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Antibacterial activity of *Lactobacillus acidophilus* and *Lactobacillus casei* against methicillin-resistant *Staphylococcus aureus* (MRSA)

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Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multidrug-resistant microorganism and the principal nosocomial pathogen worldwide. The antibacterial activity of lactic acid bacteria against MRSA from ten human clinical isolates as well as MRSA standard strain ATCC 43300 was tested *in vitro*. The Lactobacillus (Lb.) strains (*Lb. acidophilus* CL1285® and Lb. *casei* LBC80R) as pure cultures, which came from commercial food products were employed. The growth inhibitory effect produced by the antimicrobial activity of the lactic acid bacteria on the MRSA strains was tested on solid medium using agar diffusion methods as well as a using a liquid medium procedure that contained a mixture of MRSA and lactic acid bacteria cultures. In the latter instance, we were able to demonstrate that the direct interaction of lactic acid bacteria and MRSA in such a mixture led to the elimination of 99% of the MRSA cells after 24 h of their incubation at 37 °C.

1. Introduction

Bacterial infections caused by the genus *Staphylococcus* represent a grave threat to both humans and animals and they are of major concern to health authorities. Over the last few decades, methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as the principal nosocomial pathogen worldwide (Simor et al. 2001; Graffunder and Venezia 2002; Anderson et al. 2008). In addition to its impact on livestock, MRSA infections in humans have been associated with excess morbidity, mortality and increased length of hospitalization (Libert et al. 2008).

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The widespread therapeutic use of antimicrobials in humans and the administration of antimicrobials as growth promoters in food for animals have been associated with the development of resistant bacteria. Most antibiotics are administered to patients empirically before any diagnosis based on results from cultures are known (Roghmann and McGrail 2006). In one study, only 17% of MRSA-infected patients were initially given an effective antibiotic (Crum et al. 2006). Such MRSA colonies may be present in an individual's global microbial population as part of the natural balance of his/her own microbial flora (Alvarez-Olmoz and Oberherman 2001). Since they have become part of this essential ecological system, any threat to the normal balance of the entire flora has to be taken into account when attempting to eradicate MRSA (Simor et al. 2007). For example, the use of antibiotics can disrupt the balance of flora and cause antibiotic-associated diarrhea (AAD) in up to 35% of treated patients (Beausoleil et al. 2007).

Antibiotic therapies carry the risk of transforming the MRSA pathogen into multidrug-resistant strains.

S. aureus can become MRSA by the acquisition of the mecA gene, which encodes a penicillin binding protein (PBP2a) with a low affinity for β-lactams (Hackbarth et al. 1994; Niemeyer et al. 1996; Deresinski 2005; Martins and Cunha 2007). The PBP2a-producing MRSA strain is resistant not only to methicillin, oxacillin and nafcillin but also to all other β-lactam antibiotics including cephalosporins. Sequencing of the entire MRSA genome was reported (Kuroda et al. 2001) and it revealed *S. aureus*' remarkable ability to acquire genes from various organisms through a lateral gene transfer.

Most antibiotic resistance genes are carried either by plasmids or by mobile genetic elements (Kuroda et al. 2001).

MRSA colonization generally precedes MRSA infection and it plays a major role in the spread of this organism within human communities and healthcare facilities (Bradley 2007; Roghmann and McGrail 2006). MRSA is a biofilm-forming pathogen that adheres to numerous surfaces. In humans and animals, its main habitats are the nasal membranes and skin. Such colonies cause life threatening infections such as pneumonia, sepsis, osteomyelitis and infections endocarditis (Simor et al. 2007). Patients with MRSA colonization are often colonized for long periods of time. Approximately 50% of MRSA patients are still colonized after one year (Bradley 2007; Roghmann and McGrail 2006; Simor et al. 2007).

Topical mupirocin, chlorhexidine gluconate washes, oral rifampicin and doxycycline are recommended for the eradication of MRSA colonies (Simor et al. 2007). For example, in a fifteen-year study in San Diego, resistance to clindamycin dramatically increased MRSA presence in the community (Crum et al. 2006).

In the 1960s and 1970s, replacement of antibiotic-resistant bacteria with another but similar antibiotic-susceptible bacteria was used to interrupt *S. aureus* outbreaks in nurseries in order to reduce recurrent furunculosis in persistent carriers. Unfortunately, a ''nonpathogenic'' strain, *S. aureus* 502A, was not always nonpathogenic and its use fell out of favor (Tagg and Dierksen 2003; Roghmann and McGrail 2006). Many antibiotic-resistant bacteria in humans are opportunistic pathogens and are currently a normal part of our microbiological flora and therefore not easily displaced.

Treatment with selected probiotic strains may be the ultimate answer to decolonization of MRSA because they do not increase the risk of multi-drug resistance of this pathogen (Tagg and Dierksen 2003; Roghmann and McGrail 2006). The alarming increase in inappropriate antibiotic use along with bacterial resistance has led to renewed interest in ecological methods to prevent infections, which make probiotics a very interesting field for further research. For example, a patient in Japan with a decubitus ulcer colonized by MRSA was successfully treated with a probiotic *Lactobacillus* preparation (Alvarez-Olmoz and Oberherman 2001). One non-antibiotic strategy to combat the bacterial infections involves the selection and promotion of endogenous barrier flora to interfere with pathogenic bacterial adhesion (Tagg and Dierksen 2003).

Lactic acid bacteria (LAB) strains are potentially promising because they generate bactericidal bioactive peptides (bacteriocins) and enzymes that are able to control biofilm formation and the growth of the pathogens. Nisin is the best defined bacteriocin (Huttunen et al. 1995) produced by species Lactococcus that has been approved for use in food products (Hansen 1994; Millette et al. 2007).

Bacteriocins are also present in species of genus Lactobacillus. The *Lb. acidophilus* produce lactacin B or F, whereas *Lb. casei* B80 produce casein 80. (Rammesberg and Radler 1990; Klaenhammer 1993).

Certain LAB strains have been reported to be highly antagonistic to biofilm-forming *S. aureus* (Ammor et al. 2006a). The genus *Lactobacillus* has a long history of safe use, especially in the dairy industry, and it plays a major role in the transformation of fermented milk and other food products.

Over the past few decades, there has been increased impetus to introduce new *Lactobacillus*

strains into foodstuffs with the goal of exerting a beneficial health effect when ingested by humans or animals (Tagg and Dierksen 2003; Maragkoudakis et al. 2006). Four types of LAB strains have been studied as competitive inhibitors of pathogenic organisms (Massi et al. 2004). These strains are: *Lb. casei 99p* rhamnosus GG, *Lb. casei* Shirota, *Bifidobacteria brave* Yacult and *Lb. acidophilus* Johnsonni (Nomato 2005).

Beneficial effects conferred by *Lactobacilli*, including inhibition of gram negative and positive pathogenic bacteria, were described by Maragkoudakis et al. 2006, Nomato 2005. Charlier et al. 2008 reported that *Lactococcus (Lc.) lactis* had a specific antagonistic effect against *S. aureus*.

Antimicrobial activity produced by LAB strains appears to be unrelated to the acidification of the medium. LAB strains were reported to exert a strong inhibitory effect on *S. aureus* growth in milk. Several suggestions have been proposed for inhibition of *S. aureus* by LAB. These include production of bacteriocins, hydrogen peroxide, and organic acids such as lactic and acetic acid (Charlier et al. 2008; Lin et al. 2006; Hernandez et al. 2005).

In our previous study, using the European LAB strains (Schellenberg et al. 2006), we were able to demonstrate their anti-MRSA activity. The purpose of this study was to evaluate the *in vitro* antibacterial activity of the LAB *Lb. acidophilus* and *Lb. casei,* against pathogenic MRSA from human clinical isolates.

To our knowledge, no data exist on a competitive interaction between clinical MRSA isolates and LAB cultures. Furthermore, we could not find any data on the survival of clinical MRSA isolates in co-cultures after being mixed with LAB strains.

2. Materials and methods

2.1. Strain and culture conditions

All strains were stored at −20 °C in appropriate culture media with 12–15% glycerol and revived using basic microbiological techniques in strain-appropriate media. After being revived, the cultures were stored at 4 °C (1–4 days) and propagated twice before being used in antimicrobial tests. The identified Lactic Acid Bacteria (LAB) strains were of commercial origin and were provided by Bio-K+ International (Luquet 2003).

The *Lb. acidophilus* CL1285 and *Lb. casei* LBC80R strains were purified and earlier identified by standard biochimical and molecular methods comprising sequence analysis of r16s DNA with the NCBI – database (National Center for Biotechnology Information) at the Institut Pasteur (Lille, France). *Lb. acidophilus* strain CL1285 was patented (US 6607905-2003) and was earlier deposited (1994) as strain No 1-1492 in the Collection National Cultures de Microorganisms (CNCM, Institut Pasteur, Paris CEDEX 15 - according to the provisions of Budapest Treaty). The commercial product that contained the mixture of these strains LAB in a fermented milk-based or soya-based medium was also used in this study. The basic growth media for LAB were Man-Rogosa-Sharpe (MRS; Difco), M17 (Difco) and Peptonized Milk Nutrient (PMN; Sigma).

For our MRSA strain the basic growth medium (broth and agar) was Brain-Heart Infusion (BHI: Difco). For *S. aureus* the selective medium mannitol-salt agar (MSA; Difco) was used. It has been proven that only MRSA can grow on this medium, but LAB strains used in this study are unable to grow on the MSA agar plates. Since coagulase protein is an important virulence factor of *S. aureus*, MRSA strains from clinical human isolates that were coagulase positive were chosen from the collection of Dr. Karska–Wysocki. A total of ten different MRSA strains, which were isolated from individual patients with clinical infections at the Hôtel-Dieu Hospital in Montreal were used (see Table 1).

The resistance phenotype in all MRSA strains was verified by streaking diluted 24 h cultures on Mueller-Hinton agar (BBL) with 4% NaCl and 6 μg mL^{-1} oxacillin (Sigma) (Simor et al. 2001, Brown et al. 2005). The antibiotic susceptibility (Simor et al. 2001, Brown et al. 2005) of all isolated strains was tested according to the standard methodology suggested by the Canadian Committee on Antibiotic Resistance (Brown et al. 2005; Public Health Agency 2005). All strains were shown to be vancomycin sensitive, but resistant to methicillin, oxacillin, erythromycin and cefazolin. (Yazdanparast 2002).

As a positive control for the LAB antibacterial activity test, ATCC strain MRSA 43300 was used (Swenson et al. 2001; Huletsky et al. 2004). In

Table 1. Clinical MRSA Isolates.

Clinical MRSA Isolate	Clinical Infection Site
#18	Nose
#22	Calf wound
#27	Thigh wound
#36	Abdominal pus
#43	Lungs
#61	Vagina
#64	Eye
#69	Nose
#75	Tongue
#80	Wound pus

addition, one strain of *Lactococcus (Lc.) lactis spp cremoris* (from Dr. Karska-Wysocki's collection) was applied as a negative control for the expression of antibacterial activity produced by LAB strains.

2.2. Confirmation of the Presence of coa and mecA genes in Clinical MRSA Isolates

In order to confirm the identity of the clinical MRSA isolates, their genomic DNA was isolated (DNA isolation kit, Roche Applied Science) from 24 h cultures of MRSA in BHI broth in the presence of oxacillin at a concentration of 8 μg mL^{-1}. Genomic DNA was tested for the presence or absence of the mecA and coa genes by the multiplex PCR system (Novocastra primer set NCL-SA-PS, Vision Bio Systems). The PCR products were visualized (using BioRad FX system) under UV transillumination following electrophoresis on 1.5% agarose gel. As a reference, the Ladder 123 (Sigma) was incorporated into agarose gel. Figure 1 was obtained using BioRad FX System.

2.3. Antimicrobial activity

Antimicrobial activity in LAB strains was assessed using two different modified procedures as described by Jacobsen et al. (1999) and Maragkoudakis et al. (2006); an agar diffusion method on solid medium, and a liquid medium containing a mixture of LAB with MRSA cultures, respectively.

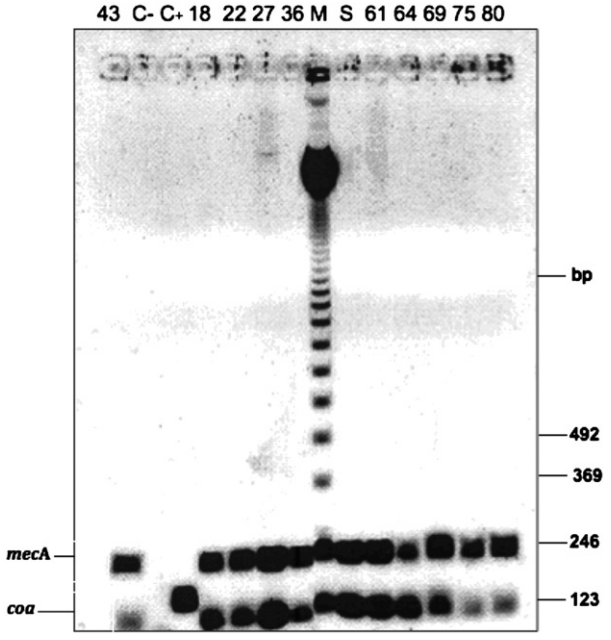

Figure 1. Multiplex PCR. The detection of the coa (117 bp) and mecA (214 bp) genes in tested MRSA clinical isolates: 43, 18, 22, 27, 36, 61, 64, 69, 75, 80. S – Standard strain MRSA ATCC 43300, M – Molecular weight marker, C+ – PCR positive control, C – PCR negative control.

The pre-culture preparation consisted in creating optimal conditions for the LAB to express their capacity to produce anti-MRSA components. Before being tested for anti-MRSA activity, the strains of LAB had been subcultured twice on Peptonized Milk Nutrient (PMN) broth to allow for the adaptation to the growth conditions. These cultures were incubated for 24 h at 37 °C.

For the preparation of MRSA strain pre-cultures, plates of soya agar with 5% sheep blood were used. After 24 h incubation at 37 °C, the cells were subcultured twice on BHI broth. The cell concentration of the pre-cultures was enumerated using the standard colony forming units (CFU) measure (Schellenberg et al. 2006).

Specifically, the agar diffusion test was divided into three steps: (1) pre-culture preparation, (2) growth of LAB cells as a spot on MRS agar for 24 h, followed by (3) preparation of MRSA solution in soft agar and addition of this solution onto the surface of Petri dishes (containing the spots of already grown LAB strains). Prepared in this way, plates were incubated for 24 or 48 h at 37 °C (depending of type MRSA strains used) and the zones of inhibited MRSA growth by LAB components were observed and measured.

The detailed procedure of agar diffusion test was as follows: a 3 μL volume of non-diluted culture of overnight (24 h at 37 °C) LAB strains raised on PMN broth were spotted on the surface of plates containing 7 mL MRS 1.2% agar. To develop the growth of LAB bacteria in the spots, the plates were incubated for 24 h at 37 °C under anaerobic conditions in gas jars using the GasPak System (BBL). The following day a 200 μL sample of an overnight pre-culture of MRSA in BHI broth medium was deposited into 7 mL of soft agar (0.7%). This soft agar contained a 1:1 mixture of BHI and MRS. After being added to soft agar, the MRSA cells were poured over the plate surfaces containing the spots of LAB. Inhibition of MRSA growth was observed if a zone free of bacterial growth formed around the spots formed by growing LAB strains.

In the case of LAB fermented food product, the pre-culture preparation step was not necessary since the food product had already been fermented by Lactobacilli previously (*Lb. casei* and *Lb. acidophilus*). Following step two (of agar diffusion method) and incubation in anaerobic conditions, the procedure for detection of anti-MRSA activity was essentially the same as described above, with the exception of the

elimination of the first step. The Petri dishes were further incubated for 24–48 h at 37 °C under aerobic conditions. The inhibition of MRSA growth diameters were then calculated and the Petri dish photographed using Alpha Imager System, and transferred to print using printer software (Alpha Ease™). Antimicrobial activity was determined after measuring the bacterial growth free inhibition zones around the spotted area.

All tests concerning the detection of antimicrobial activity were repeated three times in three independent experiments. Appropriate controls as described above in Section 2 were incorporated in each case by using the standard strain ATCC 43300 MRSA as a positive control and *Lc. lactis supp cremoris (Lc. cremoris)* as a negative control.

3. Results

3.1. Presence of MecA Gene in clinical MRSA strains isolates

The genomic identity of the clinical MRSA isolates was confirmed by the genomic DNA test using the PCR technique. The data showing the presence of mecA gene and coa gene in all clinical isolates of MRSA examined are presented in Figure 1 and confirm the methicilline resistance of all the clinical isolates.

3.2. MRSA growth inhibition illustrated by photographs

The presence of anti-MRSA activity in LAB strains was screened in the first step of our study.

The ten different strains of MRSA isolates and the MRSA standard ATCC 43300 (Swenson et al. 2001; Huletsky et al. 2004) were tested using the antimicrobial activity detection method described for the agar diffusion test in Materials and Methods. The growth inhibitory effects of *Lb. casei* or *Lb. acidophilus* against MRSA clinical isolate #43 were observed and are presented in Figure 2.

The experimental protocol was as follows: 3 μL of a 24 h culture of *Lb. casei* LBC80R was spotted on a Petri dish containing 7 mL of MRS agar and was incubated for 24 h at 37 °C under anaerobic conditions. The following day 200 μL of a 24 h culture of MRSA clinical isolate #43 was added to a 1:1 mixture of BHI broth and MRS containing 0.7% agar and poured onto the previously spotted (with LAB) Petri dish. The Petri dish was further incubated for 24 h at 37 °C under aerobic conditions and the bacterial growth diameters were calculated and Petri dish photographed. Only agents inhibiting MRSA growth formed zones of inhibition around the inoculated region with LAB. *Lb. casei* LBC80R and showed a growth inhibitory effect with an inhibitory zone of 2 cm (panel A).

A growth inhibitory effect on MRSA clinical isolate #43 was also observed with *Lb. acidophilus* CL1285 with an inhibitory zone of 3 cm (panel B). In both panels A and B, inoculation of the *Lc. lactis* spp *cremoris* culture did not produce MRSA growth inhibition as evidenced by the lack of any inhibitory zone (Figure 2, arrow).

3.3. MRSA growth inhibition by LAB fermented commercial food product

Food product was tested for the presence of antimicrobial activity against MRSA.

These products contained milk or soya-based medium fermented by *Lactobacilli* (*Lb. casei* mixed with *Lb. acidophilus*).

The procedure was as follows: the 3 μL of each product (3 μL of milk-based product contained

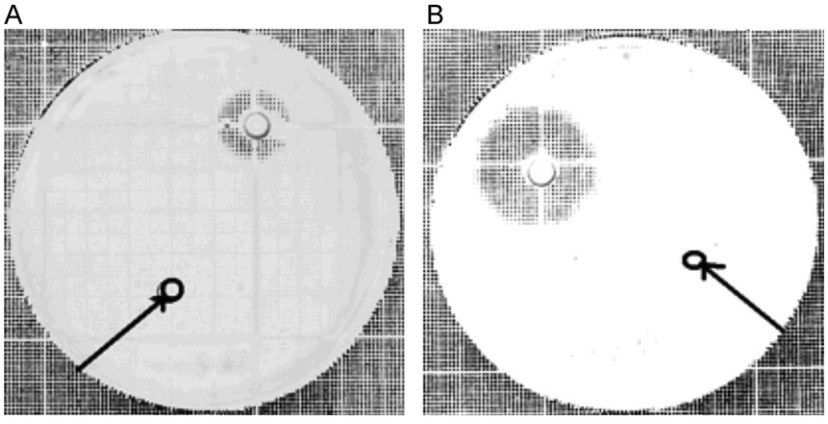

Figure 2. Antibacterial activity of LAB against MRSA strain. *Lactobacillus casei* LBC80R (Panel A) and *Lactobacillus acidophilus* CL1285 (Panel B) against clinical isolate #43. In both Petri dishes, inoculation of *Lactococcus lactis* spp. *cremoris* culture did not show any MRSA growth inhibition zones (Figure 2 arrows).

9.9×10^5 cells; 3 μL of soya-based product contained 4.5×10^5 cells) was deposited on MRS media agar plates and incubated under anaerobic conditions for 24 h at 37 °C. The strain MRSA #43 was prepared as described above. The 200 μL of the MRSA #43 or ATCC 43300 twenty four hour fresh pre-culture contained approximately 8.7×10^6 cells and was transferred into 7 mL of 0.7% soft agar and then poured on top of a layer comprised of fermented food product, which contained the LAB bacterial cells.

The antibacterial activity present in tested food products was observed during three independent experiments. The standard deviation (SD) values were calculated from the three experiments.

On milk-based medium, the inhibition MRSA growth zone diameters were 3.03 ± 0.15 cm for strain #43 and 2.83 ± 0.15 cm for MRSA strain ATCC 43300. However, on soya-based medium, the inhibition MRSA growth zone diameter was 2.43 ± 0.12 cm for strain #43 and 2.03 ± 0.25 cm for strain ATCC 43300.

The strain *Lc. lactis* spp cremoris was the control negative for these experiments since it did not exhibit the growth inhibition zones.

These results show that food products (tested on fermented milk and soya-based mediums) displayed inhibitory activity against a clinical isolate, MRSA #43 or MRSA standard strain ATCC 43300, and that the lactic acid bacteria, even in food preparations, inhibit growth of MRSA.

3.4. Determination of optimal proportions of two mixed *Lactobacillus* cultures for antimicrobial activity test

To improve the antimicrobial test, we investigated the optimal proportions of *Lb. casei* and *Lb. acidophilus* that were needed for preparing the co-cultures with the highest concentration of anti-MRSA agent(s) (Table 2).

In this experiment, the pre-culture for each strain was inoculated by 500 μL of fresh culture (after 24 h incubation at 37 °C) in separated tube of 10 mL Peptonized Milk Nutrient broth. The mixtures of these two types of strains were prepared immediately before use in the antibacterial test (using agar diffusion method). The growth inhibitory effect of mixtures comprised of different concentrations of *Lb. casei* and *Lb. acidophilus* was tested on clinical MRSA isolate #43. The MRSA growth inhibition zone diameters varied from 2.9 to 3.4 cm. The ratio that resulted in the highest inhibition zone diameters was the 1:1 ratio (volume) mixture, which consisted of approximately 64% (of total cells) *Lb. acidophilus* and approximately 36% *Lb. casei* (of total cells).

Table 2. Measure of the anti-MRSA activity, which is present in mixtures containing different ratios of LAB strains.

LAB strain Ratio	Diameters of MRSA growth inhibition zones (cm)
4 μL Lb. ac. +6 μL Lb. ca (2:3)	2.93 ± 0.40
8 μL Lb. ac. +2 μL Lb. ca (4:1)	3.27 ± 0.25
6 μL Lb. ac. +4 μL Lb. ca (3:2)	3.00 ± 0.50
5 μL Lb. ac. +5 μL Lb. ca (1:1)	3.47 ± 0.15
2 μL Lb. ac. +8 μL Lb. ca (1:4)	2.77 ± 0.21
3 μL Lc. *cremoris*	0.00

± - Standard deviation values derived from data of three independent experiments; Lb. ac - *Lactobacillus acidophilus*; Lb. ca - *Lactobacillus casei*; Lc - *Lactococcus cremoris (control)*.

These results clearly demonstrate that the relative concentrations of the LAB strains may play a significant role in favoring maximum inhibition and that the combination of *Lb. casei* and *Lb. acidophilus* is clearly inhibitory to MRSA growth.

3.5. Comparison of *Lactobacillus* antimicrobial activity and sensitivity to this activity among ten strains of MRSA

The results obtained in the next experiments (Table 3) were performed as a measure of anti-MRSA activity produced by *Lb. acidophilus* CL1285® and *Lb. casei* LBC80R against ten different MRSA clinical isolates and ATCC 43300. The inhibition of MRSA growth (as zone diameters) was seen when either *Lb. acidophilus* or *Lb. casei* were used. The extent of the inhibition varied from 1.7 to 2.9 cm for *Lb. acidophilus* and from 1.4 to 2.9 cm for *Lb. casei* depending on the MRSA clinical isolate (Table 3). These data clearly show the growth inhibition of multiple clinical MRSA isolates by *Lb. acidophilus* and *Lb. casei*. No expression of anti-MRSA activity was detected when *Lc. cremoris* was used.

3.6. Verification of MRSA growth inhibition in the presence of mixed *Lactobacillus* strains and mixed MRSA clinical isolates

In the following experiments, the growth inhibitory effect of *Lb. casei* and *Lb. acidophilus* was tested against mixtures of ten different clinical MRSA isolates.

Table 3. Diameters of MRSA growth inhibition zones of clinical isolates and MRSA ATCC 43300 by *Lb. acidophilus* LC1285 and *Lb. casei* LBC80R strains.

LAB strains	Inhibition zones diameters (cm) MRSA Strains											
	#43	#64	#75	#27	#61	#22	#18	#69	#80	#36	ATTC 43300
Lb. acidophilus CL1285®	2.93±0.12	2.53±0.15	1.93±0.12	2.43±0.12	1.70±0.26	1.97±0.25	1.93±0.12	2.07±0.12	2.03±0.06	2.97±0.06	2.77±0.21
Lb. casei LBC80R	2.07±0.12	2.10±0.10	1.43±0.21	2.03±0.06	1.93±0.12	2.13±0.15	2.27±0.25	2.43±0.12	2.47±0.06	2.90±0.10	2.30 ±0.26
Lc. Cremoris (control)	0	0	0	0	0	0	0	0	0	0	0

Notes: ± - Standard deviation values derived from data of three independent experiments; Lb. - *Lactobacillus*; Lc. - *Lactococcus cremoris (control)*; cm - Inhibition zones diameters (measured on agar dishes).

The antibacterial activity was tested using the agar diffusion method (as described in section of Materials and Methods, Sections 2,3).

We prepared pre-cultures of the following strains in separate tubes: one tube with spp. *Lb. acidophilus*, one tube with spp. *Lb. casei* and ten other tubes,each containing one of the ten different clinical MRSA strains previously described. The strains of spp. *Lb. acidophilus* and spp. *Lb. casei* were separately inoculated in the PMN medium and incubated for 24 h at 37 °C (the procedure was performed twice). To test the anti-MRSA activity by LAB strains, these two bacterial pre-cultures (spp. *Lb. acidophilus* and spp. *Lb. casei*) were mixed in a ratio 1:1 (3 μL of spp. *Lb. acidophilus*+3 μL of spp. *Lb. casei*) and immediately dropped as spots on MRS plates (3 μL by plate) and incubated for 24 h at 37 °C under anaerobic conditions. We ensured that 3 μL of these LAB mixture contained approximately 6.7×10^6 LAB cells. In parallel, but 24 h later, we separately reactivated each of the previously described clinical MRSA isolates on the blood plates and successively cultivated them on the BHI broth and incubated them for 24 h at 37 °C (repeated twice). The strain ATCC 43300 was used as positive control for the experiments.

After a second incubation, all ten MRSA strains (by aliquots of 500 μL from each strain) were mixed together in one tube to obtain a mixture. Immediately after mixing, the mixture was used in agar diffusion test.

The aliquot of 200 μL derived from the mixture of ten different clinical isolates was rapidly transferred from the tube containing the MRSA mixture to a tube containing 7 mL of 0.7% soft agar and then rapidly poured on the surface of the plate with the previously grown (during 24 h at 37 °C) spots containing the LAB strain mixture. Subsequently, the plate was incubated for 24–48 h at 37 °C as described above. The aliquot of 200 μL of the ten different clinical MRSA isolates mixed together contained approximately 1.4×10^7 MRSA cells.

When we tested this MRSA mixture with the mixture of LAB strains (*Lb. acidophilus*+*Lb. casei*, ratio 1:1), the diameters of MRSA growth inhibition zones varied from 3.20 to 3.50 cm. Using the 1:1 ratio for the mixture of LAB strains against the MRSA isolates mixture (of ten strains), it has been demonstrated that the diameters of MRSA growth inhibition zones were 3.33±0.15 cm.

The antibacterial activity of this Lactobacillus mixture was also tested against MRSA ATCC 43300 strains. The diameter of MRSA 43300 growth inhibition zones was 2.77±0.21 cm.

The above mentioned standard deviations are presented in Table 3.

These results clearly show that the LAB (alone or in combination) inhibit more than one (multiple).

MRSA clinical isolates and that the combination of *Lb. casei* and *Lb. acidophilus* at 1:1 ratio demonstrates the anti-MRSA inhibition effect.

3.7. Anti-MRSA activity expressed by LAB in liquid medium

After obtaining qualitative findings in the preceding tests of anti-MRSA activity of LAB, we proceeded with a quantitative test to get a closer look at this interesting and promising direct interaction between LAB and MRSA cells in liquid medium. In this medium, both species shared the same environment.

Two types of bacterials cultures were prepared; the first, containing pure monocultures with *Lb. acidophilus*, *Lb. casei* and MRSA strain #43 and the second containing mixtures of co-cultures with these three microorganisms.

The pure culture was also considered as a control culture in studying the direct interaction between the cells of LAB and MRSA.

The results obtained from our observation of growth development of LAB+MRSA in mixed cultures incubated together for 3 days in liquid medium were essential to establish a hypothesis if the antibacterial activity expressed by LAB against MRSA was bacteriostatic or bactericid for MRSA cells.

The experimental protocol for preparation of the pre-culture was to incubate 500 μL of bacteria in 10 ml Peptonized Milk Nutrient (PMN) at 37 °C for 24 h. The viability of monocultures over time was recorded (as shown in Table 4) under continuous culture conditions. The continuous cultures (for 3 days) were prepared as described by Schellenberg et al. (2006). In this experiment, only the volumes of the pre-culture additions were modified from 200 to 500 μL and were added to 10 mL of PMN medium.

For the preparation of continuous cultures, the bacteria came from pre-cultures incubated for 24 h at 37 °C and they were subcultured twice as described above. For the first day of continuous culture, the 500 μL of 24 h bacterial culture was transferred into 10 mL of PMN broth medium and incubated for 24 h at 37 °C. An aliquot of 500 μL from these 24 h cultures was transferred into a fresh 10 mL volume of PMN and was incubated for another 24 h at 37 °C. This culture was designated as 48 h. The third day cultures were prepared as for second day and designated as 72 h of incubation (see Table 4).

Colony forming units (CFU) were measured using standard methods (see Schellenberg et al. 2006) by counting the colonies formed on Peptonized Milk Nutrient (PMN agar 1.2%) solid medium.

Table 4 shows the number cells of lactic acid bacteria strains or MRSA strain, which were cultivated in mono-culture. The viability of these cultures was represented as a function of incubation time.

Evidently, the PMN liquid medium is appropriate for the cultivation of all of spp. of tested microorganism. In addition, their cell concentration after 24 h of incubation at 37 °C is crucial.

This information was necessary for the preparation of mixed co-cultures for the next step of experimentation. Aliquots from the 24 h incubated pure monocultures of each strains served as starting material for preparation of the mixed cultures. These cultures were prepared separately in liquid medium (PMN) by mixing 100 μL of *Lb. acidophilus* (1.3×10^6 cells), 100 μL *Lb. casei* (3.2×10^6 cells) and 100 μL MRSA #43 (3.8×10^6 cells) and adding them to 10 mL of Peptonized Milk. In this step of the experimental manipulation, it is important to precisely know the number of MRSA cells present in 10 mL of medium

Table 4. Comparison of growth and viability between LAB and MRSA cells cultivated as pure monocultures or mixed co-cultures CFU were measured in liquid medium culture.

Medium	Bacterial Culture	CFU/mL as a function of incubation time		
		24 h	48 h	72 h
PMN	Monoculture *Lb. acidophilus* LC1285	1.29×10^8	3.54×10^8	1.34×10^8
PMN	Monoculture *Lb. casei* LBC80R	3.20×10^8	2.33×10^8	2.26×10^8
PMN	Monoculture MRSA #43	3.76×10^8	3.80×10^8	2.30×10^8
MSA	Mixed culture *Lb. acidophilus* LC1285 *Lb. casei* LBC80R MRSA #43	2.99×10^3	0	0
PMN	Mixed culture *Lb. acidophilus* LC1285 *Lb. casei* LBC80R MRSA #43	3.65×10^8	2.12×10^8	1.07×10^8

LAB – Lactic Acid Bacteria; MRSA – Methicillin-Resistant *Staphylococcus aureus*; PMN – Peptonized Milk Nutrient agar (can grow both LAB and MRSA); MSA – Mannitol Salt agar (selective medium, can grow only MRSA).

after inoculation, but prior to the incubation period.

The mixed cultures were grown for 24, 48 and 72 h at 37 °C. After incubation, the number of CFU were estimated on the agar plates. The 100 μL of samples containing suspension of mixed cultures were spread in parallel on two types of media over the agar plates. To differentiate the LAB from MRSA, both species present in mixed cultures, two types of agar plates were used: PMN agar (a non-selective medium) and MSA (a selective medium for MRSA). After inoculation and before incubation, the mixture contained the LAB cells and approximately 3.8×10^6 of MRSA cells in 10 mL of the medium. In contrast, as demonstrated in Table 4, after 24 h at 37 °C incubation of the mixture, we were able to detect only about 2.99×10^3 mL^{-1} MRSA cells, while the mixture contained 3.65×10^8 mL^{-1} of LAB cells. The decrease in the number of MRSA cells reveals the presence of bactericid activity produced by LAB cells against MRSA microorganisms.

The results summarized in Table 4 show that after 24 h at 37 °C incubation of MRSA with *Lb. acidophilus* and *Lb. casei*, more than 99% of the MRSA bacteria were eliminated.

These data support the results obtained previously using the agar diffusion method but in addition prove the bactericid activity of lactic acid bacteria against MRSA cells.

4. Discussion

This study shows that the LAB *Lb. acidophilus* CL1285® and *Lb. casei* LBC80R produce antimicrobial components that can inhibit the growth and eliminate of the MRSA cells. This phenomenon was also observed when tests were performed with commercialised food products, which were fermented by the patented LAB strains *Lb. acidophilus* CL1285® and *Lb. casei* LBC80R.

To our knowledge, these results provide the first direct evidence that MRSA strains collected from a variety of patients were vulnerable to the antimicrobial action expressed by LAB when tested *in vitro*. The interaction between Lactic acid bacteria and MRSA, in mixed liquid culture, can be bactericidal for a pathogenic microorganism such as MRSA.

The objective of our research was not a comparative study of different pure LAB strains but rather to increase our knowledge on bactericidal effect produced by mixed LAB cells against MRSA when these two species are cultivated together in the same environment in liquid medium. In this situation, they can produce multiple antibacterial components, which are absent when each species has been cultivated separately as pure monoculture.

In order to prevent variability in the results, homogenous MRSA and LAB strains were chosen.

In addition, for the agar diffusion spot test, we standardized and optimized bacterial cultures and the inoculation method. This method included a preparation of conditions and cell concentrations for LAB, which can be used for elimination of the pathogenic cells from mixed co-culture (Martins and Cunha 2007).

In the course of this study, the antibacterial activities against MRSA produced by a mixture of LAB strains cultivated on milk or soya-based medium were analyzed. The analysis of the anti-MRSA activity of these patented strains increased our knowledge on the use of natural media, such as milk or soya, for multiplication of LAB strains.

The dimensions of the inhibitory zones are related to the concentration of LAB cells. They were different in milk from that seen in soya-based medium. In soya-based medium, the cell concentration, as measured by our method, was smaller than in milk-based medium. Both of these natural media were effective in the production of antibacterial agent activity. Matto et al. (2006) reported that a probiotic mixture containing multiple strains with different properties, resulted in more effective activity in the prevention of infection with pathogenic bacteria.

We also observed that the proportion of cells added to co-cultures during the preparation of a solution formed by LAB strains influenced their anti-MRSA activity (Table 2). These results (Table 2) clearly demonstrate that the *Lb. acidophilus* (64%) rather than *Lb. casei* (34%) in mixed solution expressed the best antibacterial effects.

The results presented in Table 3 compare the two types of lactic acid bacteria and their antimicrobial activity against ten strains of MRSA clinical isolates.

Table 3 also shows the level of sensitivity of each one of the ten MRSA strains to the antibacterial action of LAB *Lb. casei* or *Lb. acidophilus*. When the diameters of MRSA-inhibited zones on Petri dish assays are compared, it is evident that they are similar for all 10 different MRSA strains, which leads to the conclusion that the sensitivity of all these strains to LAB inhibitory activity is also similar.

The agar diffusion method was primarily used to study the effects on the production of anti-MRSA compounds by LAB co-cultures, which were mixed with co-cultures of ten MRSA isolates. This study

demonstrated that whether all ten MRSA isolates or a single (#43) isolate were tested with cultures of LAB strains, the sensitivity of MRSA towards antimicrobial LAB activity was similar (as comparing for ration 1:1 of *Lb. acidophilus* and *casei* mixture).

During the next step of our study, the interaction between LAB strains and one MRSA strain in the liquid medium, using well-defined cell concentrations and standardized culture conditions, was investigated. In mixed or pure cultures of LAB and MRSA, the concentration of bacterial cells was determined by standard of CFU counting (Schellenberg et al. 2006) (Table 4). For measurement of cell viability in mixed LAB and MRSA cultures, the samples from these cultures were transferred (for inoculation) on agar Petri plates containing a MRSA selective medium or on plates containing a non-selective medium for both organisms (Table 4). After incubation, the viability of LAB and MRSA was estimated (Lin et al. 2006). The data show that LAB cultures produced the antibacterial compounds reducing the number of MRSA cells more than 5 $\log_{10}$ CFU of MRSA population within 24 h at 37 °C (Table 4). This estimation was based on a comparison between the quantity of MRSA cells in monoculture (the cells bacterial concentration was $\times 10^8$ after 24 h at 37 °C incubation, Table 4) and the quantity in mixed MRSA with LAB culture (MRSA the cells concentration was $\times 10^3$ after 24 h at 37 °C incubation, Table 4).

As described, these experiments prove the bactericidal activity of LAB mixture against MRSA strain. This phenomenon can have a practical application if it can be performed in vivo …

It is difficult to compare our results with results of other studies concerning the co-culture of LAB with MRSA, since the majority of information available is on the co-culture of LAB with the *S. aureus* sensitive to methicillin (MSSA) (Huletsky et al. 2004; Millette et al. 2006). Our situation is very different.

The global activity of mixed microbial populations is determined by the presence and function of each species, which are strongly influenced by interactions among the different partners.

However, current knowledge of microbial physiology is generally based on pure culture studies and conditions, that are different from those encountered in a complex ecosystem. Consequently, performing mixed culture studies is essential to get closer to the reality of complex populations that exist in hospitalized humans.

The results presented in this paper are in contrast with those published in previous studies that reported multiple difficulties related to their experimental procedures, which attempted to demonstrate probiotic activity of LAB strains (Annuk et al. 2003; Lin et al. 2006; Ammor et al. 2006a, b; Hernandez et al. 2005; Normanno et al. 2007). Contrary to those studies, we were able to develop better methods and thus, did not observe the variability in LAB antibacterial activity which was more stable and lower than that reported by the above cited authors. In addition, the data reported in this study using the agar diffusion test was confirmed by testing cell viability in liquid medium and co-cultures containing one MRSA strain mixed with LAB strains (Table 4).

The LAB anti-pathogenic strains that were investigated in this study have been used earlier by Millette et al. (2006) and Beausoleil et al. (2007) against pathogenic organisms. The results obtained by Millette et al. (2006) showed that the whey isolated from fermentation of milk transformed by strains CL1285®*Lb. acidophilus* and *Lb. casei* (presently designated as LBC80R) was able to inhibit the growth of pathogenic bacteria such as *S. aureus* (MSSA) by 85%, *Listeria monocytogenes* by 78% and E coli 0157:H by 77%. Moreover, the same microbial anti-pathogenic bacteria, *Lb. acidophilus* and *Lb. casei* cultures, were capable of delaying the growth of these food-borne pathogens. The results obtained by Beausoleil et al. (2007), showed that daily intake of commercial Bio-K+ International Inc. product containing *Lb. acidophilus* CL1285® and *Lb. casei* LBC80R was a safe and effective means of preventing antibiotic-associated diarrhea (AAD) caused by *Clostridium difficile* in hospitalized patients.

As reported by Rodgers (2008), the food service sector now has access to microbial protective cultures, such as the availability of commercial microbial culture preparations. These include nisin-producing BS-10® (*Lb. lactis* spp. lactic) from Chr. Hansen (Denmark), BIOPROFIT™ (*Lactobacillus rhamnosus* LC705) from Bio-Gaia (Sweden), the Bovamine Meat Cultures™ from Texas Tech University (Texas, USA) active against *Salmonella* and *Escherichia coli* in meat (Anon 2006), and HOLD-BAC™ series (*Lactobacillus plantarum*, *Lb. rhamnosus*, *Lactobacillus sakei*, *Lactobacillus paracasei* and *Proprionibacterium freudenreichii* spp. *shermanii*) from DANISCO (Denmark), active against *Listeria*.

Finally, even though there is a growing demand for products marketed as "probiotics", as Rodgers' (2008) list would suggest, relevant scientific data does not always follow.

Consequently, well-documented scientific research on these products is still necessary.

5. Conclusion

Our findings lend support to the assertion that there are components produced by lactic acid bacteria (LAB) that can inhibit growth and eliminate MRSA.

These effects open the possibility for a protective use of LAB against infections with MRSA.

While using natural media and adapted cell concentrations of LAB and MRSA strains, we have standardized conditions to determine the efficiency of LAB to express their anti-MRSA activity.

For this purpose, strains of MRSA from clinical human isolates and strains of *Lactobacillus* currently used by the dairy industry were selected for study. These two microbial species are part of the flora that live on humans and animal tissues.

We believe the study will provide an opportunity to explore this natural process by letting the antagonism between LAB and MRSA play out to the advantage of human health.

We hope that our research will lead to *in vivo* studies of the inhibitory reactions similar to those described *in vitro*. This approach has merit is supported by the finding that *Clostridium difficile* diarrhea can be controlled by administering mixed cultures of *Lactobacillus* (Beausoleil et al. 2007).

At this time, there is an urgent need for a new program to control of the rapidly progressing dissemination of MRSA in the human and animal populations. Currently, the use of antibiotics destroys a patient's microbial ecology, which was established during the evolutionary process and thus allowed for the adaptation of humans and animals to life on our planet.

For this reason, the application of new food additives to dairy and other food products, which can stimulate growth of lactic acid bacteria and increase their antagonistic activity expressed by LAB (probiotic) toward most pathogenic organisms, opens new perspectives for research.

This new preventive approach offers great possibilities to put a stop to selection and dissemination of antibiotic-resistant microbial infections. What becomes very interesting, as demonstrated by our research, is that one can choose mixtures of various probiotics to better adapt their common action and obtain surprising results.

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