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Lactobacillus sakei 2a and its Concentrated Acid Extract on Inhibition of Food-Borne *Salmonella* strains

Jane Mary Lafayette Neves Gelinski[1]*, César Milton Baratto[2], Dirceu Sacaratti[3] and Bernadette Dora Gombossy de Melo Franco[4]

[1,2,3]Universidade do Oeste de Santa Catarina - Unoesc, Nucleus of Biotechnology, Paese, 198 Bairro Universitário -89.560.000 - Videira-SC, Brazil.
[4]Universidade de São Paulo - USP, FacultyofPharmaceuticalSciences, Department of Food and Experimental Nutrition - São Paulo-SP, Brazil.

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Lactic acid bacteria are used in food production to provide desirable organoleptic characteristics, and can also act as biopreservatives, controlling the growth of undesirable microorganisms. In this study, we examined the antimicrobial action of *Lactobacillus sakei* 2a and its concentrated acid extract against food-borne *Salmonella* spp. The extract was obtained by acid extraction from culture broth of *L. sakei* 2a and was designated extract 2a. We determined that extract 2a had significant activity (approximately 500 AU ml^{-1}). We used different antimicrobial substances alone or in combination with extract 2a to evaluate the inhibitory activity of the various treatments on a pool of five *Salmonella* strains. The pathogen *Listeria monocytogenes* Scott A Cm^r Em^r was used as an indicator strain of inhibitory activity. In summary, all antimicrobials substances that were tested showed an inhibitory effect against the growth of *Salmonella*, andthis action was enhanced in the presence of extract 2a. Moreover, among the treatments applied, the combination of extract 2a and 0.1% lactic acid exhibited the most potent inhibitory effect towards the pool of *Salmonella* strains. Our findings indicate that *L. sakei* 2a and extract 2a, especially in combination with other antimicrobials, present potential technological application in the control of salmonellae in foods.

Key words: Bacteriocin. *Lactobacillus,* Antimicrobials, *Listeria monocytogenes*, food-borne *Salmonella*.

The use of lactic acid bacteria (LAB) in preparation of foodstuffs can provide desirable organoleptic characteristics, including enhanced taste, smell, and overall appearance. In addition, some LAB inhibit pathogenic or spoilage microorganisms through their metabolic products, such as lactic acid, hydrogen peroxide, and bacteriocins[1,2]. The main focus of research involving LAB and bacteriocin in meat products is the inhibition of pathogens, such as *Listeria monocytogenes*, and the germination of *Clostridium botulinum* spores[3-7]. However, it has also been established that the use of a bacteriocin alone is insufficient to guarantee the safety of a food product. Thus, various technologies for inhibition of undesirable microorganisms, which were applied individually in the past, have been

* To whom all correspondence should be addressed.
E-mail: jgelinski@yahoo.com.br
jane.gelinski@unoesc.edu.br

increasingly used in combination with other processes of preservation, such as decreasing the pH of a final food product followed by storing the product in packages with modified atmosphere during refrigeration[8-9].

In the food industry, the gram negative *Salmonella* is one of the most important pathogens which remains one of the leading causes of human foodborne. It has previously been shown that bacteriocins of lactic acid bacteria are inactive against gram-negative bacteria, unless other agents that act on the outer membrane of cells are included in a combination treatment[10-11],such as ion sequestering agents[12-18]. For example, EDTA-Ethylenediamine tetraacetic acid a divalent cation chelator in the lipopolysaccharide present on cells[16,17], rendering the cells sensitive to substances such as bacteriocins that do not normally have an effect on gram-negative bacteria.

Relevant to this study, *Lactobacillus sakei* 2a is a strain producer of the antilisterial bacteriocin 2a[19], and previous studies have demonstrated that bacteriocin 2a acts by forming pores in the membrane of target cells[6]. In the present study, as a preliminary evaluation, we examined the antimicrobial action of a crude extract obtained by acid extraction from *L. sakei* 2a when it is combined with different antimicrobial substances, as well direct action of *L. sakei* 2a in experiments of co-cultive of *Salmonella* strains in order to verify their potential for inhibiting food-borne *Salmonella* strains.

MATERIALS AND METHODS

Strains and cultivation conditions

Salmonella strains

In a previous study[20]five strains of *Salmonella* were isolated from Brazilian sausages:*Salmonella* serotype Derby SD2, *S.*serotype Enteritidis SE3, *S.*serotype Hadar SH4, *S.*serotype Panama SP5, and *S.* serotype Typhimurium ST6. These cultures were maintained in Brain-Heart Infusion (BHI; Oxoid, UK) agar culture medium at 4°C. For use in experiments, the cultures were grown in BHI broth (Oxoid, UK) on a shaker set to 140 rpm at 37°C for 18-24 h. The five strains were grouped in a pool (*Salmonella*-pool) for use in experiments after being grown separately in BHI broth at 37°C for 18 h, or until they reached an optical density of 0.1 at 600 nm. The number of CFUs in this culture was confirmed through plate counting in BHI agar after it was incubated at 37°C for 48 h (10^4 CFU ml^{-1}/culture). Each isolated culture was harvested (10,000x*g*,10 min) and suspended in 0.05 M Tris-HCl buffer (pH 7.2), and all cultures were subsequently mixed to form the *Salmonella*-pool consisting of 5 x 10^4 CFU ml^{-1}.

L. monocytogenes Scott A Cm^r Em^r[21]. A culture of *L. monocytogenes* Scott A that is resistant to the antibiotics erythromycin and chloramphenicol was used in this study to prove that extract 2a could be more inhibiting than a potent antibiotic. The indicator culture was tested for bacteriocin activity in plate diffusion tests. Prior to its inclusion in experiments, the culture was grown for 24 h at 37°C in Tryptic Soy Broth with a 0.6% yeast extract supplement (Oxoid), containing 5 µg ml^{-1} chloramphenicol, and 0.5 µg ml^{-1} erythromycin (both reagents from Sigma-Aldrich Chemical Co., Germany). The culture was then spread on Tryptic Soy Agar (Difco Laboratories, USA) containing the appropriate antibiotics for maintenance of the culture in refrigerated conditions (4°C) or for immediate use (37°C).

L. sakei 2a

The *L. sakei* 2a culture originally isolated from Brazilian sausage[19] was cultivated in De Man, Rogosa, and Sharpe (MRS; Oxoid) broth and was incubated anaerobically at 30°C (Anaerogen system, Oxoid). Prior to their use in experiments, the cultures were thawed and grown in a similar composition of MRS broth supplemented with 0.5% of glucose (to avoid excess lactic acid production). Aliquots of each culture were cultivated in their appropriate culture media and were stored at -20°C after addition of glycerol 20% (v/v) to each aliquot.

Concentrated acid extract of a culture of *L. sakei* 2a

The 2a extract was obtained by a previously described method of acid extraction[22] with the following modifications:3.5 L of MRS broth were inoculated with 1% (v/v) of an 18-h culture of *L. sakei* 2a grown in MRS broth. After 36 h of cultivation at 30°C in a fermenter (New Brunswick Scientific, USA) in aerobic conditions (this condition was used only for extract

production) on a shaker set to 140 rpm, the culture was heated to 70°C in 30 min to inactivate cells and proteases. An 1.0-ml aliquot was inoculated in 15 ml of MRS broth and was incubated at 30°C for 24 h under anaerobic conditions to confirm that all cells were inactivated (i.e., no multiplication was observed). At the same time, the initial pH of the inactivated culture was adjusted to 5.5 with a 2 M NaOH solution (Synth, Brazil). The culture was subsequently centrifuged at 15,000x *g* under refrigeration for 40 min, and a 1-ml aliquot of the supernatant (cell-free culture medium) was collected to check the antimicrobial activity of the component(s) that were not adsorbed to the cells. The pellet (i.e., containing the inactivated cells) was washed twice with 5 mM 2-(N-morpholino) ethanesulfonic acid buffer (Synth) with a pH of 6.5, and resuspended in 300 ml of 100 mM NaCl (Synth), and the pH of the solution was further adjusted to 2.0 with a 5.0% phosphoric acid solution. After being left at 4°C with shaking for 24 h, the suspension was again centrifuged (same parameters), and the supernatant was dialyzed against 10 mM phosphate buffer (Synth) with a pH of 7.0 for 24 h, using dialysis membranes for molecules above 2 kDa. The pH of this solution was adjusted to 5.5 and a 1-ml aliquot was subsequently collected for quantification of this crude extract.

The sample of the acid extract of a culture of *L. sakei* 2a was partially concentrated in a LSL Secfroid lyophilizer (Lyolab BII, Switzerland) under a vacuum for 48 h with a condensation temperature of -80°C. The sample was subdivided into 1.0-ml aliquots (no solvent was necessary) and stored at -70°C until use. This concentrated acid extract of a culture of *L. sakei* 2a was designated as "extract 2a."

Antimicrobial agents

EDTA-Ethylenediamine tetraacetic acid (Sigma-Aldrich) was used in the co-cultivation experiments involving pools of *Salmonella* strains with *L. sakei* 2a in BHI medium in aerobiosis at 37°C at the final concentrations of 10 or 20 mM. When extract 2a was used in combination with EDTA, the concentration of the latter was 1.0 mM only.

Lactic acid (sodium lactate) - lactic acid was used at 0.1%; Chicken egg white lysozyme (EC 3.2.1.17) - lysozyme was used at 10 µg ml^{-1} (both reagents were from Sigma-Aldrich);

Citric acid (sodium citrate, Sigma-Aldrich) - citric acid was used at concentrations of 10 or 20 mM was utilized in the experiments of co-cultivation of *Salmonella* strains with *L. sakei* 2a.

Nisin (Nisaplin™; Danisco, England) – nisin was used as the bacteriocin standard at a concentration of 100 IU ml^{-1}; this inhibitor of *L. monocytogenes*, which was a good indicator strain for bacteriocin 2a activity, was used herein according to the protocol described by Rosa *et al.*,[6].

The effect of the action of each antimicrobial with extract 2a on *Salmonella* strains was measured after 30 min of exposure of the *Salmonella*-pool (with $5x10^7$ $CFUml^{-1}$). Agar plates containing either XLD were prepared as mentioned above. MRS agar was also prepared when in co-cultivation experiments with *L. sakei* 2a.

All experiments were performed in triplicate and the results were expressed as $CFUml^{-1}$ and presented as the median of two independent measures.

Antimicrobial activity of *L. sakei* and extract 2a by well diffusion method

Cell-free supernatants of *L. sakei* 2a, which were previously cultivated in MRS medium, were collected by centrifugation. The supernatants were neutralized to a pH of 6.0 with 2.0 N NaOH, sterilized by filtration through a 0.45-µm filter (Millipore), and tested by the well-diffusion method[1,23] to screen for their antimicrobial activity, using the *L. monocytogenes* Scott A Cm^rEm^r strain as the indicator organism. The reciprocal of the greatest inhibitory dilution yielding an inhibition zone of at least 2.0 mm (excluding the diameter of the well) was used to calculate arbitrary units (AU) per milliliter of bacteriocin activity. The same procedure was carried out to measure antimicrobial activity during the process of acid extraction of the supernatants used to produce extract 2a. A solution of nisin, at a concentration of 100 IU (international unit) per milliliter diluted in 0.02 N HCl, pH 5.0, was used as a positive control, whereas sterile culture medium or solution of 0.02 N HCl, pH 5.0 were used as a negative control when tested supernatants of *L. sakei* 2a or extract 2a, respectively.

Co-cultivation experiments

Experiments of co-cultivation were performed using pools of *Salmonella* strains with *L. sakei* 2a (10^4 CFU ml^{-1}/culture/microorganism) only and/or in the presence of EDTA or citric acid. These substances are normally used in food preservation. The experiments were performed in BHI medium at 37°C in aerobiosis/24 hours. Agar plates containing either XLD (Xylose Lysin Desoxycholate) agar (Oxoid) or MRS agar were prepared with aliquots from each culture to differentiate and quantify the number of CFUs of *Salmonella* or *L. sakei* 2a, respectively.

Statistical analyses

The statistical analyses were conducted using either one-way or two-way ANOVA procedures depending on the experimental design. Statistical differences in samples were tested for at $p < 0.05$. Duncan's multiple-range test (DMRT) was used to differentiate between the mean values. All the analyses were done with SPSS (11.0) software. All determinations were performed in triplicate.

Fig. 1. Agar diffusion test showing antimicrobial activity of the extract 2a against *Listeria monocytogenes* Scott A Cm^r Em^r one narrow = extract 2a with 500 AU/ml no dilution (+) = positive control (nisin 100 IU/ml), and (-) = negative control (0.2N HCl, pH 5.0) Extract 2a at different dilutions are marked next to each well: 1/2; 1/4, 1/8, 1/12, 1/16 and 1/20

RESULTS

Antimicrobial activity of extract 2a

We determined that the extract 2a exhibited antimicrobial activity against *L. monocytogenes* Scott A Cm^r, Em^r, which was used as the indicator strain of activity. Specifically, extract 2a had approximately 500 AU mL^{-1} of antimicrobial activity (Fig. 1).

Multiplication of the *Salmonella*-pool in co-cultivation with *L. sakei* 2a and/or EDTA

Culture of the *Salmonella*-pool (non-treated group) showed a normal curve of multiplication during 12 h of growth (curve a; Fig. 2). However, when the *Salmonella*-pool was co-cultivated with *L. sa*kei 2a in the same conditions, the sample showed a two-log reduction in the concentration of the pathogen (curve b; Fig. 2). Moreover, when the *Salmonella*-pool was cultivated in the presence of EDTA at two different concentrations (10 or 20 mM), its growth was inhibited up to five logarithmic cycles (in 12 h of cultivation), compared with the growth of the *Salmonella*-pool with both *L. sakei* 2a and EDTA (10 or 20 mM) resulted in elimination non-treated group (curves c and d; Fig. 2). Based on these results, the EDTA was effective at both 10 and 20 mM, as depicted by the curves "c" and "d" in Fig. 2. Finally, co-cultivation of the of the pathogen after only ~3 h of cultivation (curves e and f;

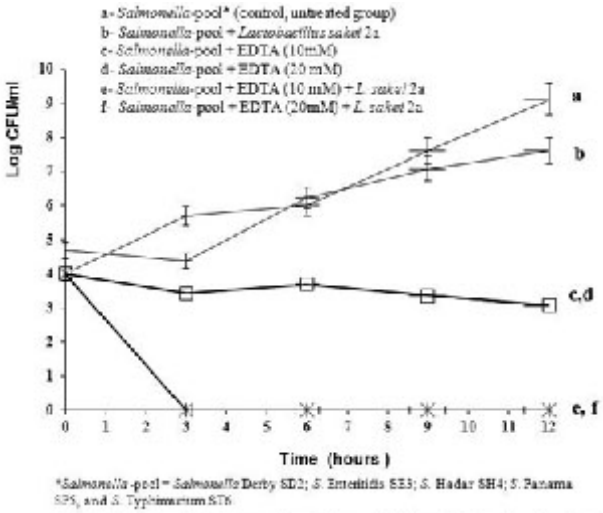

Fig. 2. Multiplication of the *Salmonella* - pool in co-cultivation with *Lactobacillus saket* 2a only and or in the presence of EDTA in BHI broth at 37°C. *Error bars* respect the standard deviation

Fig. 2). The application of multiple ANOVA with Tukey resulted $p<0.05$, showing a statistically significant difference between the treatments used in the experiments. When each treatment was compared to the *Salmonella*-pool (non-treated group) the results indicated a significant difference for the c, d, e, and f treatments.

Multiplication of the *Salmonella*-pool in the presence of extract 2a and/or EDTA

Fig. 3 shows the actions of EDTA (1.0 mM) and/or extract 2a on the *Salmonella*-pool that had an initial concentration of 7.68 on a log-scale. These data indicate that the extract 2a had an inhibitory effect on the *Salmonella*-pool that was potentiated by EDTA, as evidenced by the 3.5-log reduction in its concentration compared with the level of the non-treated group (bars a, b, and c). Moreover, the combination of the *Salmonella*-pool, nisin, and EDTA resulted in a 1.5-log reduction in the concentration (bar d). Also of note, the extract 2a alone was more efficient in reducing the *Salmonella*-pool than the nisin alone treatment

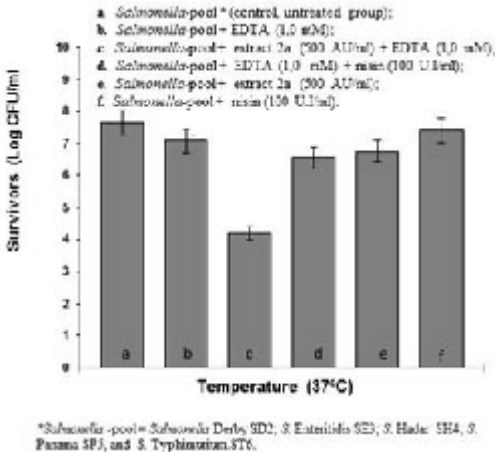

Fig. 3. Multiplication of the *Salmonella* - pool in the presence of EDTA and extract 2. *Error bars* respect the standard deviation

(curves e and f, respectively). When all treatments were compared to the *Salmonella*-pool (non-treated group),the results indicated a negative significant difference only for the *Salmonella*-pool in treatment "c"that inhibited the growth of the pathogen in rates close to theother treatments. Based on these findings, the treatment with the best action against the*Salmonella*-pool was the combination including extract 2a and EDTA.The treatments b, d, e, and f were ineffective to the *Salmonella*-pool.

Effect of citric acid on the multiplication of the *Salmonella*-poolco-cultivated with *L. sakei* 2a

As shown in Fig.4, we found a 1.4- to 6-log reduction in the concentration of the *Salmonella*-pool when it was co-cultivatedwith *L. sakei* 2a alone or in combination with citric acid (at concentrations of either 10 or 20 mM). Compared with the growth of the non-treated group, the *Salmonella*-pool co-cultured with *L. sakei* 2a exhibited a 1.4-log reduction in the concentration of pathogen (curves a and b,

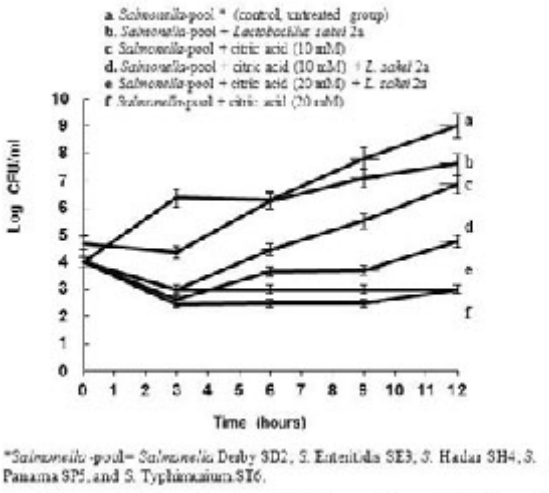

Fig. 4. Effect of citric acid on the multiplication of the pool of *Salmonella* strains co-cultivated with *L. sakei* 2a. Error *bars* represent the standard deviation

respectively; Fig. 4). Moreover, upon co-culturing the *Salmonella*-pool with citric acid (10 mM) only or with citric acid (10 mM) and *L. sakei* 2a, the mixtures exhibited reductions of 2.20- and 4.23-log, respectively (curves c and d, respectively). Moreover, we observed a 6.0-log reduction in the concentration of the *Salmonella*-pool cultivated with either citric acid (20 mM) and *L. sakei* 2a or citric acid (20 mM) only (curves e and f, respectively; Fig. 4).

The application of multiple tests of ANOVA with Tukey resulted $p < 0.05$, showing a statistically significant difference between the treatments used in the experiments. Results also indicated a significant difference for treatment d, e, and f in relation to the control group *(Salmonella*-pool, non-treated group). But there was no significant difference for groups b and c.

Combined effect of citric acid, EDTA, lysozyme or lactic acid with extract 2a on the survival of *Salmonella*-pool

We found that the combination of citric acid (10 mM) and extract 2a (500 AU ml^{-1}) reduced the concentration of the pathogenic*Salmonella*-pool by approximately 2.5-log compared with either the non-treated group or the group treated with citric acid only (bars a, b,c, and d;Fig. 5). We also found that the *Salmonella*-poolwas reduced by about 2-log upon being cultivated with the combination extract 2a (500 AU ml-1), EDTA (1.0 mM), and lysozyme (10 µg ml^{-1}) (bars b and c; Fig. 6). This inhibitory action of the pool was similar to that of the combination treatment of lactic acid (0.1%), EDTA, and lysozyme, revealing a reduction of 2-log (data not shown)

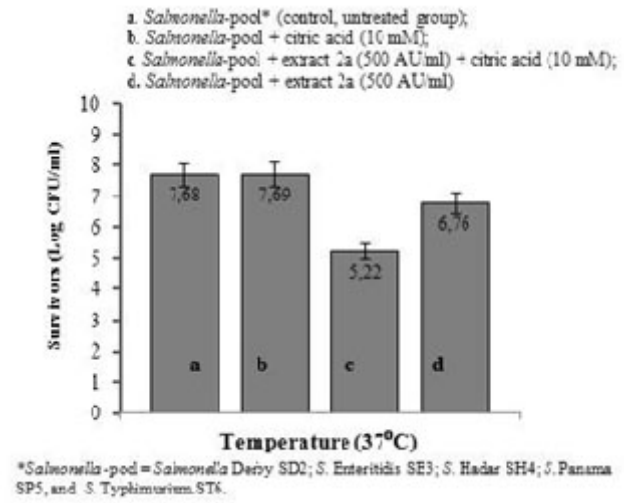

Fig. 5. Effect of citric acid extract 2a, or citric acid combined to extract 2a on the multiplication of the pool of *Salmonella* strains. Error *bars* represent the standard diviation

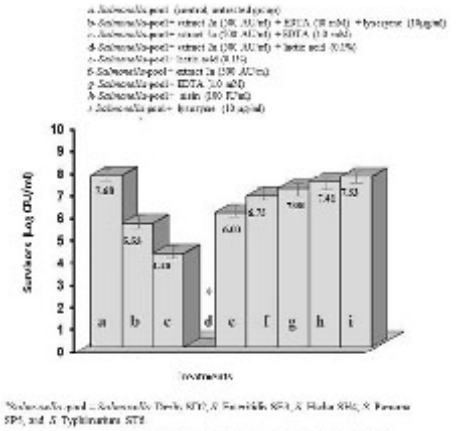

Fig. 6. Combined effect of the action of extract 2a, EDTA, lysozyme, lactic acid, and extract 2a on the multiplication of pool of *Salmonella* strains. Error bars represent the standard deviation

compared with the level of the non-treated group. These combinations were more efficient in decreasing the *Salmonella*-pool concentration than the isolated action of each antimicrobial agent (bars e, g, andi; Fig. 6). Also of note, the combination lactic acid and extract 2a treatment resulted in a total elimination of the *Salmonella*-pool (no CFU into selective agar plate) (bar d; Fig. 6). The test of multiple ANOVA with Tukey resulted $p<0.05$, showing a statistically significant difference between the treatments used in the experiments. When comparing the *Salmonella*-pool (non-treated group) with the other treatments, the results indicate a significant difference for treatments b, c, d, e, f, and g and there was no significant difference for the treatments h and i (Fig. 6).

DISCUSSION

Lactic acid bacteria and their metabolites (organic acids, bacteriocins, etc.) have received research attention as alternatives in various food technologies for inhibition of undesirable microorganisms[9,24-26]. In gram-negative pathogens

like the *Salmonella* spp., bacteriocins can act on bacteria by being used in combination with substances such as organic acids and chelators.

The extract 2a that was obtained from *L. sakei* 2a in the present study inhibited the growth of the strains of *Salmonella* grouped in a pool. We also verified that the inhibitory activity of concentrated acid extract 2a combined to other antimicrobials (which were at the limit concentration recommended for use against pathogens in foods) was more efficient than nisin used as a control on inhibition of the *Salmonella*-pool. Stevens *et al.*,[10,11] observed that the use of nisin at concentration of 50 µg/ml in combination to EDTA (20mM) was sufficient at reduction of 3-6 log of different *Salmonella* serotypes. This and other studies[9,12-14] show that there is not a defined standard concentration at using nisin, when the objective is the action of bacterial pathogens. Thus, different concentrations of nisin are tested (50 IU ml^{-1} to 2,500 IU ml^{-1}). These variations are due to differences in the systems studied, and the types of treatments used with the target microorganism. In our study, we used the inhibitory agents EDTA, citric acid, lactic acid, and lysozyme as facilitating agents to be used in combination with the extract 2a on *Salmonella*. We found that, at the concentrations used in our study, these reagents interfered with the multiplication of the *Salmonella*-pool. In particular, the most effective action against *Salmonella* was the combination treatment including extract 2a and EDTA (which caused a 3.5-log reduction), whereas the combination nisin/EDTA treatment was less effective (causing a 1.0-log reduction). However, previous studies have revealed that in experiments that used a concentration of nisin (50 IU ml^{-1}) in combination with EDTA (20 mM) resulted in an average of a 3.0-logreduction in different *Salmonella* serotypes[10].

In this study, the co-cultivation experiments revealed that mixing *Salmonella* and *L. sakei* 2a resulted in a log reduction of the CFUs of the *Salmonella*-pool. This reduction could be attributed to a number of different factors, such as organic acids and/or other antimicrobial products including hydrogen peroxide[27].We also obtained two distinct findings regarding the combinations including lactic acid. The first revealed a total elimination of the *Salmonella*-pool upon treatment with both extract 2a and lactic acid; the second revealed a 3.7-log reduction in the concentration of the pathogenic pool upon treatment with both lysozyme and lactic acid. Thus, the combination treatment of lysozyme and lactic acid was insufficient to completely eliminate the pool of *Salmonella* strains, but it significantly reduced the concentration of the *Salmonella*-pool. Finally, the combination treatment including extract 2a and lactic acid efficiently eliminated the pathogenic *Salmonella*-pool in the conditions used in this study.

Carvalho *et al.*,[28] purified acid extract of *L. sakei* 2a and obtained sakacin P and two other different antimicrobial compounds active against *Listeria monocytogenes*, but not to *Salmonella* Enteriditis ATCC 13076 or *S.* Typhimurium ATCC 14028. They conclude that sakacin P activity can be potentiated by other antimicrobial proteins.

Based both on previous studies[6,29] and on the protocol used to obtain extract 2a in our study, we conclude that extract 2a contained antimicrobial activity against food-borne *Salmonella* strains. In addition, the antimicrobial activity of extract 2a is potentiated with the action of lactic acid. Thus, our findings also indicate that *L. sakei* 2a and extract 2a, especially in combination with other antimicrobials, presents potential technological application in the control of salmonellae in foods. However, according to Gálvez *et al.*,[30] the effectiveness of substances like bacteriocins requires careful analysis before they can be applied to selected target bacteria in food systems.

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