EVALUATION OF ANTIMICROBIAL ACTIVITY OF
Streptococcus thermophilus, Lactobacillus viridescens AND
Lactobacillus sakei

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ABSTRACT – Bacteriocins are peptides, synthesized on the ribosome of bacteria that show specific antimicrobial activity against a range of bacteria. Among the bacteria producing bacteriocin, the lactic acid bacteria have special emphasis for producing bacteriocins that are not toxic and are easily broken in the mammalian stomach. Therefore, this study evaluated the potential of strains of Streptococcus thermophilus, Lactobacillus viridescens, Lactobacillus sakei for the production of bacteriocins. Fermentations were performed in de Man, Rogosa and Sharpe (MRS) broth. After this, the broth was done lyophilization, being subsequently evaluated the inhibitory potential compared lyophilized and non lyophilized broth by the diffusion method on agar plates, where it was found inhibitory effect compared to the pathogenic microorganisms Escherichia coli and Staphylococcus aureus. The results confirmed the potential of lactic acid bacteria tested for the production of bacteriocins with inhibitory effects against the tested pathogenic microorganisms.

KEYWORDS: Bacteriocin, lactic acid bacteria, antimicrobial, pathogenic microorganism.

1. INTRODUCTION.

The lactic acid bacteria (LAB) are characterized as Gram-positive coccus or bacillus, non-aerobic, but aerotolerant, capable of fermenting carbohydrates for energy and lactic acid production (Parada et al., 2007). LAB are generally known as safe (Generally Recognized as Safe - GRAS), and has an important role in the preservation of food and fermented products. They can be used as competitive naturally microflora or as specific enzymes under controlled conditions (Cintas et al., 2001, apud Parada et al., 2007). Some of these bacteria produce substances of protein structure (both proteins and polypeptides) called bacteriocins, which in small amounts are very active against pathogenic microorganisms (Klaenhammer, 1993; Beasley et al., 2004).

Bacteriocins differ antibiotics by having a spectrum of antimicrobial activity against closely related species, while antibiotics have effect against a wide spectrum of microorganisms. It is estimated that most of the bacteria existent can produce some kind of bacteriocin (Beasley et al., 2004; Rodriguez et al., 2000; Nascimento et al., 2008; Tagg et. al., 1976).

Bacteriocins generally have low molecular weight (rarely more than 10 kDa) and undergoing post-translational modification, moreover, can be easily degraded by proteolytic enzymes, especially proteases in the gastrointestinal tract of mammals, which make them safe for human consumption (Rodriguez et al., 2000).
Different mechanisms of action have been proposed for bacteriocins, including the alteration of enzyme activity, inhibition of spore germination and inactivation of anionic carriers by forming pores selective and nonselective (ABEE, 1995; Martinez e de Matins, 2006, apud PARADA, 2007). Bacteriocins may function through different mechanisms to exert an antimicrobial effect, but the cell membrane is usually target of bacteriocins action by formation of pores or rupture of the membrane with consequent leakage of intracellular material and cell death (Reddy et al., 2004).

The main application of bacteriocins is intended for the food industry (Cleveland et al., 2001; Zamfir et al, 2000). In industrialized countries, it has been found that about 30% of the population showed to be infected with pathogenic bacteria and fungi foodborne (Galvez et al., 2007). In addition, there is great concern regarding the use of chemical preservatives in food and risks associated with them, therefore, the biopreservation technique of food becomes a very attractive alternative to chemical preservatives currently used (Nascimento et al., 2008; Benitez, 2010; Machado et al., 2011).

Besides the food field, there is a strong potential for the use of bacteriocins as an alternative to existing antibiotics. The accelerated growth and spread of multi-resistant pathogenic bacteria have forced researchers and companies to seek alternative methods for the treatment of infections (Benitez, 2010). Moreover, bacteriocin can also be used as alternatives to agrochemicals used for controlling plant diseases. Gupta and Srivastava (2014), have studied the application of an antimicrobial peptide produced by Lactobacillus plantarum to combat the growth of spoilage fungi in grains.

In view of the above, this study aimed to evaluate the bacteriocins production potential of strains of lactic acid bacteria Streptococcus thermophilus, Lactobacillus sakei and Lactobacillus viridescens and check the inhibition strength of bacteriocins produced front to pathogenic microorganisms Escherichia coli and Staphylococcus aureus, common in food contamination.

2. MATERIALS AND METHODS.

The tests were performed with three strains of lactic acid bacteria, Streptococcus thermophiles, Lactobacillus viridescens and Lactobacillus sakei. For production of bacteriocins, fermentation was carried out in De Man, Rogosa and Sharpe (MRS) broth using for this Erlemeyers containing 250 ml of broth. The Erlenmeyer flasks containing the broth were autoclaved, and then inoculated with 2 ml of the active LAB strain in a concentration of 3,0x10⁸ UFC/mL, as in Mac Farland scale (Oplustil, 2004). Fermentation was performed in shaker with temperature control, maintained at 37 °C under constant agitation of 120 RPM. To check the effects of fermentation time on the inhibition of pathogens, samples were taken after 24 h and 48 h. Fermentations were performed in duplicate, totaling 12 samples.

After fermentation, the samples were centrifuged at 4 °C for 25 min at 6000 RPM to separate the cells and solids from the fermentation broth. After that, samples were collected from the centrifuged fermented broth (CFB) for verification of antimicrobial activity. Were also taken 50 mL samples of CFB for the solution concentration. The concentration was performed by lyophilization of the samples for 72 h. Then it was weighed 1.00 g of lyophilized samples and diluted into 2 ml of sterile saline solution to form a concentrated broth (CB).

The antimicrobial activity of the samples was tested with two bacteria: Staphylococcus aureus (ATCC 25923) and Escherichia coli (ATCC 25922) which were kept in Brain Heart Infusion (BHI) broth added 10% glycerol at -20 °C. For the tests, the bacteria from the stock culture were sown into nutrient agar plates and incubated for 24 h at 36 °C.

For inoculum preparation, 3 to 4 colonies of the bacteria were scraped from the Petri dish with the aid of a sterile strap and suspended in 5 ml of sterile saline solution. The turbidity of the solution was checked for adjustment of 0.5 McFarland scale (1,5x10⁸ CFU/mL) (Oplustil, 2004). The culture medium was prepared according to the manufacturer's instructions, and sterilized by autoclaving for 15 min at 120 °C. Muller-Hinton agar was added to petri dishes to give a thickness of 4 to 6 mm being done orifices about 7 mm in diameter. It was left spacing at least 15 mm between the well and the
other, keeping a maximum of 4 wells per plate. The prepared bacterial inoculum was sown on the surface of agar Muller-Hinton with the aid of a sterile swab. Aliquots of 60 µL of the CFB and CB samples, previously sterilized by filtration through a membrane of 22 µm were placed in each well. This procedure was performed in triplicate.

The sown plates were incubated in triplicate at 35 °C for 24 h in bacteriological incubator. After this period the plates homogeneity was observed and was read the bacterial inhibition halos. This technique consists in measuring the diameter in millimeters with a ruler. The inhibition strength is measured according to the methodology proposed by Aslim et al. (2005), in which the inhibition halos are classified according to their diameter, as follows:

– Neutral, no inhibition;
+ Weak, inhibition zone of 8,0-10,0 mm;
++ Intermediate, inhibition zone of 10,0-20,0 mm;
+++ Strong, inhibition zone of 20,0-30,0 mm.

3. RESULTS AND DISCUSSION.

After the fermentation period, it was observed that the fermentation was normal, there is good cell growth and appropriate turbidity of the medium in all samples.

Regarding the antimicrobial activity test performed in CFB samples, only one of the plates containing metabolites relating to the fermented broth by Lactobacillus viridescens, showed the formation of inhibition zone with 9 mm diameter around the well excavated in the sown plate of Staphylococcus aureus, which is classified as weak in relation to its inhibition force. In other plates there was no formation of inhibition zone.

The antimicrobial activity test performed in CB samples showed better results. It was possible to obtain a visible halo inhibition for the majority of tests performed. As example shown in Figure 1.

Some samples formed cloudy inhibition halo, indicating that there was an early microbial growth around the well excavated followed by the death of microorganisms around the well. Other samples showed clear inhibition zone (Figure 1), demonstrating that it wasn’t possible bacterial growth occur at any time near the well containing the produced metabolites. The results are shown in Table 1.

The results presented in Table 1 demonstrate that all three strains LAB tested were capable of producing metabolites which show antimicrobial activity against Staphylococcus aureus, and the metabolites produced by Lactobacillus viridescens showed the highest inhibition values and were the only ones to produce an halo that completely inhibited bacterial growth. All metabolites produced were classified with inhibition force as intermediate against Staphylococcus aureus.

Figure 1 – Agar plate with excavated well containing inhibition halo.

Source: Author, (2016).
In inhibition test against *Escherichia coli*, it is clear that the metabolites produced by *Lactobacillus sakei* achieved similar results to those observed against *Staphylococcus aureus*. The metabolites produced by *Streptococcus thermophilus* showed no antimicrobial activity against *Escherichia coli*. As for the metabolites produced by *Lactobacillus viridescens*, showed better results against *Escherichia coli*, reaching complete inhibition of bacteria and getting strong rating for the inhibition force.

The analysis of the results also show that fermentations performed in 48 h produced inhibition zone larger than the fermentations in 24 h. This indicates that the fermentations in 48 h probably have a higher concentration of metabolites with antimicrobial effect on the fermented broth.

### Table 1 - Inhibition zone measurement results.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fermentation time (h)</th>
<th>Staphylococcus aureus</th>
<th>Escherichia coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>Standard deviation</td>
</tr>
<tr>
<td><em>Lactobacillus sakei</em></td>
<td>24</td>
<td>12,17*</td>
<td>1,33</td>
</tr>
<tr>
<td><em>Lactobacillus sakei</em></td>
<td>48</td>
<td>14,00*</td>
<td>1,55</td>
</tr>
<tr>
<td><em>Lactobacillus viridescens</em></td>
<td>24</td>
<td>15,83</td>
<td>1,17</td>
</tr>
<tr>
<td><em>Lactobacillus viridescens</em></td>
<td>48</td>
<td>17,83</td>
<td>1,17</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>24</td>
<td>11,83*</td>
<td>1,17</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>48</td>
<td>15,17*</td>
<td>1,33</td>
</tr>
</tbody>
</table>

* Results that show partial inhibition.

### 4. CONCLUSIONS.

The results demonstrate the potential of LAB strains studied to produce metabolites with antimicrobial effect against the tested pathogenic microorganisms. The metabolites produced by *Lactobacillus viridescens* showed best performance among all the samples produced, achieving have inhibitory effect against Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*). This result demonstrates the potential of these metabolites for use as inhibiting growth agent of pathogenic microorganisms. Further studies to confirm that the metabolites with antimicrobial effect these are bacteriocins are needed.

It can also be conclude that the fermentation broth, under conditions where this study was conducted, showed no sufficient metabolite concentration to inhibit the growth of pathogenic microorganisms, requiring concentration. A study involving the purification and concentration of metabolites obtained can provide even better results than those observed in this work.

### 5. ACKNOWLEDGEMENTS.

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### 6. BIBLIOGRAPHIC REFERENCES.
