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Full Length Research PaperAmanda Rafaela Carneiro de Mesquita

Activity of metabolites produced by new strains of *Lactobacillus* in modified de Man, Rogosa and Sharpe (MRS) medium against multidrug-resistant bacteria

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The emergence of nearly untreatable infections caused by multidrug-resistant bacteria has led to a new public health concern in which a need for development of alternative non-antibiotic strategies has become urgent. The activity of metabolites produced by new strains of *Lactobacillus* against multidrug-resistant bacteria was investigated. The objective of this work was to isolate and identify lactobacilli from artisanal kefir by 16S rRNA gene sequencing as well as to evaluate the effect of the growth of *Lactobacillus* species in the Man Rogosa and Sharpe broth, supplemented with Tween 80 at concentrations of 0.6, 0.4, and 0.2 %. Cell Free Supernatants (CFSs) were obtained from these lactobacilli cultures and with them, organic acids (lactic acid and acetic acid) and ethanol were quantified by HPLC followed by the determination of their antimicrobial activities against eight strains of *Staphylococcus aureus*-MRSA and *Klebsiella pneumonia* KPC strains. The GenBank BLAST analysis revealed that, the isolated lactobacilli belonged to *Lactobacillus paracasei* (n=4) and *Lactobacillus plantarum* (n=5) strains. Increasing concentrations of Tween 80 did not affect the growth of *Lactobacillus* species–significantly when compared to their controls (MRS broth). HPLC analysis of CFSs showed concentrations greater than 18.0, 4.0 and 1.0 g/L for lactic acid, acetic acid and ethanol, respectively. All CFSs were able to inhibit all pathogenic microorganisms evaluated. The percentage of inhibition was on average greater than 88% for MRSA and KPC strains. The antimicrobial activity was dependent on the CFSs tested. Based on these experimental conditions, organic acids and ethanol are likely to be responsible for this antimicrobial activity.

Key words: Antibacterial activity, lactobacilli, cell free supernatants, Tween 80.

INTRODUCTION

The nosocomial infections caused by multidrug-resistant microorganisms (MDR) are among the most serious problems of clinical medicine and pose a major public health concern (Cecchini et al., 2015). Methicillin resistant *Staphylococcus aureus* (MRSA) and extended spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* are

the main bacteria associated with nosocomial infections (Fuzi, 2016). MDR develop numerous strategies of resistance to antimicrobial agents and host defenses. Therefore, patients infected by these microorganisms have increased length of hospital stay and higher mortality rate compared to individuals infected with susceptible strains (Spicknall et al., 2013).

According to Cecchini et al. (2015), MDR infections are expected to reach 40 % increase by 2050 that could lead to 10 million deaths per year worldwide. An estimate of between 100 million and 30 billion dollars is being spent annually to treat these kinds of infections, for which therapy is limited to the use of few antimicrobial agents that are often ineffective. In this context, new searches for alternative therapeutic regimen, including the use of probiotics, are needed in order to handle complex infections (Arqués et al., 2015; Alexandre et al., 2014; Alexandre et al., 2013).

Probiotics are defined as living microorganisms, which when administered in adequate amounts, bring a health benefit to the host (Gasbarrini et al., 2016). The probiotics and their byproducts are being used either as prophylactic agents to prevent or delay colonization as well as to combat infections (Şanlıbaba and Güçer, 2015; Prado et al., 2015 Alexandre et al., 2014).

Among the probiotic bacteria, *Lactobacillus* species have received increasing attention because of their specific role in maintaining human health (Gasbarrini et al., 2016). The beneficial action of lactobacilli has been widely described in several reports which focus on their viable cells and cell free supernatants (CFS) (Mariam et al., 2014; Lau and Liong, 2014). The CFS is a well-known source of bioactive compounds such as antioxidants, bacteriocins, surfactants, organic acids, H_2O_2, CO_2, and low molecular weight peptides (Lau and Liong, 2014; Sgibnev and Kremleva, 2016). Their biosynthesis depends on the factors linked to growth such as pH, temperature, O_2 tension, and culture medium composition (Zalán et al., 2010).

Lactobacilli are fastidious microorganisms because they need specific compounds for their growth that are not normally present in other culture media. One of these compounds is Tween 80 that is present Man Rogosa and Sharpe medium (De Man et al., 1960). Tween 80 is a water-soluble ester of oleic acid, which enhances growth and changes the fatty acid composition of cytoplasmic membrane of lactobacilli, making them resistant to environmental stresses (Endo et al., 2006; Li et al., 2011; Broadbent et al., 2014).

In this way, several studies were published relating the presence of Tween 80 in the culture medium to the membrane composition of lactobacilli (Nikkila et al., 1995; Endo et al., 2006; Broadbent et al., 2014). However, the effect of different concentrations of this surfactant on the metabolism of carbohydrates as well as the antibacterial activity of CFSs against MRSA and KPC strains are still under explored (Mariam et al., 2014; Arqués et al., 2015).

Based on the description above, the aim of this study was (i): To isolate and identify *Lactobacillus* sp. from kefir; (ii) To evaluate the effect of Man Rogosa and Sharpe medium supplemented with 0.6, 0.4 and 0.2 % Tween 80 on growth of *Lactobacillus* sp.; (iii) To obtain cell free supernatants (CFSs) from these cultures; (iv) To quantify lactic acid, acetic acid and ethanol as well as to determine their antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumoniae* producing carbapenemase (KPC) strains.

MATERIALS AND METHODS

Isolation of Lactobacilli and phenotypic characterization

Kefir samples of different sources (milk and water) were serially diluted in sterile 0.9 % saline, seeded on Man Rogosa and Sharpe agar, MRS (Lactobacilli MRS Agar, Difco™) and incubated at 30°C for 72 h under 5 % CO_2. Colonies of different morphologies were isolated in MRS plates and characterized by the methodology described in the Bergey's Manual of Determinative Bacteriology (Hammes and Hertel, 2009). Gram-positive, non-sporulating, catalase negative, and rod-shaped bacteria were assumed as being Lactobacilli, and therefore sub-cultured onto MRS agar and then stored into MRS broth with 20 % (v/v) glycerol at -80°C.

Identification of selected Lactobacilli

Lactobacilli isolates (n=9) were identified by Polymerase Chain Reaction (PCR), according to Dos Santos et al., 2015, DNA was extracted using ZR Fungal/bacterial DNA kit (ZymoReasearch, Irving, CA, USA). Taxonomical identification was confirmed by sequencing of the PCR- amplified 16S rRNA using the universal pair of primer 8F (5′-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3′) and the reverse primer 1512R (5′-GTG AAG CTTACG GYTAGC TTG TTA CGA CTT-3′), where Y means C + T and M means A + C. In order, to differentiate *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*, an average 400 base pair fragments from 16 to 23S intergenic spacing region were also PCR-amplified and sequenced, using the pair of primers Lu-5 (5′-CTA GCGGGT GCG ACT TTG TT-3′) and Lac-2 (5′-CCT CTT CGC TCG CCG CTA CT-3′).

All PCR reactions were done in a final volume of 25 µL, each containing 0.125 µL of Taq DNA polymerase (New England Biolabs) and 5 pmol of each primer. Sequencing reactions were performed for the forward and reverse strands using the DYEnamic™ET Cycle Sequencing kit (GE Healthcare, Piscataway, NJ, USA) and the DNA sequencing analyzer MegaBACE (GE Healthcare, Little Chalfont, UK). BLAST tool and *Lactobacillus* genome reference sequences (*L. casei* ATCC 334 – AF121200, *L. paracasei* JCM1181- AF182724, *L. plantarum* ATCC 14917 – AF080101, *L. pentosus* ATCC 8041 – U97134, from National Center for Biotechnology Information (NCBI)) were used for identification analysis. Additional sequence alignments were

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performed using APE (A plasmid editor V 2.0). *L. plantarum* ATCC 8014, *L. rhamnosus* ATCC 9595, and *L. casei* ATCC 7469 strains were used as control organisms in the identification process.

Preparation of modified Man Rogosa and Sharpe (MRS) media, standardization of inoculum and growth of *Lactobacillus* sp.

Lactobacillus species were previously cultured onto MRS agar and incubated at 30°C for 48 h. Single colonies were selected and inoculated into MRS broth supplemented with Tween 80 at concentrations of 0.2, 0.4, and 0.6 % (Sigma-Aldrich) (MRS/TW) to obtain turbidities comparable to the 0.5 McFarland standard (10^8 CFU/mL).

Afterwards, these bacterial suspensions were diluted into their respective broths (MRS/TW) to obtain a final inoculum of 10^6 Colony Forming Units per milliliter (CFU/mL). All cultures were incubated at 30°C and samples were analyzed after 6, 9, 12, 24, 48, and 72 h to determine their growth over time. Viable cells of lactobacilli were enumerated and results were expressed as $\log_{10}$ CFU/mL. The MRS broth was used as a control. Parameters related to growth as doubling time (g) and specific growth rates (μ) were calculated (Brizuela et al., 2001; Georgieva et al., 2014).

Organic acids and ethanol analysis by High Performance Liquid Chromatography-HPLC

Lactobacilli cultures in MRS/TW 0.6 % broth were incubated for 48 h and centrifuged at 1,300 g for 15 min. The supernatants were filtered through a 0.22 μm polytetrafluoroethylene membrane (Chromafil®). The resulting CFS_S were diluted 1:10 in sterile HPLC-grade water followed by the detection and quantification of lactic acid, acetic acid and ethanol.

The analysis was performed on a High Performance Liquid Chromatography column coupled with a refractive-index detector (HPLC-RID) (Agilent, 1200 series), equipped with a binary pump and a diode array detector. Separations were achieved with a column Aminex HPX-87H (300 mm x 7.8 mm, Bio-Rad). Mobile phase was composed of a 5.0 mM sulfuric acid solution at a flow rate of 0.6 mL/min at 35°C. Running time was 25 min and the injection volume of the samples was 20 μL. The uninoculated MRS broth served as a negative control. The identification of compounds was performed by using DL-lactic acid, acetic acid (Sigma-Aldrich), and ethanol (Merck) standards to compare their retention times with those found in the literature (Sluiter et al., 2008), while the quantification was performed by external calibration with the standards. All samples were analyzed in triplicate.

Determination of the antibacterial activity of cell free supernatants CFS_s

The antibacterial activity of CFS_s was determined against seven *Staphylococcus aureus* methicillin resistant (MRSA) (LFBM 13, LFBM 14, LFBM 17, LFBM 29, LFBM 30, LFBM 34, LFBM 35) and *Klebsiella pneumoniae* producing carbapenemase (LFBM 01, LFBM 02, LFBM 03, LFBM 04, LFBM 05, LFBM 06, LFBM 08) strains. These microorganisms were obtained from stock cultures and maintained in our laboratory (Laboratório de Fisiologia e Bioquímica de Microorganismos (LFBM).

All microrganisms used in this study showed a resistance phenotype to several antimicrobial agents such as beta-lactams, aminoglycosides, macrolides, fluoroquinolones, tetracycline, chloramphenicol and carbapenems, previously determined by the broth dilution method. *Staphylococcus aureus* ATCC 33591 and *Klebsiella pneumoniae* ATCC BAA 1705 were included in this study as resistant control strains.

These microorganisms were cultured into Mueller Hinton broth. The CFSs were deposited in sterile 96-wells microplates, and five microliters of test microorganism suspensions were inoculated in each well to give a final concentration of 10^4 CFU/mL. The growth inhibition was demonstrated by optical density at 630 nm using a microplate reader (Thermo plate – TP Reader®) after 24 h incubation at 35°C. Considering the total growth (100 %) in the control wells (MRS broth inoculated), the percentage of growth reduction was attributed to the remaining wells (CFS + bacterium). Negative controls were included after the neutralization of CFSs with 1N NaOH solution (to pH 7.0). All experiments were performed in triplicate.

Statistical analysis

Data were expressed by mean ± standard deviation. Analysis was achieved by using the statistical software Graph pad Prism version 5.0. Differences between means were evaluated using one-way and two-way ANOVA. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Lactobacilli identification

The Lactobacilli isolates presented unique genetic profiles based on 16S rDNA and 16–23S intergenic spacer region identification. The sequence identities were 99 % similar to *L. paracasei* (GenBank accession N° AF182724) and 99% similar to *L. plantarum* (GenBank accession N° AF080101). These strains were designated as *L. paracasei* (LFBM 01, LFBM 05, LFBM 06, and LFBM 10) and *L. plantarum* (LFBM 02, LFBM 04, LFBM 07, LFBM 08 and LFBM 09).

L. paracasei and *L. plantarum* are known to be associated with a variety of fermented food such as milk and kefir (Prado et al., 2015).

Growth of the *Lactobacillus* species

The growth curves of *Lactobacillus* species exposed to different concentrations of MRS/TW are showed in Figures 1, 2, 3 and 4.

The different concentrations of Tween 80 (0.2, 0.4 and 0.6%) present in the MRS medium did not significantly affect the growth of *L. paracasei* and *L. plantarum* strains, nor did it affect those of *Lactobacillus rhamnosus* ATCC 9595 when compared to their controls or to each other, $p<0.05$. A discrete growth increase of the *Lactobacillus plantarum* LFBM 02, LFBM 04 and LFBM09 strains in MRS (control) was observed at 24 h. However, this growth was not statistically significant when compared to their respective growth in the MRS/TW media, $p<0.05$ Figure 2 and 3.

The Lactobacilli cultures that remained in the exponential phase for 24 h (0 to 24 h) were used to estimate the linear regression equation. The *Lactobacillus* species showed specific growth rate and doubling time

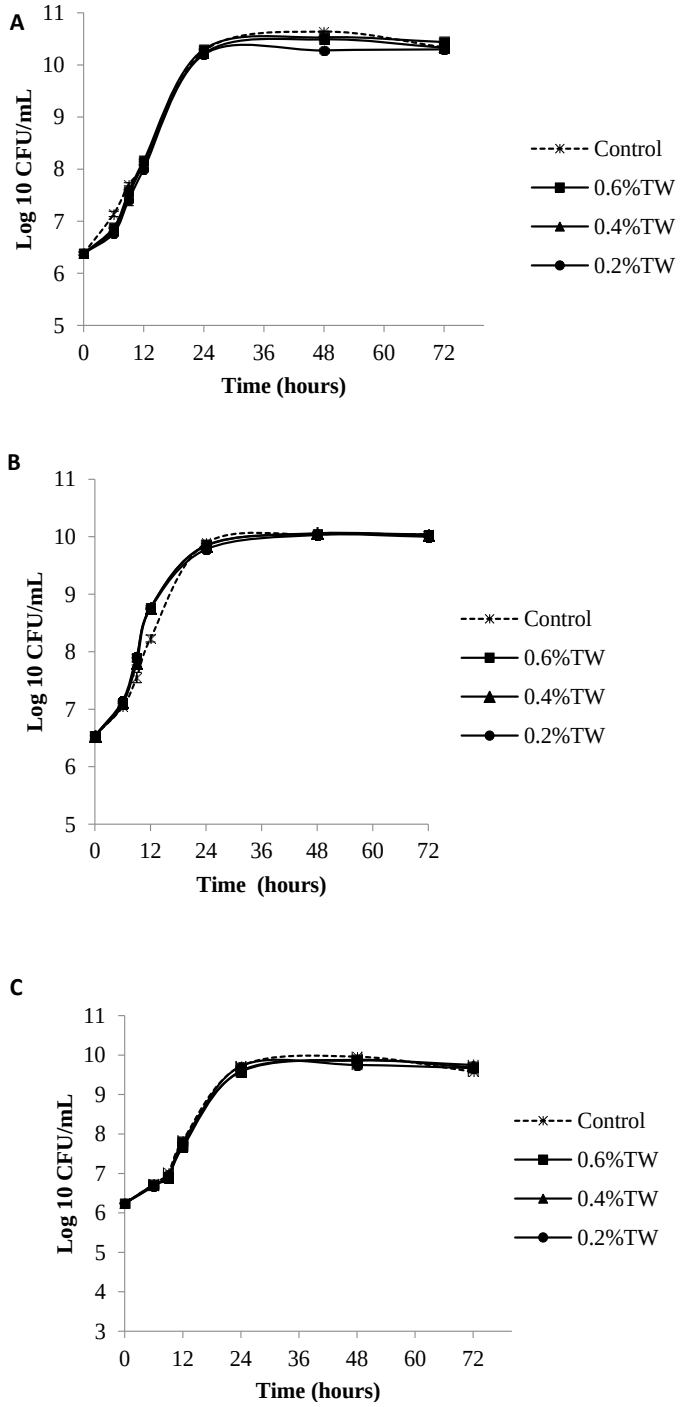

Figure 1. The growth of three strains of *Lactobacillus paracasei* in Man Rogosa and Sharpe-MRS control broths (✳) and in the MRS broths supplemented with Tween 80 (TW) at 0.6% (■), 0.4% (▲) and 0.2% (●); (A) LFBM 01, (B) LFBM 05, (C) LFBM 06.

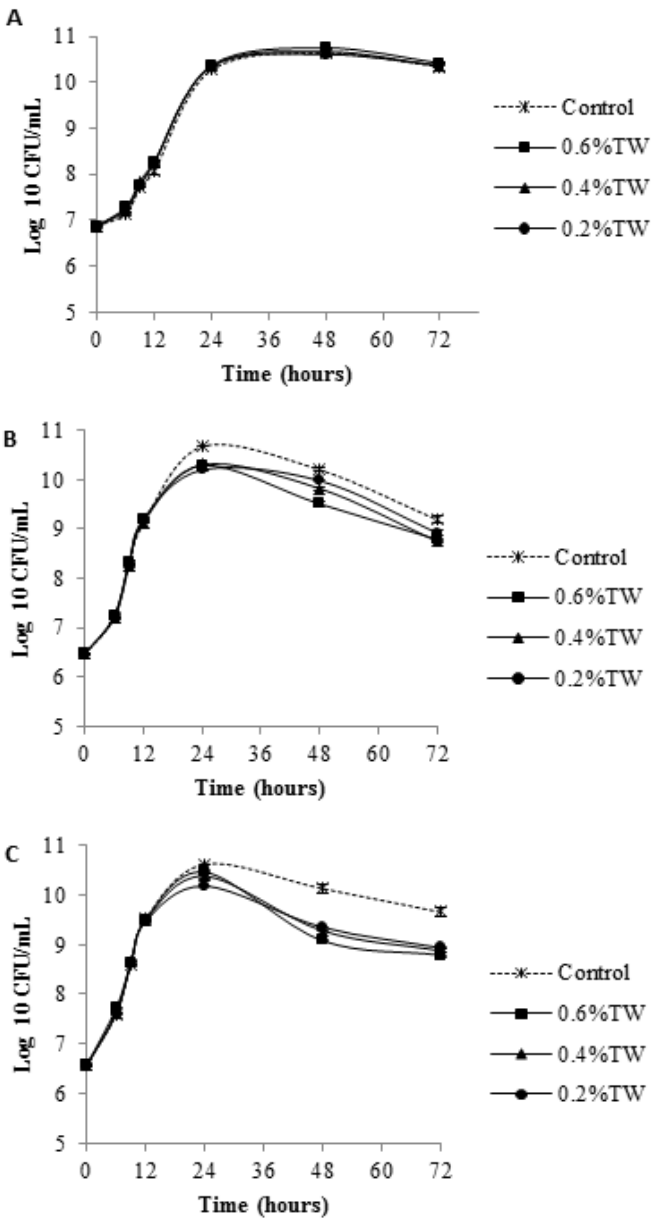

Figure 2. The growth of *Lactobacilli* in Man Rogosa and Sharpe-MRS control broths (✳) and in the MRS broths supplemented with Tween 80 (TW) at 0.6% (■), 0.4% (▲) and 0.2% (●); (A) *L. paracasei* LFBM 10, (B) *L. plantarum* LFBM 02, (C) *L. plantarum* LFBM 04.

values between 0.12 to 0.21 h^{-1} and 1.38 to 2.44 h, respectively, Table 1 and 2. When these values were compared to control (MRS), no statistical difference was observed $p<0.05$. These results are in accordance with some studies that reported a doubling time of about 1 h for lactobacilli (Brizuela et al., 2001; Ayeni et al., 2011; Rezvani et al., 2016).

It is known that, the growth of lactobacilli can be strongly affected by Tween 80 which is often included in the culture media as a growth factor for fastidious bacteria as well as for lactic acid bacteria and *Mycobacterium tuberculosis* (Li et al., 2011; Ghodbane et al., 2014). The oleic acid present in Tween 80 is incorporated into the lipid membranes of bacteria altering the fatty acid composition, fluidity and its permeability

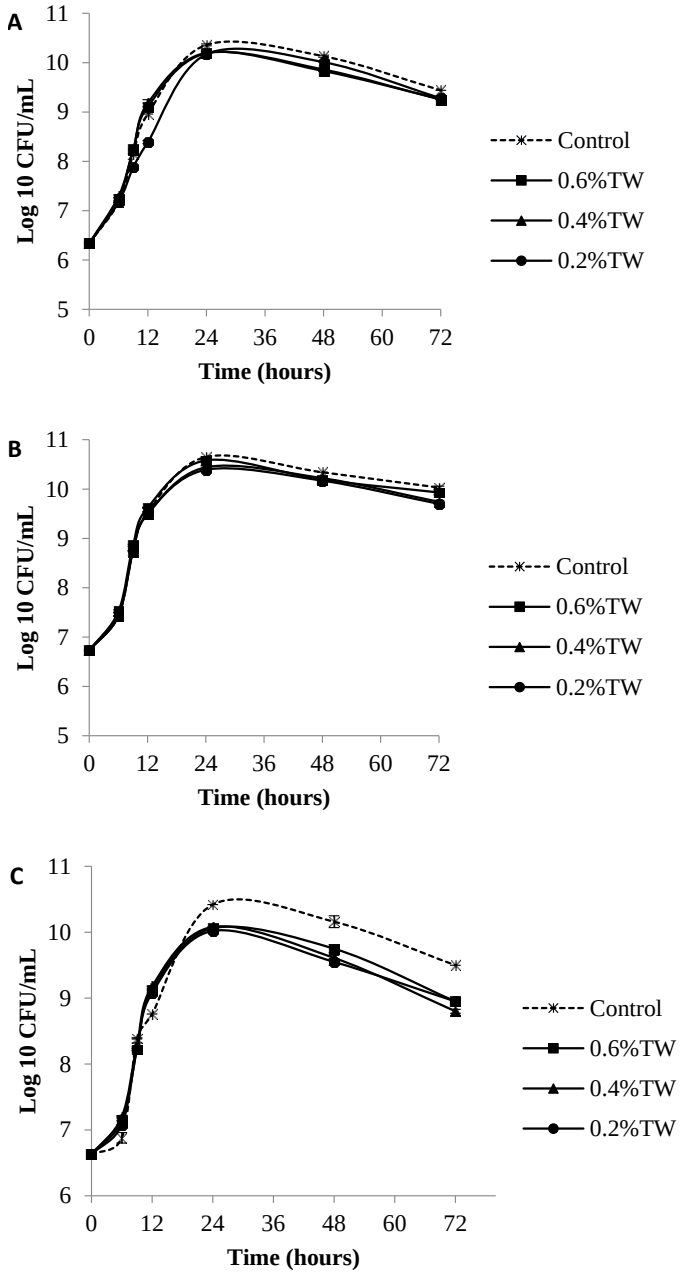

Figure 3. The growth of three strains of *Lactobacillus plantarum* in Man Rogosa and Sharpe- MRS control broths (✳) and in the MRS broths supplemented with Tween 80 (TW) at 0.6% (■), 0.4% (▲), and 0.2% (●); (A) LFBM 07, (B) LFBM 08, (C) LFBM 09.

(Nikkila et al., 1995; Kankaanpää et al., 2004). In addition, these fatty acids can still replace the requirement for biotin and be used as a carbon source by some lactobacilli (Li at al., 2011).

In Lactobacilli, the oleic acid is incorporated into membranes through methylenation to form dihydrosterculic acid (9,10-methyleneoctadecanoic acid; C19:0cyc9) (Nikkila et al., 1995) which leads to their increasing tolerance to low pH, high bile salts and NaCl

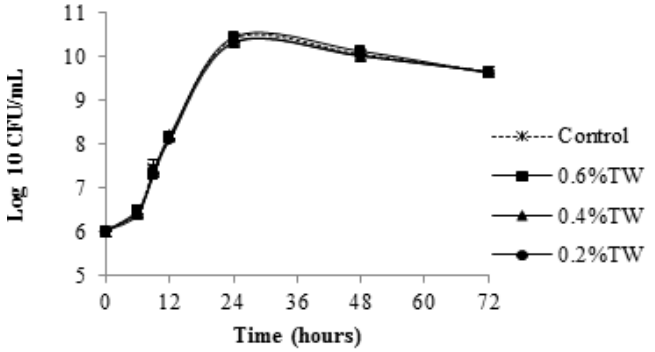

Figure 4. The growth of *Lactobacillus rhamnosus* ATCC 9595 in Man Rogosa and Sharpe-MRS control broths (✳) and in the MRS broths supplemented with Tween 80 (TW) at 0.6% (■), 0.4%(▲), and 0.2% (●).

concentrations, as well as their protection against oxidative stress (Li et al., 2011; Hayek and Ibrahim, 2013). The presence of oleic acid in the culture media also stimulates glucosyl transferase secretion as well as accumulation of glycine and betaine, which are amino acids that preserve the structure and function of cellular proteins in environments at high osmolarity level (Guillot et al., 2000; Jacques et al., 1985).

Organics acids and ethanol production

In this work, lactic acid was the major metabolite produced by *Lactobacillus* species whose values ranged from 18.59 to 23.32 g/L, Figure 5. Acetic acid and ethanol were both detected in low quantities Figure 6 and 7. Concentrations greater than 4.0 and 1.0 g/L were produced for acetic acid and ethanol, respectively. We verified that the production of organic acids and ethanol was dependent on the lactobacilli isolated. These results did not present significant differences when compared to each other $p < 0.05$.

Although there is no consensus on the amount of lactic acid that can be produced by lactobacilli, values similar to those found in this study were published by Broadbent et al. (2014) and Ayeni et al. (2011). In addition, the variation of organic acid amounts is dependent on several factors, such as culture conditions, medium composition and in particular, the species of *Lactobacillus* (Srivastava et al., 2015; Zalán et al., 2010). Srivastava et al. (2015) and Coelho et al. (2011) attributed a positive correlation between the presence of Tween 80 in several culture media and the lactic acid production by *Lactobacillus* species. This surfactant promotes the migration of nutrients into the cell by increasing its metabolism, by releasing intracellular enzymes and consequently increasing the production of byproducts (Broadbent et al., 2014).

According to Coelho et al. (2011), higher concentrations

Table 1. Doubling time and specific growth rates of four *Lactobacillus paracasei* strains and *Lactobacillus rhamnosus* ATCC 9595 grown in MRS and MRS supplemented with 0.6%, 0.4 and 0.2% Tween 80.

Strains	Doubling time (g) and growth rates (μ)	Control MRS	MRS supplemented with Tween 80 (TW)		
			TW 0.6%	TW 0.4%	TW 0.2%
L. paracasei LFBM 01	g (h)	1.74 ± 0.04	1.65 ± 0.01	1.67 ± 0.03	1.63 ± 0.04
	μ (h^{-1})	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
L. paracasei LFBM 05	g (h)	1.91 ± 0.03	2.32 ± 0.05	2.22 ± 0.05	2.44 ± 0.02
	μ (h^{-1})	0.15 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
L. paracasei LFBM 06	g (h)	1.68 ± 0.01	1.69 ± 0.02	1.69 ± 0.01	1.65 ± 0.02
	μ (h^{-1})	0.18 ± 0.01	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
L. paracasei LFBM 10	g (h)	1.77 ± 0.01	1.74 ± 0.04	1.81 ± 0.01	1.76 ± 0.01
	μ (h^{-1})	0.17 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
L. rhamnosus ATCC 9595	g (h)	1.38 ± 0.04	1.38 ± 0.03	1.40 ± 0.04	1.39 ± 0.02
	μ (h^{-1})	0.20 ± 0.01	0.21 ± 0.01	0.21 ± 0.01	0.21 ± 0.01

g = doubling time, μ= specific growth rates, TW= Tween 80, LFBM: Laboratório de Fisiologia e Bioquímica de Micro-organismos. Results are expressed by means and standard deviation of two independent trials.

Table 2. Doubling time and specific growth rates of five *Lactobacillus plantarum* grown in MRS and MRS supplemented with 0.6%, 0.4% and 0.2% Tween 80.

Strains	Doubling time (g) and growth rates (μ)	Control MRS	MRS supplemented with Tween 80 (TW)		
			TW 0.6%	TW 0.4%	TW 0.2%
L. plantarum LFBM 02	g (h)	1.73 ± 0.01	1.90 ± 0.00	1.90 ± 0.01	1.95 ± 0.01
	μ (h^{-1})	0.17 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.15 ± 0.00
L. plantarum LFBM 04	g (h)	1.87 ± 0.08	1.86 ± 0.00	1.91 ± 0.03	1.95 ± 0.07
	μ (h^{-1})	0.16 ± 0.01	0.16 ± 0.00	0.15 ± 0.00	0.15 ± 0.00
L. plantarum LFBM 07	g (h)	1.80 ± 0.00	1.89 ± 0.01	1.88 ± 0.01	1.89 ± 0.01
	μ (h^{-1})	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00
L. plantarum LFBM 08	g (h)	1.50 ± 0.01	1.88 ± 0.00	1.95 ± 0.02	1.98 ± 0.01
	μ (h^{-1})	0.16 ± 0.00	0.16 ± 0.00	0.15 ± 0.00	0.15 ± 0.00
L. plantarum LFBM 09	g (h)	1.95 ± 0.01	2.15 ± 0.01	2.13 ± 0.01	2.17 ± 0.01
	μ (h^{-1})	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.00	0.14 ± 0.00

g = doubling time, μ= specific growth rates, TW: Tween 80, LFBM: Laboratório de Fisiologia e Bioquímica de Micro-organismos. Results are expressed by means and standard deviation of two independent trials.

of Tween 80 (higher than 1.4 w/v) were able to decrease the lactic acid production. This observation is likely due to the destruction of the cell membrane and loss of its function, caused by the solubility of lipid bilayer (Qi et al., 2009) as well as by the reduction of lactate dehydrogenase activity (Nagarjun et al., 2005).

In a study conducted by Broadbent et al., 2014, it was observed that the lactic acid production by *Lactobacillus casei* ATCC 334 was influenced mainly by Tween 80 concentration, much more than medium pH or cyclopropane synthase activity, enzyme responsible for the conversion of oleic acid (present in Tween 80) to dihydrosterculic acid. However, when this biosynthesis occurred at a pH of 3.8, the cyclopropane synthase (Cfa) was required in order to protect the cytoplasmic membrane against acid stress. These authors constructed an ATCC 334 *cfa* knockout mutant and found out that the Cfa inactivation had a negative effect on lactobacilli metabolism, at low pH, mainly on the lactic acid production.

Antimicrobial activity of CFS_S

The results of the antimicrobial activity of CFSs against eight MRSA and KPC strains are presented in Figures 9 and 10. All CFSs from ten lactobacilli cultures showed a stronger inhibitory effect on the growth of test microorganisms. These inhibitory percentuales ranged from 65 to 97%. When CFSs were compared to each other no significant difference was observed, except for the CFS from *L. plantarum* 09, which has showed to be

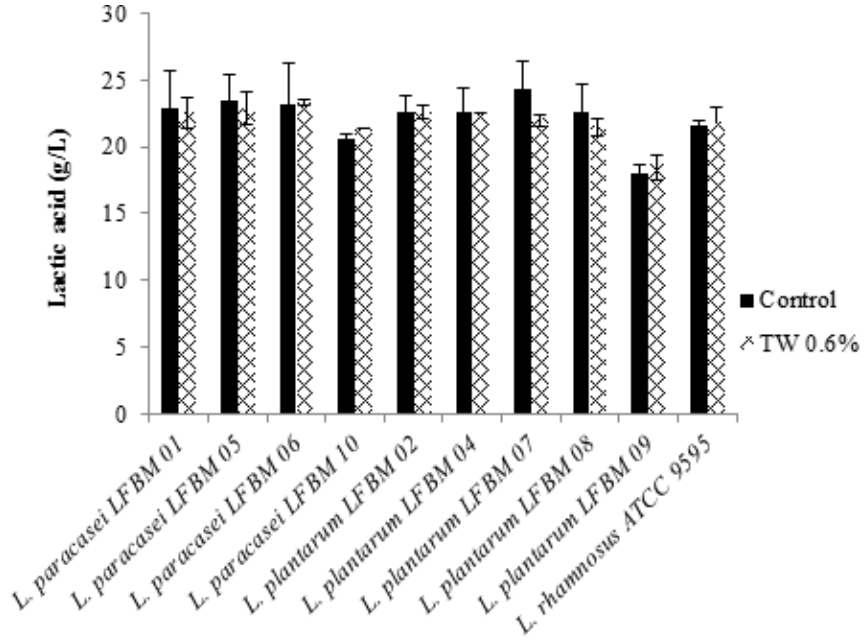

Figure 5. Lactic acid production by ten lactobacilli in Man Rogosa and Sharpe medium-MRS (control) as well as in the MRS supplemented with 0.6% Tween 80 (TW). The analysis was performed with cell free supernatants.

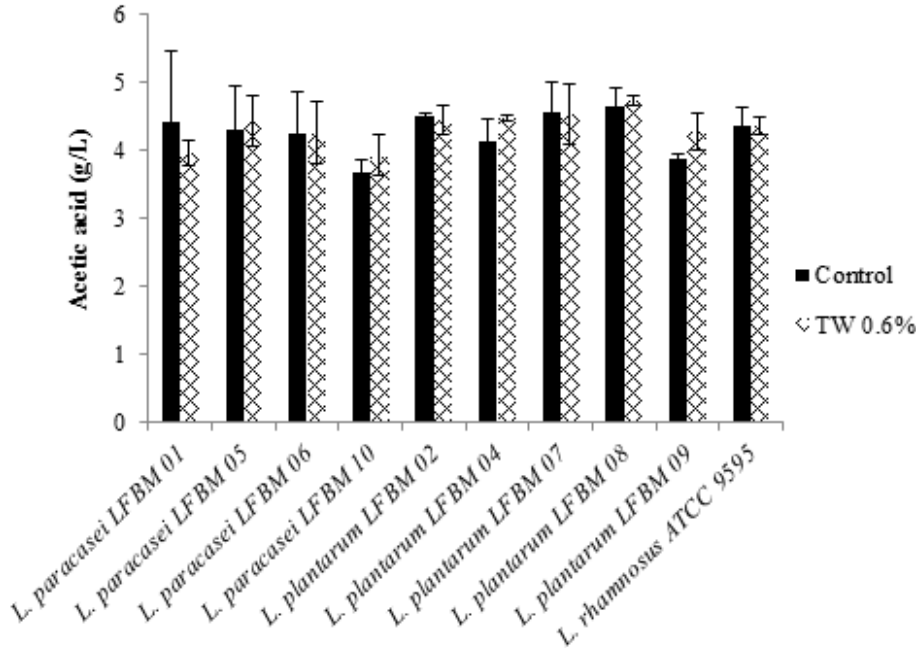

Figure 6. Acetic acid production by ten lactobacilli in Man Rogosa and Sharpe broths (MRS) as well as in the MRS supplemented with 0.6% Tween 80 (TW). The analysis was performed with cell free supernatants.

less active ($p<0.05$). This antimicrobial activity was dependent on the CFSs tested.

In the present study, a pH decrease was observed after the incubation into MRS/TW broth for 48 h, Figure 8. This fact suggests that the anti-MRSA and anti-KPC activities of CFSs are closely related to the production of organic

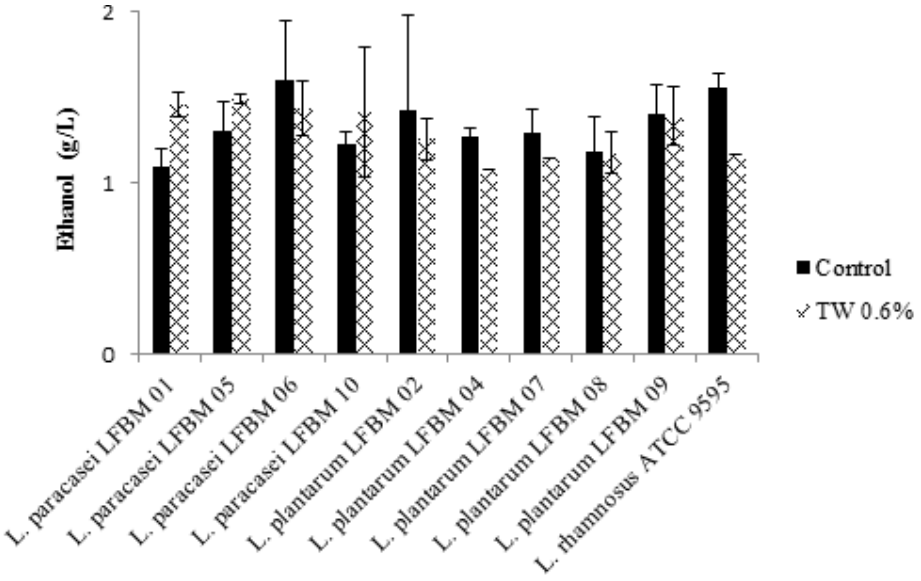

Figure 7. Ethanol production by ten lactobacilli in Man Rogosa and Sharpe broth (MRS) as well as in the MRS supplemented with 0.6% Tween 80 (TW). The analysis was performed with cell free supernatants.

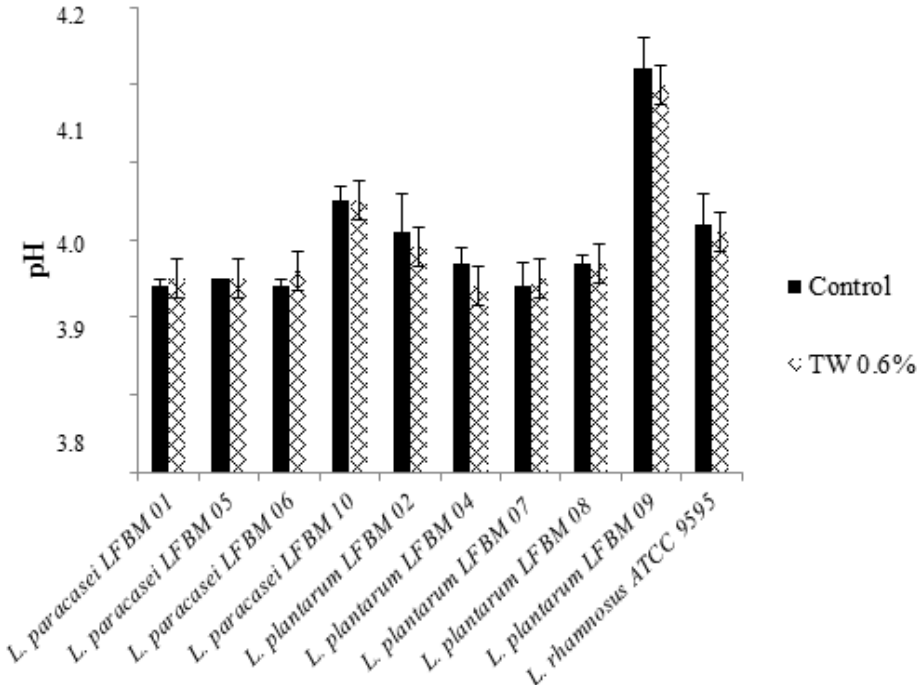

Figure 8. pH values of cell free supernatant of ten lactobacilli cultures in Man Rogosa and Sharpe broth-MRS (control) as well as in the MRS supplemented with 0.6% Tween 80 (TW).

acids, mainly lactic acid. After the neutralization of CFSs, a complete loss of the antibacterial activity was observed. This fact supports that these extracellular antibacterial agents may have an acid nature. The *Lactobacillus* genus has the ability to produce large amounts of organic acids through the fermentation of carbohydrates besides carbon dioxide, H_2O_2, surfactants and bacteriocins, all with antimicrobial activity (Lau and Liong et al., 2014; Di

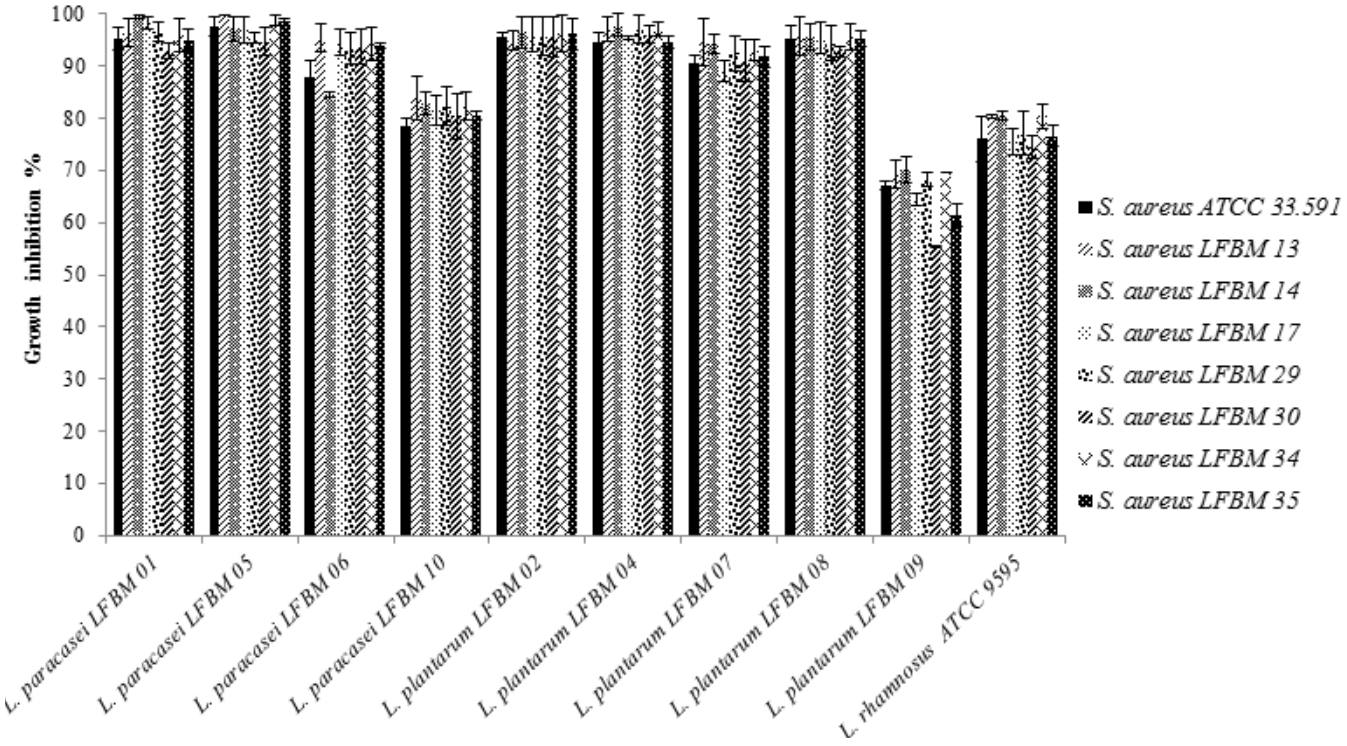

Figure 9. Antibacterial activity of cell free supernatants from ten lactobacilli cultures against eight methicillin resistant *Staphylococcus aureus*–MRSA strains.

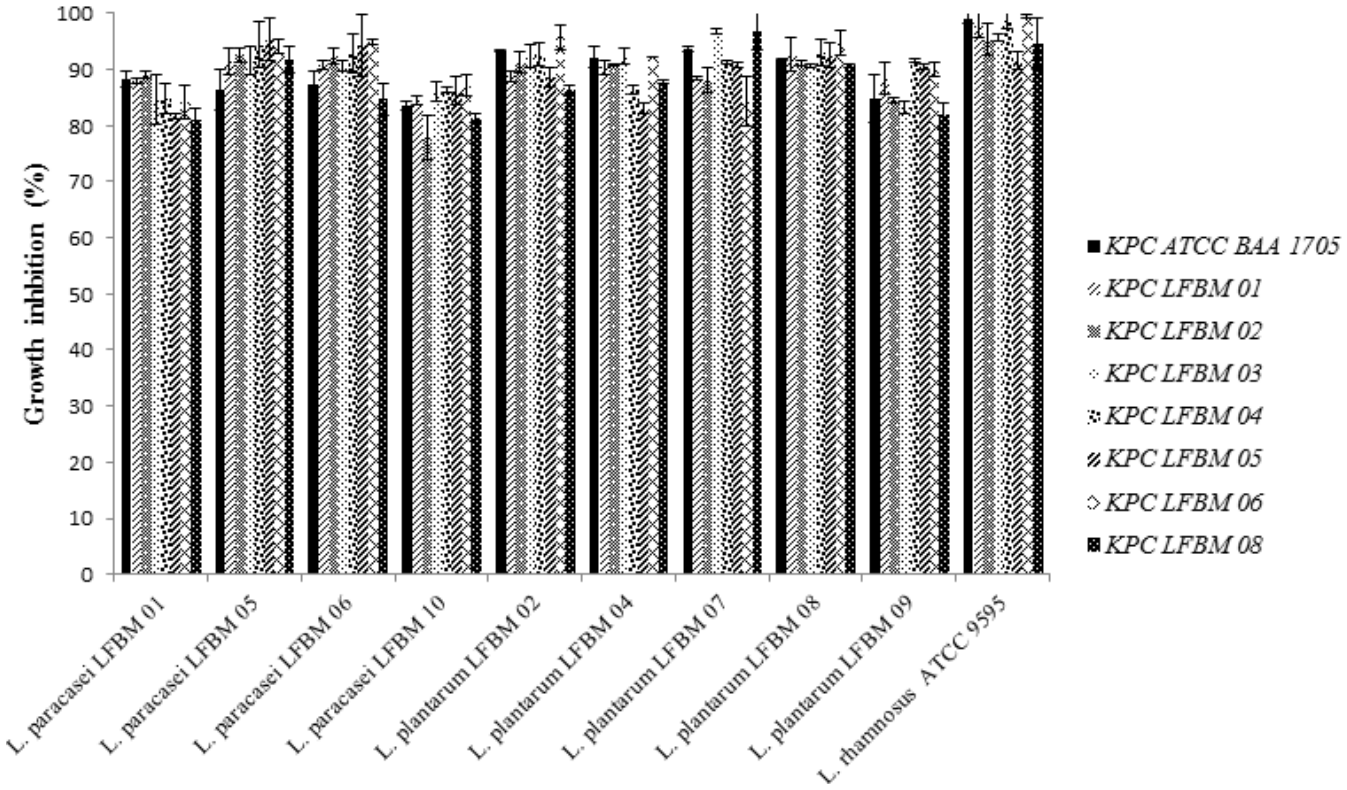

Figure 10. Antibacterial activity of cell free supernatants from ten lactobacilli cultures against eight *Klebsiella pneumoniae* producing KPC carbapenemase strains.

Cerbo et al., 2016; Sgibenev et al., 2016).

The possibility of a bacteriocin being responsible for this activity was ruled out for two reasons: (i) The activity of most bacteriocins drastically decreases during the stationary growth phase (Taheri et al., 2012) (ii) Bacteriocins are unstable in environments below pH 4.0 (Todorov and Dicks, 2005). According to Todorov and Dicks (2005), the supplementation of Tween 80 in the growth medium reduced the level of ST28MS and ST26MS bacteriocins by *L. plantarum*. These authors also observed that these bacteriocins were chemically unstable at low pH levels and that a significant decrease and consecutive loss of their activities occurred after the logarithmic phase. The loss of activity was attributed to different mechanisms such as proteolytic degradation, protein aggregation, adsorption to cell surfaces, and feedback regulation. Moreover, a similar activity pattern was also observed for lactacin B, helveticin J, mesenterocin 5 and enterocin 1146.

The antimicrobial activity of CFSs could be related to the presence of H_2O_2, However, we have used the MRS medium in which lactobacilli do not produce this metabolite. Although the MRS medium is widely used for the growth of lactobacilli, it is less suitable for the studies involving H_2O_2 production, due to the presence of manganese that can catalyze the breakdown of H_2O_2. Thus, some alternative media that lack manganese have been developed, such as MRS without manganese and LAPTg medium. Moreover, the H_2O_2 presence in CFSs would have little or no in inhibition in cases when catalase producer organisms, such as *Staphylococcus aureus*, are being evaluated (Pridmore et al., 2008; Martín and Suarez, 2010).

Several studies have been published on antimicrobial activity of lactic acid produced by lactobacilli against pathogen microorganisms (Şanlıbaba and Güçer, 2015; Arqués et al., 2015; Sgibnev and Kremleva, 2016). The antimicrobial activity of organic acids is directly related to the pH reduction and its ability to dissociate. The undissociated forms of these acids are presumed to penetrate the bacterial membrane and dissociate within the cell. As bacteria maintain a neutral cytoplasm pH, the efflux of protons will consume cellular ATP and result in energy depletion (Wang et al., 2015).

The ability to produce large quantities of organic acids, mainly lactic acid through the fermentation of carbohydrates and consequent pH decrease, are fundamental for the antimicrobial activity of *Lactobacillus* species.

Conclusions

According to our results, we conclude that the products from the metabolism of lactobacilli isolate were responsible for the antibacterial activity observed in our study. These important activities against both MRSA and KPC-producing strains may be an important tool towards new searches for therapy regimen against infections caused by these microorganisms.

It is possible that lactic acid, which was produced in higher amounts, may have played an important role on this activity together with acetic acid and ethanol. Moreover, the presence of other bioactive compounds present in the culture media may have acted synergistically to achieve such activities. Thus, further studies are necessary before new therapies can be implemented.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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