possess. The antimicrobial ability of the potential probiotic strain *Lactobacillus acidophilus* La-14 against some enteropathogens, such as *Listeria monocytogenes*, was assayed in this study. The overnight culture of *L. acidophilus* La-14 showed strong inhibition action towards *Listeria* spp. (Table 1). Moreover, treated supernatant (without peroxide and lactic acid) also showed antipathogen activity.

These observations suggest that *L. acidophilus* La-14 produced bacteriocins to inhibit the test pathogens. Some authors have reported that production of bacteriocins by lactobacilli is relatively common, and may contribute to their colonization of habitats and their competitive edge over other bacteria (Garriga *et al.*, 1993). The antimicrobial activity of lactic acid bacteria may be due to a number of factors including decreased pH levels, competition for substrates, and the production of substances with a bactericidal or bacteriostatic action, including bacteriocins (Parente and Riccardi, 1994).

Our results (Table 2) suggest that bacteriocin produced by *L. acidophilus* La-14 does not belong to group IV bacteriocins (Klaenhammer, 1988; Heng *et al.*, 2007) and the carbohydrate or lipids are not involved in the structure of the active molecule or molecular complex. According to De Vuyst and Vandamme (1994), most bacteriocins are polypeptides. Some exceptions are those classified in group IV (Klaenhammer, 1988; Heng *et al.*, 2007), such as carnocin 54, produced by *Leuconostoc carnosum* (Keppler *et al.*, 1994), which is example of amylase-sensitive bacteriocins.

The bacteriocin produced by *L. acidophilus* La-14 was not affected by the presence of selected chemicals (Table 2). In a similar experiment with bacteriocins produced by *P. acidilactici* HA-6111-2 and HA-5692-3 (Albano *et al.*, 2007), exposure to Triton-100 or Triton X-114 caused a reduction in bacteriocins activity. Similar results were also reported for plantaricin 423 (Verellen *et al.*, 1998), pediocin AcH (Biswas *et al.*, 1991), lactacin B (Barefoot and Klaenhammer, 1984) and lactocin 705 (Vignolo *et al.*, 1995). However, the effect of SDS or Triton X-100 seems to be bacteriocin dependent, as the activity of plantaricin C19 (Atrih *et al.*, 2001), pediocin ST18 (Todorov and Dicks, 2005d), plantaricin ST31 (Todorov *et al.*, 1999), and bozacin B14 (Ivanova *et al.*, 2000) did not decrease when treated with these compounds.

The bacteriocin produced by *L. acidophilus* La-14 was stable at pH rangeing from 2.0 to 12.0 (Table 2). This is a remarkable finding, as several other studies have shown a reduced activity of bacteriocins exposed to pH 12.0, such as *P. acidilactici* HA-6111-2 and HA-5692-3 (Albano *et al.*, 2007), and pediocin PA-1 (Bhunia *et al.*, 1988; Gonzales and Kunka, 1987). The loss of activity may be ascribed to proteolytic degradation or protein aggregation (Aasen *et al.*, 2000; Parente and Riccardi, 1994; Parente *et al.*, 1994; De Vuyst *et al.*, 1996).

The bacteriocin produced by *L. acidophilus* La-14 was thermostable (Table 2). The antimicrobial activity of pediocin PA-1 was unaffected by heating at 80°C for 60 min, and at 100°C for 10 min, and the effect of 121°C for 15 min was controversial, as values of residual activity of 6% and 60% have been reported (Yang and Ray, 1994). Purified pediocin PA-1 at pH 5 remained stable when stored at 4°C and at 25°C, but not at pH 7.0. The peptide remained stable at -20°C, independent of storage at pH 5.0 or 7.0 (Fimland *et al.*, 2002). These authors have shown that heat resistance of pediocin PA-1 produced by *P. parvulus* was pH dependent. At pH 6.0, 84% activity was lost when heated at 121°C for 15 min. No activity was recorded when the same experiment was done with pediocin PA-1 adjusted to pH 7.0 and 8.0. However, at pH 4.0, only 11 % of the activity was lost. Pediocin is more heat sensitive at lower pH. The same results were recorded for other pediocins and enterocins (Bhunia *et al.*, 1988; Moreno *et al.*, 2003).

Based on results of inhibition of *L. monocytogenes* ScottA by bacteriocin produced by *L. acidophilus* La-14, most probably this bacteriocin exhibit bacteriostatic mode of action. Possibly, development of bacteriocin resistance in *L. monocytogens* ScottA is the reason for the detection of viable cells at 12 and 14 h. Other reason for the reduction of efficacy of bacteriocin produced by *L. acidophilus* La-14 against *L. monocytogens* Scott A may be the protein degradation by proteolytic enzymes, bacteriocin aggregation or simply full utilisation of the added bacteriocin in the inhibitory process.

When stationary phase cells of *L. monocytogenes* ScottA (10^7-10^8 CFU/ml) were treated with the bacteriocin produced by *L. acidophilus* La-14, low levels (10^1-10^2 CFU/ml) of viable cells of test microoganism were detected. No significant changes in cell numbers of *L. monocytogenes* ScottA were recorded in the untreated (control) sample. Previously, a similar effect regarding bacteriocins HA-6111-2 and HA-5692-3 produced by *P. acidilactici* to *E. faecium* HKLHS was reported by Albano *et al* (2007).

No adsorption of the bacteriocin to the producer cells was recorded. Similar observation were reported for plantaricin ST31 (Todorov *et al.*, 1999), pediocin ST18 (Todorov and Dicks, 2005d), bacteriocins HA-6111-2 and HA-5692-3 (Albano *et al.*, 2007) and bozacin B14 (Ivanova *et al.*, 2000).

Patients taking probiotics are often treated for other illnesses. It is thus important to determine the effect of medicaments on the growth of probiotic strains.

Only two antibiotics (Amoxil and Urotrobel) and the non-antibiotic drug Atlansil inhibited growth of *L. acidophilus* La-14 in a MIC of <0.5 mg/ml, 5.0 mg/ml and 2.5 mg/ml, respectively (Table 3). Growth of *L. acidophilus* La-14 was not inhibited by other medicaments belonging to different generic groups, including non-steroidal anti-inflammatory drugs (NSAID) containing diclofenac potassium or ibuprofen arginine, and drugs containing sodium or potassium diclofenac (Table 3). A previous study reported that sodium diclofenac inhibited the growth of *L. plantarum* ST8KF and ST341LD, *Enterococcus faecium* ST311LD and *Leuconostoc mesenteroides* subsp. *mesenteroides* ST33LD and that dimenhydrinate was inhibitory to *Lactobacillus plantarum* ST8KF (Todorov and Dicks, 2008). In another study,

potassium diclofenac and ibuprofen inhibited the growth of *Lactococcus lactis* subsp. *lactis* HV219 (Todorov *et al.*, 2007). Anti-inflammatory drugs, moderate diuretics and neuroleptics containing potassium or sodium diclofenac, ibuprofen, triamterene hydrochlorothiazide and thioridazine hydrochloride acted as inhibitors of the growth of *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei* and *Lactobacillus pentosus* strains isolated from boza and evaluated as probiotics (Todorov *et al.*, 2008).

Dimenhydrinanat inhibited the growth of *Lactobacillus rhamnosus* ST462BZ and *Lactobacillus plantarum* ST664BZ (Todorov *et al.*, 2008). It is, however, important to mention that the concentration of these substances is critical for their inhibitory mode of action on the probiotic LAB. As shown by Carvalho *et al*. (2009) *L. casei* Shirota and *L. casei* LC01 were inhibited by non-steroidal anti-inflammatory drugs (NSAID) containing diclofenac potassium or ibuprofen arginine. In addition, *L. casei* Shirota was affected by selective serotonin reuptake inhibitors (SSRI) antidepressant containing paroxetine and antiarrhythmic medication containing amiodarone. *L. casei* LC01 was inhibited by hypolipidemic medication containing simvastatin. The levels of MIC for these drugs on the growth of *L. casei* Shirota and *L. casei* LC01 were reported (Carvalho *et al*., 2009). It is important to point out that *L. acidophilus* La-14 showed good resistance to several drugs, and may be applied in combination with them in the treatment of several medical cases.

Botes *et al.* (2008b) reported that *L. casei* Shirota was inhibited by several commercial antibiotics (ciprofloxacin, amoxicillin, cefadroxil, roxithromycin, doxycycline and norfloxacin). Anti-inflammatory drugs containing meloxican (Coxflam), Ibuprofen (Dolocyl, Adco-Ibuprofen), potassium diclofenac (Cataflam) and prednisolone (Preflam) also inhibited the growth, in a lesser extent.

Pinmed, that contains paracetamol, codeine phosphate and promethazine HCl, misclassified as an analgesic instead of an antitussive agent, was also inhibitory to *L. casei* Shirota. The same authors also reported the inhibitory effect of Pynmed (Botes *et al.*, 2008b), which is more likely due to the presence of alcohol in the formulation than to the drug itself. An important point is that in the study of Botes *et al.* (2008b) the MIC of the active drugs were not determined, hampering the correct evaluation of their activity against *L. casei* Shirota in the human body, especially when used on a daily basis by patients with chronic diseases. The correct evaluation of possible interactions between medicaments and probiotic bacteria depends on the determination of MIC of these medicaments.

In another study by Botes *et al*. (2008a), a similar experiment was performed (Botes *et al*., 2008b) to verify the effect of drugs over the same probiotic strains (*E. mundtii* ST4SA and *L. plantarum* 423) and using the same commercial probiotic strains as controls (*L. casei* Shirota, *L. johnsonii* La1 and *L. rhamnosus* GG). As previously reported (Botes *et al*., 2008a), the authors (Botes *et al.*, 2008b) evaluated the effect of commercial antibiotics and non-antibiotic drugs on a few probiotic strains, without establishing the MIC of this agents. The authors studied the effect of selected drugs on the adhesion to Caco-2 cell line to evaluate *E. mundtii* ST4SA and *L. plantarum* 423 as probiotics.

The mechanism of the inhibitory effect against probiotic LAB and other GIT-related bacteria needs to be related to the chemical composition of drugs. A simple recommendation would be not to apply a drug presenting an inhibitory effect on the probiotic LAB at the same time, since the drug will have a negative effect on the probiotic cells, resulting in decreased viability.

The application of drugs along with probiotic cultures needs to be reconsidered, regarding the possibility of a negative interaction. The drug MIC on the survival and growth of probiotic bacteria is an important cross point. This type of drug must not be taken by the patient permanently**.** The daily dose for this drug needs to be linked with the MIC against probiotic LAB. Especially important are drugs used in the treatment of chronic diseases. Some of the drugs tested in this study showed an MIC of 2.5 mg/ml (Atlansil, an antiarrythmic drug normally used for long courses of treatment). Administration of these drugs needs caution, when done together with probiotic cultures, especially with *L. acidophilus* La-14, since they are applied on a daily basis and an accumulation of the active substances in the GIT is highly possible. However, this will also increase the inhibitory effect of the drug on *L. acidophilus* La-14 and therefore result in a reduction of the viability of the probiotic strain.

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