








## Nisin and $\epsilon$ -poly-L-lysine as natural antimicrobials towards spoilage-associated *Lactobacillus plantarum*

Fernanda Cristina Kandalski Bortolotto<sup>1</sup>  Maria Helena da Rosa Farfan<sup>1</sup>   
Nathalia Cristina Kleinke Jede<sup>1</sup>  Gabriela Maia Danielski<sup>1</sup>  Renata Ernlund Freitas de Macedo<sup>1\*</sup> 

<sup>1</sup>Programa de Pós-Graduação em Ciência Animal, Escola de Ciências da Vida, Pontifícia Universidade Católica do Paraná (PUCPR), 80215-901, Curitiba, PR, Brasil. E-mail: renata.macedo@pucpr.br. \*Corresponding author.

**ABSTRACT:** Sausages are highly susceptible to microbial spoilage. Lactic acid bacteria (LAB) is the main group of spoilage bacteria in vacuum packed cooked sausages. To control microbial growth natural antimicrobials have been used as food preservatives. The aim of this study was to identify strains of lactic acid bacteria isolated from spoiled commercial Calabresa sausages and use them in an in vitro challenge with the natural antimicrobials, nisin (NI) and  $\epsilon$ -poly-L-lysine ( $\epsilon$ -PL). Mass spectrometry identification of LAB isolated from sausages using MALDI-TOF revealed a predominance of *L. plantarum* in the LAB population. RAPD-PCR of *L. plantarum* strains showed four different genetic profiles. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of NI and  $\epsilon$ -PL, alone and in combination, against a pool of different profiles *L. plantarum* were determined. MIC of NI and  $\epsilon$ -PL were 0.468 mg/L and 75 mg/L, respectively, whereas MBC of NI and  $\epsilon$ -PL were 12.48 mg/L and 150 mg/L, respectively. The combined effect of NI and  $\epsilon$ -PL was determined using concentrations at 1/4 and 1/8 of individual MICs. Synergistic effect was confirmed at both concentrations showing a fractional inhibitory concentration index of 0.5 and 0.2, respectively. The combination of NI and  $\epsilon$ -PL at a small concentration of 0.05 mg/L and 9.375 mg/L, respectively, showed inhibitory effect towards spoilage *L. plantarum*. Results show the potential of the combined use of NI and  $\epsilon$ -PL to control sausage spoilage-associated with lactobacilli.

**Key words:** meat product, lactic acid bacteria, natural antimicrobials, shelf life.

## Combinação de nisina e $\epsilon$ -poly-L-lysina como antimicrobianos naturais contra *Lactobacillus plantarum* associado à deterioração

**RESUMO:** Linguiças são altamente susceptíveis à deterioração microbiana. As bactérias ácido lácticas (BAL) são o principal grupo de bactérias deteriorantes em linguiças cozidas embaladas a vácuo. Para controlar o crescimento microbiano, antimicrobianos naturais têm sido utilizados como conservantes de alimentos. O objetivo deste estudo foi identificar cepas de bactérias ácido lácticas isoladas de linguiças Calabresa em deterioração e utilizá-las como modelo in vitro com os antimicrobianos naturais nisina (NI) e  $\epsilon$ -poly-L-lysina ( $\epsilon$ -PL). A identificação por espectrometria de massa das BAL isoladas das linguiças usando a técnica de MALDI-TOF revelou predominância de *L. plantarum*. A PCR-RAPD revelou quatro perfis genéticos diferentes. A concentração inibitória mínima (CIM) e a concentração bactericida mínima (CBM) de NI e  $\epsilon$ -PL, individualmente e em combinação, contra o pool de *L. plantarum* foram determinadas. O valor de CIM de NI e  $\epsilon$ -PL foi 0,468 mg/L e 75 mg/L, respectivamente, e de CBM de NI e  $\epsilon$ -PL foi 12,48 mg/L e 150 mg/L, respectivamente. O efeito da combinação de NI e  $\epsilon$ -PL foi determinada usando concentrações de 1/4 e 1/8 dos CIMs individuais. O efeito sinérgico foi confirmado em ambas concentrações mostrando um índice de concentração inibitório fracionado de 0,5 e 0,2, respectivamente. A combinação de NI e  $\epsilon$ -PL em baixa concentração, 0,05 mg/L e 9,375 mg/L, respectivamente, mostrou efeito inibitório contra *L. plantarum*. Os resultados demonstram o potencial do uso combinado de NI e  $\epsilon$ -PL para o controle de lactobacilos associados à deterioração em linguiça.

**Palavras-chave:** produto cárneo, bactérias ácido-lácticas, antimicrobianos naturais, vida útil.

## INTRODUCTION

Sausages represent an important segment in the processed meat market worldwide. Calabresa sausage is a cooked sausage widely incorporated in the Brazilian diet. It is one of the main ingredients of the Brazilian traditional dish *Feijoada* and the Calabresa pizza.

Despite undergoing a cooking process, which reduces the microbial load of cooked meat products, some groups of bacteria, mainly LAB, are able to survive this process, remaining viable in the final product. The microbial deterioration of this type of product is favored by its characteristics as high-water content, pH (6.6-6.8) and nutritional composition (FORSYTHE, 2013). In vacuum-packed meat

products, non-putrefactive LAB is the most important group associated with spoilage (HOLLEY & GILL, 2005; NYCHAS et al., 2008).

A common practice in many retailers of vacuum-packed cooked meat products in Brazil is the storage at room temperature, which leads to the reduction of the shelf life and the onset of signs of spoilage even before the expiration date.

Following the trend of using natural antimicrobials to control microbial growth in food, bacteriocins such as nisin have been applied for controlling a broad spectrum of Gram-positive bacteria, LAB and also sporogenic *Bacillus* and *Clostridium* (RILEY & WERTZ, 2002; CLEVELAND et al., 2001; LERAYER et al., 2015; COTTER et al., 2005). Its effect can be potentiated in combination with organic acids or other natural preservatives (ARAUZ et al., 2009).

$\epsilon$ -poly-L-lysine ( $\epsilon$ -PL) is a cationic peptide composed of 25–35 L-lysine residues, produced by *Streptomyces albulus lysinopolymerus* and used as a food preservative in Japan, Korea, the United States, and China (SHIH et al., 2006).  $\epsilon$ -PL can interact with cell membrane phospholipid heads, causing conformational changes in membrane and forming free vesicles or micelles, leaves a gap through which  $\epsilon$ -PL can access cytoplasm and cause even more damage to the cell (HYLDGAARD et al., 2014; NAJJAR et al., 2007).

The synergistic effect of NI associated with  $\epsilon$ -PL against some Gram-positive pathogens was reported (NAJJAR et al., 2007; LIU et al., 2015). However, the inhibitory effect of these antimicrobials towards spoilage-associated LAB has not been assessed.

The aim of this study was to identify LAB strains isolated from commercial *Calabresa* sausage, stored at room temperature and presenting signs of lactic spoilage, and, thereafter, evaluate the antimicrobial effect of NI and  $\epsilon$ -PL, individually and in combination, towards these LAB strains.

## MATERIALS AND METHODS

### Materials

Nisin Z (NI 2.5%; 1,045 IU/ mg) and  $\epsilon$ -poly-L-lysine (99.5%) were purchased from Handary Ltd (Brussels, Belgium).

### Isolation of LAB and phenotypic characterization

LAB strains were isolated from *Calabresa* sausages from nine different batches produced by a medium scale processing facility in Brazil, collected in three different retailers. Samples were packaged under vacuum and stored at retail at room temperature.

Samples were collected within 20 to 60 d of shelf life and presented visual signs of spoilage (slime, presence of exudate and loss of vacuum). Lactic spoilage was confirmed in laboratory by measuring samples pH (digital pHmeter Model HI 99163, Hanna, Portugal), and counting LAB population on MRS agar (ACUMEDIA, Lansing, USA). The pH of samples ranged from 5.23 to 6.0, (below the normal pH 6.6 for cooked sausage), whereas LAB count varied from 5.86 to 8.30 log CFU/g.

To isolate the dominant microbiota of LAB from sausages, samples (25 g) were aseptically transferred to sterile plastic bags containing 225 mL of 0.1% peptone water (HIMEDIA, Mumbai, India) and homogenized in a stomacher (IUL Instruments, Barcelona, Spain). From the first dilution (1:10), subsequent dilutions were performed and plated in MRS agar (ACUMEDIA, Lansing, USA) plates incubated at 37 °C for 48 h under restricted oxygen conditions (Anaerocult, MERCK, Germany). Colonies were counted and 10% of the colonies were picked up from the highest dilution of agar medium and subjected to cell morphology, Gram staining and catalase activity. One presumptive LAB strain (Gram-positive, catalase-negative, non-motile rods) from each sausage sample was selected for spectrometric identification (MALDI-TOF) and molecular analysis (RAPD-PCR).

### Identification of LAB using MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry

Identification by MALDI-TOF was performed in duplicate by using a VITEK® MS Spectrometer (*bioMérieux*, Marcy l'Etoile, France). Strains of LAB were grown on trypticase soy agar with 5 % sheep blood (*bioMérieux*) at 37 °C for 48 hours. A thin layer of colony growth was applied directly to the target slide using a 1  $\mu$ L loop. Two spots on the target plate were inoculated for each isolate tested with VITEK® MS. The spots were overlaid with 1  $\mu$ L matrix solution ( $\alpha$ -cyano-4-hydroxycinnamic acid; *bioMérieux*) and allowed to dry before mass spectrometry analysis. The double spotting for the VITEK® MS was required for the algorithm used by the Durham research laboratory to interpret the output of the SARAMIS database (version 3.62). The result was considered acceptable when the confidence score was greater than 90% for the first spot and the second spot result agreed with the first. *Escherichia coli* ATCC®8739™ was used for every acquisition group on the target slide to calibrate the mass spectrometer. Matrix solution without micro-organisms was used as the negative control.

#### DNA extraction of LAB

For the determination of the genetic profile of the *Lactobacillus* strains, previously identified using MALDI-TOF, bacterial DNA was extracted according to NEUMANN & POSPIECH (1995). 2 mL of MRS broth containing each strain grown overnight at 37 °C were centrifuged for 10 min at 3,000 g. Cell pellets were resuspended in 500  $\mu$ L of extraction buffer (SET) (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5) and lysozyme (1 mg/mL) and incubated at 37 °C for 1h. Then 75  $\mu$ L of 10% SDS and 0.5 mg/mL proteinase K were added and incubated at 55 °C for 2h. 285  $\mu$ L of NaCl (5M) and 1 mL of chloroform were added and incubated at room temperature for 30 min with frequent inversion. Solution was then centrifuged at 4,500 g for 15 min at room temperature, the pellet was discarded and the aqueous phase was transferred to a new tube. An equal volume of ice-cold isopropanol was added to aqueous phase and stored at -20 °C overnight. After centrifugation at 4,500 g for 5 min, pellets were dried at room temperature and resuspended in 100  $\mu$ L of Tris-EDTA buffer + RNase and stored at -20 °C until use. DNA was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, MA, USA).

#### RAPD-PCR

Primer OPA-3 (5'-AGTCAGCCAC-3') was used for RAPD-PCR of *L. plantarum* strains (QUERE et al., 1997). RAPD was performed in a volume of 25  $\mu$ L containing 3 mM MgCl<sub>2</sub>, 1X Buffer, 0.25 mM Dntp (Invitrogen), 1U Taq (Invitrogen), 3  $\mu$ M primer and 2  $\mu$ L of resuspended bacterial DNA. RAPD-PCR amplification was performed in a DNA Engine Systems (PTC 200, Bio Rad, Foster City, USA) with initial denaturation at 95 °C for 5 min, followed by 45 cycles at 94 °C of 1 min, 36 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 7 min. Amplification products were separated on a 1.5% agarose gel by 70V electrophoresis and stained with ethidium bromide solution. A strain of *E. coli* ATCC®25922™ was used in each reaction as a positive control.

RAPD banding patterns were analyzed using PyElph 1.4 software (Bio-Rad Laboratories Inc., Hercules, CA, USA). The Dice correlation coefficient was used to calculate similarities in RAPD patterns; dendrograms were obtained by the not weighted pair group method with arithmetic averages (UPGMA). To avoid over representation of clones in the preparation of the inoculum, four strains of *Lactobacillus plantarum* were selected among those showing different genetic profiles.

#### Determination of Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

A pool of four *L. plantarum* strains, with different genetic profiles, was prepared by inoculating 100  $\mu$ L of each strain at 6 log CFU/mL, grown in MRS broth for 24 h at 37 °C, into 7.5 mL of MRS broth.

The MIC of NI and  $\epsilon$ -PL was determined using the tube dilution technique (CLSI, 2018). Treatments were performed in screw-capped tubes containing MRS broth, 80  $\mu$ L of the pool of *Lactobacillus spp.* and 13 different concentrations of NI, from 0.156 mg/L (163.02 IU/ L= 0.16302 IU/ mL) to 24.96 mg/L (26,083 IU/ L= 26.083 IU/ mL) and  $\epsilon$ -PL, from 9.3 mg/L to 150 mg/L. Tubes were incubated at 37 °C for 24 h. Positive control contained 7.2 mL MRS broth and 80  $\mu$ L bacterial inoculum.  $\epsilon$ -PL negative control contained 6.8 mL MRS broth and 1.2 mL  $\epsilon$ -PL stock solution (150 mg/L) and NI negative control contained 7.2 mL MRS broth and 800  $\mu$ L NI stock solution (26.083 IU/ mL). MIC was defined as the lowest antimicrobial concentration where no increase in turbidity (visible growth) and optical density (600 nm) were observed (CLSI, 2018).

Aliquots (1.0 mL) from the tubes with no visible growth were pour plated on MRS agar and incubated at 37 °C for 48 h in anaerobiosis. MBC was considered as the lowest antimicrobial concentration where no colony growth was observed after subculture on MRS agar.

#### Determination of Fractional Inhibitory Concentration Index (FICI) of NI and $\epsilon$ -PL

MIC of NI and  $\epsilon$ -PL were reduced by 1/2, 1/4 and 1/8 and tested in combination, following the same procedure as previously described. MIC of combination was defined as the lowest antimicrobial concentration where no increase in turbidity (visible growth) and optical density (600 nm) were observed. The type of interaction between NI and  $\epsilon$ -PL was evaluated by calculating the Fractional Inhibitory Concentration Index (FICI) (GUTIERREZ et al., 2008).  $FICI = FIC_{NI} + FIC_{\epsilon-PL}$ , where  $FIC_{NI} = MIC_{NI} \text{ in combination} / MIC_{NI}$  and  $FIC_{\epsilon-PL} = MIC_{\epsilon-PL} \text{ in combination} / MIC_{\epsilon-PL}$ .

The interactions between antimicrobials were interpreted as follows: synergistic effect:  $FICI < 0.5$ ; additive effect:  $0.5 \leq FICI \leq 1$ ; no interactive effect:  $1 < FICI \leq 4$ ; antagonistic effect:  $FICI > 4$ .

#### Statistical Analysis

Antimicrobial activity assay was conducted in triplicate and results were analyzed by one-way ANOVA with means compared by Tukey test



( $P < 0.05$ ) using Statgraphics Centurion XVI v. 16.1.11 (Statpoint Technologies, Warrenton, VA, USA).

## RESULTS AND DISCUSSION

### Identification of LAB isolates

Identification of LAB strains using MALDI-TOF revealed eight strains of *Lactobacillus plantarum* and one strain of *L. curvatus*. RAPD-PCR of these *Lactobacillus* spp. strains revealed five different patterns corresponding to different strains (Figure 1). *Lactobacillus plantarum* strains showed four different genetic profiles (clusters I to IV).

*Lactobacillus plantarum* is a highly heterogeneous species of versatile lactic acid bacteria (LAB), which are reported in many different ecological niches such as vegetables, meat, fish, and dairy products (SIEZEN et al, 2010). Among *Lactobacillus* spp., *L. plantarum* usually predominates causing fermentation and spoilage in many types of food, specially at temperatures above 10 °C (CHOI & PARK, 2000). High proliferation of LAB results in a large number of metabolic products present in samples such as lactic acid, which lowers pH of the product and in some species, carbon dioxide, which causes vacuum lost in package. *L. plantarum* is the species associated with deterioration of cooked meat products packaged under vacuum and kept at room temperature (HOLLEY & GILL, 2005).

### MIC and MBC of NI and $\epsilon$ -PL

MIC of NI and  $\epsilon$ -PL were 0.468 mg/L (0.48906 IU/mL) and 75 mg/L (75  $\mu$ g/mL), respectively. MBC of NI and  $\epsilon$ -PL were 12.48 mg/L (13.04 IU/mL) and 150 mg/L (150  $\mu$ g/mL), respectively (Table 1).

### FICI of NI and $\epsilon$ -PL

Synergistic inhibitory effect of NI and  $\epsilon$ -PL against *L. plantarum* was observed using combinations of 1/4 and 1/8 of MIC of both compounds (Table 2), showing FIC indices of 0.5 and 0.2, respectively. FICI is a widely used method for analysis of multiple compound interactions (NAJJAR et al., 2007).

NAJJAR et al. (2007) studied the combination of NI (nisin A) and  $\epsilon$ -PL against Gram-positive food-borne pathogens *Bacillus cereus* and *Listeria monocytogenes*. They reported MIC of NI and  $\epsilon$ -PL of 300 IU/mL and 15  $\mu$ g/mL, respectively, for *L. monocytogenes* and a FICI of 0.83, indicating an additive effect. For *Bacillus cereus*, they found MIC of 100 IU/mL NI and 5  $\mu$ g/mL  $\epsilon$ -PL; respectively, and a FICI of 0.56, indicating a synergistic activity. In the present study, the MIC of NI for *L. plantarum* strains was 0.489 IU/mL (0.468 mg/L), showing quite lower values than those reported by the authors. Conversely, the MIC of  $\epsilon$ -PL was higher (75 mg/L) than that reported by the authors.

Another Gram-positive species such as *Bacillus subtilis* (ATCC 10242) and *Staphylococcus*

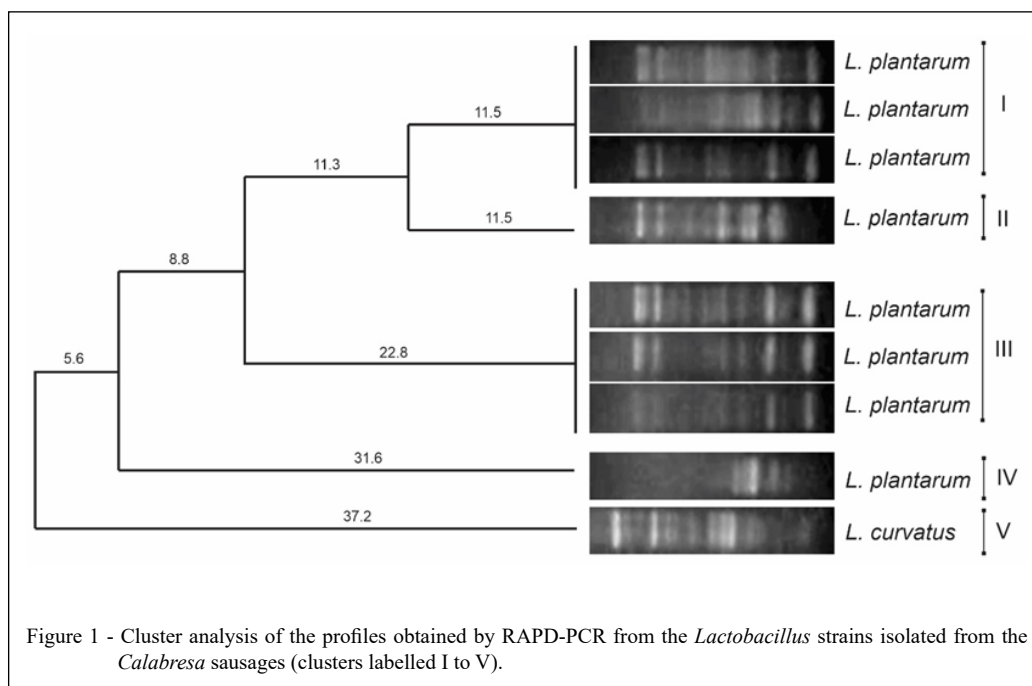


Figure 1 - Cluster analysis of the profiles obtained by RAPD-PCR from the *Lactobacillus* strains isolated from the Calabresa sausages (clusters labelled I to V).

Table 1 - Results of different concentrations of NI and  $\epsilon$ -PL against the pool of *Lactobacillus plantarum*.

-----Nisin Z-----				----- $\epsilon$ -poly-L-lysine-----			
Treatment (mg/L)	Visible growth	Colony Growth	DO <sub>600nm</sub> (n=3)	Treatment (mg/L)	Visible growth	Colony Growth	DO <sub>600nm</sub> (n=3)
C+	Yes	ND	1.46 ± 0.00 <sup>a</sup>	C+	Yes	ND	1.32 ± 0.01 <sup>a</sup>
C-	No	ND	0.18 ± 0.03 <sup>c</sup>	C-	No	ND	0.07 ± 0.00 <sup>c</sup>
0.156	Yes	ND	ND	9.3	Yes	Yes	ND
0.234	Yes	ND	ND	12.5	Yes	Yes	ND
0.312	Yes	ND	0.68 ± 0.02 <sup>b</sup>	15	Yes	Yes	ND
0.468	No	Yes	0.25 ± 0.00 <sup>cd</sup>	20	Yes	Yes	ND
0.624	No	Yes	0.24 ± 0.01 <sup>cd</sup>	25	Yes	Yes	ND
0.780	No	Yes	0.32 ± 0.03 <sup>c</sup>	35	Yes	Yes	ND
0.936	No	Yes	0.32 ± 0.03 <sup>c</sup>	50	Yes	Yes	1.34 ± 0.00 <sup>a</sup>
1.56	No	Yes	ND	65	Yes	Yes	1.06 ± 0.01 <sup>b</sup>
2.34	No	Yes	ND	75	No	Yes	0.07 ± 0.00 <sup>c</sup>
3.12	No	Yes	ND	85	No	Yes	0.08 ± 0.00 <sup>c</sup>
6.24	No	Yes	ND	100	No	Yes	0.09 ± 0.00 <sup>c</sup>
12.48	No	No	ND	125	No	Yes	0.09 ± 0.00 <sup>c</sup>
24.96	No	No	ND	150	No	No	0.07 ± 0.00 <sup>c</sup>

DO<sub>600nm</sub> mean ± standard error. C+ = positive control. C- = negative control. <sup>a, b, c</sup> Means with different letters in the same column indicate significant differences between treatments (P < 0.05). ND = not determined.

*aureus* (ATCC 10141) and a Gram-negative *E. coli* (ATCC®25923™) were challenged with NI and  $\epsilon$ -PL, individually and in combination. MIC of NI ranged from none to 250 mg/L and MIC of  $\epsilon$ -PL varied from 15.63 mg/L to 62.5 mg/L. FICI ranged from 0.26 to 0.50 (LIU et al., 2015), which was similar to that reported in the present study.

The inhibitory effect of NI towards lactobacilli was reported in strains isolated from kimchi (traditional Korean fermented vegetables) (CHOI & PARK, 2000). Authors found most of *L. plantarum* strains sensitive to 100 IU/mL, which is the recommended concentration for use in food in many countries.

In *Lactobacillus* species associated with dental caries (TONG et al., 2010), MIC of NI ranged from <1,250 IU/mL to >625 IU/mL for *L. acidophilus* ATCC®4356™, <625 IU/mL to >312.5 IU/mL for *L. casei* ATCC®393™, and <78.125 IU/mL and >39.0625 IU/mL for *L. fermenti* ATCC®9338™. MBC ranges against those strains were <2,500 IU/mL and >1,250 IU/mL for *L. acidophilus*; <1,250 IU/mL and >625 IU/mL for *L. casei* and <156.25 IU/mL and >78.125 IU/mL for *L. fermenti*.

To our knowledge, the inhibitory effect of  $\epsilon$ -PL individually or in combination with NI towards lactobacilli has not been reported until this present study.

Although, most bacteria tested are Gram-positive (*Listeria* sp., *Bacillus* sp., *Staphylococcus* sp., *Lactobacillus* sp.), the resistance to antimicrobials vary among different genera and different species in the same genus. Nisin is a cationic peptide which acts in the cell membrane of bacteria via pores formation to interfere with cell wall biosynthesis (LIU et al., 2015). It recognizes and binds anionic lipids in the target membrane, inducing temporary membrane perturbations and assembling into a pore. Nisin can also interact with high-affinity pyrophosphate binding sites on the membrane bound cell wall precursor lipid II (TONG et al., 2010). The pore formation will result in an efflux of small cytoplasmic compounds or the collapse of vital ion gradients, leading to cell death. The affinity of nisin for lipid II is much higher than the affinity of nisin for membranes containing anionic lipids (BIERBAUM & SAHL, 2009). The different affinities for cell membranes may explain the different concentrations of NI needed to inhibit different bacteria in the studies reported.

The concentration of antimicrobials to inhibit the target bacteria is also affected by the form of application and the matrix tested because food compounds may interact with antimicrobials and affect their action. Concentration of natural antimicrobials needed to achieve an inhibitory effect

Table 2 - Effect of the combination of different concentrations of NI and  $\epsilon$ -PL against *Lactobacillus plantarum*.

Treatments	-----Concentration (mg/L)-----		Visible growth	FICI	Effect
	NI	$\epsilon$ -PL			
½ MIC NI + ½ MIC $\epsilon$ -PL	0.234	37.5	No	1	Additive
¼ MIC NI + ¼ MIC $\epsilon$ -PL	0.117	18.75	No	0.5	Synergistic
⅛ MIC NI + ⅛ MIC $\epsilon$ -PL	0.050	9.375	No	0.2	Synergistic

Tests were incubated at 37 °C for 24h. FICI < 0.5 indicates a synergic effect; 0.5 ≤ FICI ≤ 1 represents an additive effect; 1 < FICI ≤ 4 has no interactive effect; and FICI > 4 indicates an antagonistic effect.

*in vitro* is usually lower than that in food matrices (HYLDGAARD et al, 2015). Using a solution sprayed on the surface of ready-to-eat seafood (minced tuna and salmon roe) containing NI and  $\epsilon$ -PL, the concentrations to achieve a synergistic effect against *L. monocytogenes* was 12.5 ppm NI (500 pm NI compound containing 2.5% NI) and 1,000 ppm  $\epsilon$ -PL (2,000 pm  $\epsilon$ -PL compound containing 50%  $\epsilon$ -PL), as reported by Takahashi et al. (2012). Although, the authors have reported the effect of NI +  $\epsilon$ -PL in a food matrix, the inhibitory concentration was high. If the *in vitro* synergistic concentration of NI +  $\epsilon$ -PL (IFIC) reported in the present study is increased 100-fold for future use in a meat product to inhibit lactic spoilage, the concentration of antimicrobials would be 5 ppm (mg/ kg) of NI and 937.50 ppm (mg/ kg) of  $\epsilon$ -PL.

The combination of NI and  $\epsilon$ -PL synergistically enhance the permeability of the bacterial cell membrane, likely reflecting the inhibition of both Na<sup>+</sup>K<sup>+</sup> and Ca<sup>++</sup>Mg<sup>++</sup> ATPase activities through these compounds. Recent findings indicate that  $\epsilon$ -PL can interact to DNA through insertion into the double helix structure, thereby hindering DNA replication. After nisin has damaged the cell structure,  $\epsilon$ -PL can easily enter the cells, interacts with DNA, and eventually hinders DNA replication (LIU et al, 2015; LIU et al, 2015).

## CONCLUSION

This study showed the synergistic effect of NI and  $\epsilon$ -PL to control spoilage *L. plantarum*, at low concentrations (0.05 mg/L NI and 9.375 mg/L  $\epsilon$ -PL). Therefore, the combination of these natural antimicrobials shows potential to be further used in meat matrices to control lactic acid spoilage bacteria, increasing the shelf life of the product and helping the food industry to offer safer and more sustainable foods to the consumer.

## ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasil - Finance code 001.

## DECLARATION OF CONFLICT OF INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## AUTHORS' CONTRIBUTIONS

FCKB and REFM conceived and designed experiments. FCKB, MHRF, NCKJ and GMD performed the experiments and carried out the lab analyses. FCKB and REFM performed statistical analyses of experimental data. FCKB and REFM prepared the draft of the manuscript. All authors critically revised the manuscript and approved the final version.

## REFERENCES

- ARAUZ, L. J., et al. Nisin biotechnological production and application: a review. **Trends in Food Science and Technology**, v.120, p.146-154, 2009. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0924224409000594>>. Accessed Feb. 22, 2019. doi: 10.1016/j.tifs.2009.01.056.
- BIERBAUM, G.; SAHL, H. G. Lantibiotics: mode of action, biosynthesis and bioengineering. **Current Pharmaceutical Biotechnology**, v.10, p.2-18, 2009. Available from: <<https://www.ingentaconnect.com/content/ben/cpb/2009/0000010/0000001/art00002>>. Accessed: Dec. 15, 2019. doi: 10.2174/138920109787048616.
- CHOI, M. H.; PARK, Y. H. Selective control of lactobacilli in kimchi with nisin. **Letters in Applied Microbiology**, v.30, p.173-177, 2000. Available from: <<https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1046/j.1472-765x.2000.00719.x>>. Accessed: Jun. 14, 2019. doi: 10.1046/j.1472-765x.2000.00719.x.
- CLSI, Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 11th. Ed. CLSI standard M07. Wayne, PA (2018).

Available from: <<https://www.researchgate.net/file.PostFileLoader.html?id=>>. Accessed: Mar. 30, 2018. doi: 610.688.0700www.clsi.org.

CLEVELAND J., et al. Bacteriocins: safe natural antimicrobials for food preservation. **International Journal of Food Microbiology**, v.71, p.1-20, 2001. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0168160501005608>>. Accessed: Apr. 29, 2019. doi: 10.1016/S0168-1605(01)00560-8.

COTTER P. D., et al. Bacteriocins: developing innate immunity for food. **Nature Reviews/ Microbiology**, v.3, p.773-788, 2005. Available from: <<https://www.nature.com/articles/nrmicro1273>>. Accessed: Jun. 06, 2018.

FORSYTHE, Stephen, J. **Microbiologia da Segurança dos Alimentos**. 2 ed. Porto Alegre: Artmed, 2013. p.146-150.

GUTIERREZ J.; et al. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. **International Journal of Food Microbiology**, v.124, p.91-97, 2008. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0168160508001177>>. Accessed: Sep. 20, 2018. doi: 10.1016/j.ijfoodmicro.2008.02.028.

HOLLEY R.; GILL C. O. Use of modified atmosphere to meat and meat products. Speech. **III Congresso Brasileiro de Ciência e Tecnologia de Carnes**. 27 – 29, September, 2005.

HYLDGAARD M., et al. The antimicrobial mechanism of action of epsilon-poly-L-lysine. **Applied and Environmental Microbiology**, v.80 (24), p.7758-7770, 2014. Available from: <<https://aem.asm.org/content/80/24/7758.short>>. Accessed: May, 20, 2018. doi: 10.1128/AEM.02204-14.

HYLDGAARD, M. et al. Binary combination of epsilon-poly-L-lysine and iso Eugenol affect progression of spoilage microbiota in fresh turkey meat, and delay onset of spoilage in *Pseudomonas putida* challenged meat. **Int J Food Microbiol**, v.215, p.131-142, 2015. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0168160515301276>>. Accessed: Dec. 10, 2019. doi: 10.1016/j.ijfoodmicro.2015.09.014.

LERAYER, A. L. S., et al. Action of nisin on sporogenic bacteria of *Bacillus* gender. **Tecnolat/ ITAL. ITAL Artigos Técnicos**. 2015.

LIU, H., et al. The antimicrobial effect and synergistic antibacterial mechanism of the combination of  $\epsilon$ -Polylysine and Nisin against *Bacillus subtilis*. **Food Control**, v.47, p.444-450, 2015. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0956713514004356>>. Accessed: Jun. 14, 2019. doi: 10.1016/j.foodcont.2014.07.050.

LIU, F. et al. Synergistic antibacterial effect of the combination of c-Polylysine and nisin against *Enterococcus faecalis*. **Journal of Food Protection**, v.78, p.2200-2206, 2015. Available from: <<https://meridian.allenpress.com/jfp/article-abstract/78/12/2200/174528/Synergistic-Antibacterial-Effect-of-the?redirectedFrom=fulltext>> Accessed: nov. 15, 2019. <https://doi.org/10.4315/0362-028X.JFP-15-220>.

NAJJAR. M. B., et al.  $\epsilon$ -Poly-L-lysine and nisin A act synergistically against Gram-positive food borne pathogens *Bacillus cereus* and *Listeria monocytogenes*. **Letters Applied in Microbiology**, v.45, p.8-13, 2007. Available from: <<https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/j.1472-765X.2007.02157.x>> Accessed: Apr. 29, 2019. doi: <https://doi.org/10.1111/j.1472-765X.2007.02157.x>.

NEUMANN B.; POSPIECH A. A versatile quick-prep of genomic DNA from Gram-positive bacteria. **Trends in Genetics**, v.11, p.217-218, 1995. Available from: <<http://www.sciencedirect.com/science/article/pii/S0168952500890526>>. Accessed: Feb. 22, 2017. doi: 10.1016/S0168-9525(00)89052-6.

NYCHAS, G. J. E., et al. Meat spoilage during distribution. **Meat Science**, v.78, p.77-89, 2008. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0309174007002185>>. Accessed: Apr. 29, 2019. doi: 10.1016/j.meatsci.2007.06.020.

QUERE, F, et al. DNA probe and PCR-specific reaction for *Lactobacillus plantarum*. **Journal of Applied Microbiology**, v.82, p.783-790, 1997. Available from: <<https://sfamjournals.onlinelibrary.wiley.com/doi/abs/10.1046/j.1365-2672.1997.00157.x>>. Accessed: May, 04, 2018. doi: 10.1046/j.1365-2672.1997.00157.x.

RILEY, M. A.; WERTZ, J. E. Bacteriocin diversity ecological and evolutionary perspectives. **Biochimie**, v.84, p.57-364, 2002. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0300908402014219>>. Accessed: May, 20, 2019. doi: 10.1016/S0300-9084(02)01421-9.

SHIH I. L., et al. Microbial synthesis of poly (epsilon-lysine) and its various applications. **Bioresource Technology**, v.97(9), p.1148-1159, 2006. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0960852404003049>>. Accessed: Apr. 21, 2019. doi: 10.1016/j.biortech.2004.08.012.

SIEZEN, R. J., et al. Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from various environmental niches. **Environmental Microbiology**, v.12 (3), p.758-773, 2010. Available from: <[https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/j.1462-2920.2009.02119.x?casa\\_token=RsmCAAAAnTOgAAAAA%3AppwaQ5SI90\\_0vmoLsp56UD2qHbmqZLR4hn bH7QiORNoPJMwJuWP1vbzP-WYaju7au7nyg9ej5XcXmokX](https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/j.1462-2920.2009.02119.x?casa_token=RsmCAAAAnTOgAAAAA%3AppwaQ5SI90_0vmoLsp56UD2qHbmqZLR4hn bH7QiORNoPJMwJuWP1vbzP-WYaju7au7nyg9ej5XcXmokX)>. Accessed: May, 04, 2018. doi: 10.1111/j.1462-2920.2009.02119.x.

TAKAHASHI, H., et al. Effect of paired antimicrobial combinations on *L. monocytogenes* growth inhibition in ready-to-eat seafood products. **Food Control**, v.26, p.397-400, 2012. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0956713512000795>>. Accessed: Jun. 14, 2019. doi: 10.1016/j.foodcont.2012.02.005.

TONG, Z., et al. Nisin inhibits dental caries-associated microorganism in vitro. **Peptides**, v.31, p.2003-2008, 2010. Available from: <<https://www.sciencedirect.com/science/article/abs/pii/S0196978110003281>>. Accessed: Jul. 08, 2019. doi: 10.1016/j.peptides.2010.07.016.