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Potential Use of Lactic Acid Bacteria with Pathogen Inhibitory Capacity as a Biopreservative Agent for Chorizo

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Abstract: The biopreservation of meat products is of great interest due to the demand for products with low or minimal chemical additives. Lactic acid bacteria (LAB) have been used as protective cultures for many centuries. The objective of this work was to characterize 10 native LAB isolated from meat masses with biopreservative potential for meat products. The isolates were subjected to viability tests with different concentrations of NaCl, nitrite, and nitrate salts, pHs, and temperature conditions. Antibiotic resistance and type of lactic acid isomer were tested. In addition, the isolates were tested against seven pathogens, and inhibitory substances were identified by diffusion in agar wells. Finally, two isolates, *Lb. plantarum* (SB17) and *Lb. sakei* (SB3) were tested as protective cultures of chorizo in a model. As a result, the viability at different concentrations of NaCl and nitrate and nitrate salts were obtained. pH and temperature exerted a negative effect on the growth of some of the isolates. Pathogens were inhibited mainly by the presence of organic acids; *P. aurius* was the most susceptible, and *S. typhimurium* and *S. marcescens* were the most resistant. The strains SB17 and SB3 had similar effects on chorizo, and time exerted a deleterious effect on microbiological quality and pH. The results indicated that the 10 isolates show promising characteristics for the preservation of cooked meat products, with the strain *Lb. plantarum* (SB17) being the most promising.

Keywords: characterization; cell-free extract; HPLC; inhibitory substances; protein precipitation

1. Introduction

Lactic acid bacteria (LAB) are generally considered safe and have been associated with the production of fermented foods for centuries [1,2]. This group of bacteria can inhibit the growth of pathogenic organisms in different foods of plant or animal origin [3,4], and it is possible to isolate native strains from fermented dairy, meat and vegetable products, and alcoholic beverages. LAB improve characteristics such as aromas, flavor, acidity, and protein digestibility and can benefit the appearance of the product [5,6]. The use of LAB as protective cultures is based on the production of secondary metabolites such as lactic acid and bacteriocins, substances that are considered biopreservatives [1,7]. In addition to their ability to survive or resist extreme and diverse conditions, they are thermostable and have the competitive characteristics necessary for their use as bioprotective and biopreservative cultures [1,8,9].

Meat products are prone to bacterial contamination because they contain compounds that favor bacterial growth [2,10]. These microorganisms are considered spoilage bacteria (that damage or degrade the product) or pathogenic bacteria (that cause symptoms) [11,12]. Foodborne pathogens include *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Serratia marcescens*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*, which can grow in refrigerated foods [11,13,14].

One method of preservation that has long been used is the application of chemical preservatives, such as nitrite and nitrate salts, which have a negative effect on human

health [15–18]. Currently, there is great consumer demand for high-quality food that is free of chemical preservatives; therefore, there is a need to find alternative preservation methods. One potential preservation method is the use of LAB as a biopreservative; in Colombia, the use of LAB is mostly focused on dairy products and is restricted in fermented meat products [11,14,19]. For this reason, this work aims to characterize native LAB isolated from the meat masses of chorizo and sausage-type products that have biopreservative potential for meat products.

2. Materials and Methods

For this work, 10 native strains of LAB isolated from chorizo and sausage-type meat masses were selected and tested. The strains were deposited in the microorganism, and filamentous fungi strain bank of the Microorganism and Applied Biotechnology Research Group (MIBIA) of the Universidad del Valle, where the tests were carried out, Table 1 shows the identification of the isolates.

Table 1. Identification of the native LAB isolates used.

LAB Strain	Identification	LAB Strain	Identification
SB3	*Lactobacillus sakei* (MW073315)	SB112	*Lactobacillus buchneri* (99.8%) [1]
SB5	*Lactobacillus sakei* (MW073316)	CB26	*Weissella confusa* (MW073317)
SB17	*Lactobacillus plantarum* (MW073312)	C12	*Enterococcus faecalis* (MW073313)
SB99	*Lactococcus lactis* (96%) [1]	C34	*Pediococcus acidilactici* (82%) [1]
SB100	*Weissella confusa* (MW073314)	C54	*Lactococcus garvieae* (MW073318)

The numbers in parentheses indicate the GenBank code. [1] Identification according to biochemical test results (API 50CHL).

2.1. Viability at Different Concentrations of NaCl, Nitrate and Nitrate Salts and Different pH Values and Temperatures

The LAB were tested for viability under different conditions by modifying the MRS broth as follows: pHs of 6, 4, and 3 from acetic acid (3M); NaCl concentrations of 1, 2, and 3%; and nitrite and nitrate salts at 100, 81, 60, and 30 ppm. The medium was inoculated with 1 mL of the bacterial strain and incubated for 16–24 h at 35 °C. The number of viable microorganisms was estimated by plate counting with serial dilutions; this test was performed in triplicate for each strain used, and only plates with counts between 30 and 300 colonies were considered. The number of colonies was multiplied by the inverse of the dilution to obtain the CFU/mL (Jurado & Gúzman, 2015). Using the same method, the strains were incubated for 24 h at 35 °C in MRS broth and subjected to temperatures of 40, 50, 60, and 80 °C for 15 min, followed by microorganism counting.

ANOVAs were performed for pH, NaCl concentrations, and nitrite and nitrate salts with heterogeneous variances, and single-factor ANOVA was performed for the temperature variable.

2.2. Antibiotic Resistance

Antibiotic resistance was determined using the Kirby Bauer method, for which 4 antibiotics (Sigma) were used: amoxicillin (30 μg), ampicillin (10 μg), penicillin (10 IU), and tetracycline (30 μg). The bacterial strains were cultured in 9 mL of MRS broth and then diluted to 10^{-3} (Macfarland 0.5) and inoculated by depletion on MRS agar. The discs were then overlayed with the antibiotics and incubated at 35 °C for 24 h. After the incubation period, the discs were examined to determine the presence or absence of inhibition halos (the translucent zone formed around the antibiotic discs) [20].

2.3. Lactic Acid Isomers

LAB are characterized by the production of different lactic acid isomers through the fermentation of glucose. During this process, they can produce L(+) dextrorotatory lactic acid, D(−) levorotatory lactic acid, or a racemic mixture of both D and L. The

strains were determined by the type of lactic acid isomers they produced, following the method recommended by Betancourt Botero et al., 2013 [21]. This method consisted of an ester-specific enzymatic oxidation-reduction reaction catalyzed by the enzyme lactate dehydrogenase, which oxidizes lactate to pyruvate by simultaneously reducing the L(+) and D(−) isomer to a NAD (NADH) form, which produces a coupling reaction that reduces the tetrazolium salt under diaphoresis, forming a reddish, insoluble compound. With this method, it was possible to differentiate the strains that produced D(−) lactic acid, which had intense reddish halos, from those that produced a racemic mixture (DL), which had less intense reddish halos. The strains that exclusively produced L(+) isomers were distinguished by the absence of color and were recommended for human consumption.

2.4. Antimicrobial Activity Test

The antimicrobial activity of the LAB isolates was determined using the modified disk diffusion method. The commercial strains used were *Listeria monocytogenes* ATCC 19111, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Serratia marcescens* ATCC 13880, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, and *Proteus vulgaris* ATCC 9484, all of which were activated according to the manufacturer's instructions.

The LAB strains were cultured in MRS broth for 24 h at 35 °C and then diluted in 9 mL of water with 0.1% peptone to 10^{-3} (adjusted to the MacFarland scale 0.5). This last dilution was seeded in MRS agar by surface seeding with a sterile swab and incubated at 35 °C for 16 h. Additionally, pathogenic bacteria were cultured in 9 mL of brain heart infusion (BHI) broth for 24 h at 35 °C and diluted to 10^{-3} in peptone water. A total of 100 microliters of this dilution was removed and sown on Mueller Hinton agar with a sterile swab. From the LAB cultures, which were incubated for 16 h, discs with diameters of 5 mm were extracted and placed in Mueller Hinton boxes inoculated with pathogens at evenly spaced locations and incubated for 24 h at 35 °C. This test was performed in duplicate. A disc of MRS agar without inoculum was also placed as a negative control. The antimicrobial activity was determined according to the diameter of the clear zones surrounding the LAB discs [13]. An ANOVA using a generalized linear model with heterogeneous variances was performed in R version 3.32.

2.5. Identification of Inhibitory Substances

2.5.1. Identification of the Inhibitory Substance by the Agar Well Diffusion Method

To determine the production of inhibitory substances during the growth of the isolates, the method of Angmo et al. 2016 was modified [22]. Isolates were grown in 20 mL of MRS broth for 48 h at 35 °C and then refrigerated for 3 days. Once the incubation time was complete, the samples were centrifuged ($10,000 \times g$ for 10 min at 4 °C), and the supernatant was sterilized by filtration using 0.22 μm syringe filters (Millipore, MERCK, Burlington, MA, USA) and placed in sterile containers. The cell-free extract (CFE) was treated as follows: fraction A: CFE of the 10 isolates; fraction B: CFE-N with the pH neutralized (pH ≥ 6) by 1 M calcium bicarbonate, which was used to determine inhibition by acids; fraction C: MRS broth as control.

Next, 5 mm-deep wells were made in Müller Hinton plates inoculated with the *S. aureus*, *S. marcescens*, and *L. monocytogenes* strains previously cultured in 9 mL of BHI, and 90 μL aliquots of fractions A, B, and C were added to the wells. The wells were incubated for 48 h at 35 °C, and observations were performed at 24 and 48 h to examine clear zones of inhibition around the wells.

2.5.2. Qualitative and Quantitative Estimation of Organic Acids Produced by the Isolates through HPLC

The isolates were cultured in 20 mL of MRS broth for 48 h at 35 °C. The supernatant was sterilized by filtration using 0.22 μm syringe filters (Millipore, Merck) and deposited in sterile vials at 4 °C for further analysis. The concentration of organic acids in the samples was determined using the high-performance liquid chromatography (HPLC) technique in

a Waters®Breeze HPLC chromatograph (Waters, Milford, MA, USA), which performed dual detection by means of 2 detectors: 1 for the refractive index (RI) and the other for ultraviolet (UV). An Aminex®HPX-87 (Bio-Rad, Hercules, CA, USA) column and a mobile phase of 5 mM H_2SO_4 were used. Organic acids such as citric, succinic, lactic, acetic, and propionic acids were detected [23].

2.5.3. Purification and Enzyme Assay of Crude Cell Extracts

To obtain proteins, the cell-free supernatant with neutralized pH (fraction B) was precipitated with ammonium sulfate reaching 80% saturation, left to dialyze for 12 h at 4 °C and then centrifuged (4000 rpm for 30 min at 4 °C). The precipitate was dissolved in 1 M phosphate buffer (pH 6.0), and the presence and concentration of proteins was estimated by the Lowry method with the standard curve of albumin. Enzyme activity was determined by the agar diffusion method described previously [22,24].

2.6. Meat Mass Testing
Production of Cooked Chorizo

The manufacturing chorizo was carried out at the MIBIA laboratory following the industrial formulation for meat sausage products; see Table 2. In this test, 6 treatments were carried out using two inoculums, SB3 and SB17. For these treatments, 30 g of chorizo-type meat mass was inoculated with 3% of the bacterial strain, with a viability of 106 CFU per mL. This mass was subjected to 80 °C for 15 min, followed by a thermal shock at 4 °C. Once this process was completed, microbiological control was carried out, the pH of the meat product was measured, and 1 g of the sausage was removed and homogenized in 9 mL of water with a Stomacher MiniMix.

Table 2. Formulation for the manufacture of chorizo-type sausage.

INGREDIENT	Control (Nitrite Salt) (C2 + N)	Treatment 1 and 2	Control (−Nitrite Salt) (C1 − N)	Treatments 3 and 4
Water (ice)	48.17	48.17	48.26	48.26
Pulverized garlic	4.82	4.82	4.83	4.83
Cassava starch	8.03	8.03	8.04	8.04
Ascorbic acid	0.96	0.96	0.97	0.97
Beef (ground)	289.02	289.02	289.58	289.58
Scallions	48.17	48.17	48.26	48.26
Orange coloring	0.32	0.32	0.32	0.32
Chorizo seasoning	6.74	6.74	6.76	6.76
Monosodium glutamate	0.80	0.80	0.80	0.80
Pork fat	81.89	81.89	82.05	82.05
Liquid smoke (mL)	1.45	1.45	1.45	1.45
Nitrous salts **	0.96	0.96	0.00	0.00
Soy protein	6.42	6.42	6.44	6.44
Refined salt	2.25	2.25	2.25	2.25
Inoculum	0.00	15.00	0.00	15.00

Two controls were performed in the presence ** and absence of nitrite and nitrate salts; two treatments in the presence of LAB, T1 ** and T3 containing strain SB17 and T2 ** and T4 containing strain SB3 in the presence and absence of nitrite and nitrate salts, respectively.

The pH was measured at room temperature using an Orion 710-A instrument (Bozkurt and Erkmen, 2002). From these mixtures, 10^{-4} dilutions were made. A total of 100 μL of the dilution was inoculated in Petri dishes with culture medium, potato dextrose agar (PDA), plate count agar (PCA), MRS with aniline blue, MacConkey agar, and violet red bile agar (VRBA). The cultures were incubated at 28 and 35 °C for 24 h in triplicate. The chorizo was stored at 4 °C under anaerobic conditions and monitored for 40 days.

Microbiological control was carried out according to Colombian Food Industries Technical Standard NTC 1325 (2008) for noncanned processed meat products and took into account the method recommended by the Colombian Technical Standards (NTC 1325,

2008; NTC 4092, 2009) for microbiological counting. The data were analyzed by means of a one-factor ANOVA model in R version 3.32.

This test had 2 controls and 2 treatments, as shown in Table 2.

3. Results

3.1. Viability at Different Concentrations of NaCl, Nitrite and Nitrate Salts, pH Values and Temperatures

3.1.1. Tolerance of LAB to Different NaCl Concentrations

The isolates were able to grow and survive in concentrations of 1, 2, and 3% NaCl; the results are shown in Figure 1A. Statistically, there were significant differences between strains in their responses to salt concentrations ($p < 0.0001$): strains SB3 and SB100 presented the lowest tolerance of NaCl, and strain C12 presented the highest tolerance.
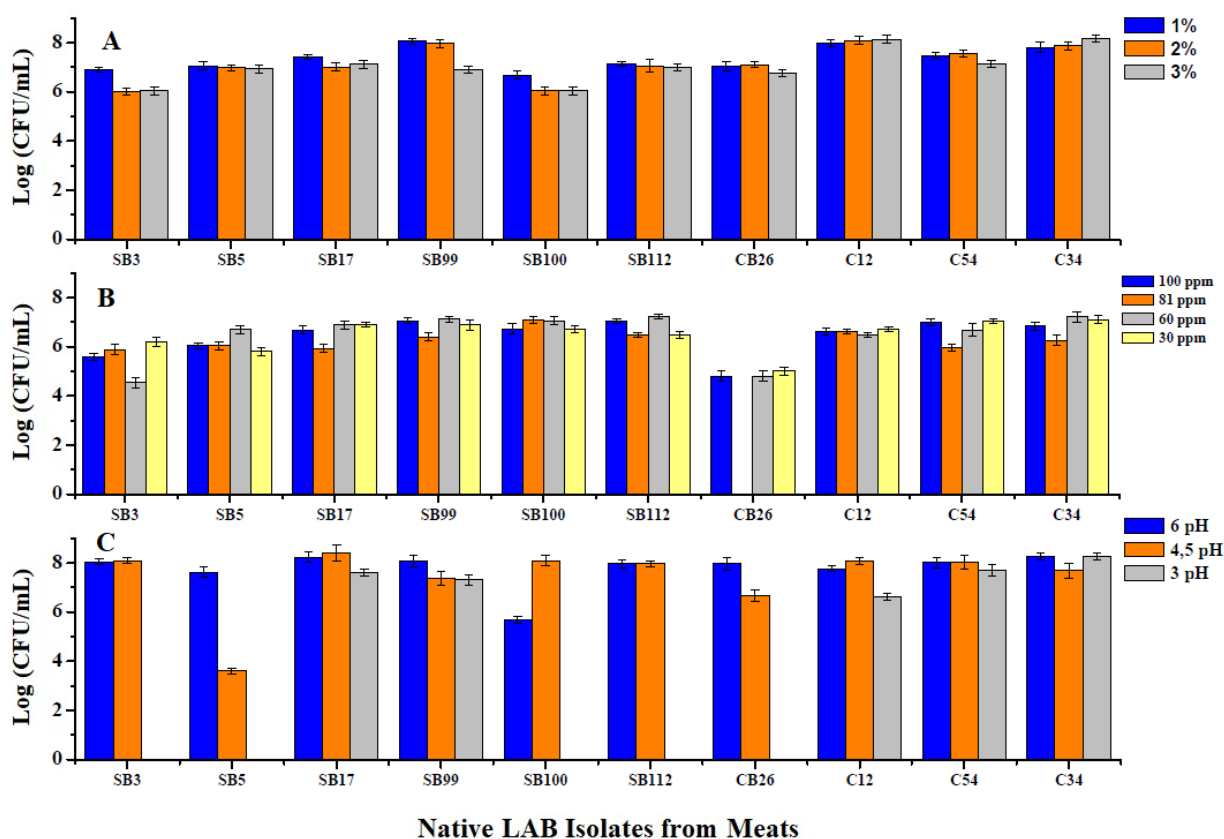
Figure 1. Viability of the 10 native LAB at different concentrations of NaCl (**A**), nitrite and nitrate salts (**B**), and different pH values (**C**).

3.1.2. Tolerance of LAB to Different Concentrations of Nitrate and Nitrate Salts

Regarding the concentration of nitrate and nitrate salts, growth was influenced by nitrate salt, but there were no significant differences in the tolerance of different concentrations ($p = 0.9138$). However, there were significant differences ($p = 0.001$) between strains in the tolerance of different concentrations of nitrite and nitrate salts. Strain SB112 presented higher growth at the concentrations evaluated. Strain CB26 was inhibited at the nitrite salt concentration of 81 ppm. The results are shown in Figure 1B.

3.1.3. Viability at Different pH Concentrations

The isolates presented better growth at a pH of 6, although the results were not significantly different from those at a pH 4 ($p = 0.1453$). Growth under these two treatment conditions differed from growth at pH 3 ($p < 0.005$), at which five of the isolates were not viable, as shown in Figure 1C.

3.1.4. Viability at Different Temperatures

When the strains were subjected to heat shock for 15 min at 50 °C, strains C12, C54, C34, CB26, SB99, and SB112 were viable and showed no significant differences in growth ($p = 0.154$) in growth. None of the strains were viable at temperatures of 60 and 80 °C.

3.1.5. Antibiotic Resistance and Type of Lactic Acid

Table 3 shows the type of lactic acid and the antibiotic resistance and susceptibility of the isolates, highlighting the resistance of strains SB3, SB112, and CB26 to tetracycline.

Table 3. Antibiotic resistance of bacterial isolates and type of lactic acid produced.

Strain	Tetracycline	Amoxicillin	Penicillin	Ampicillin	LAT
SB3	R	S	S	S	DL
SB5	S	S	S	S	DL
SB17	S	S	S	S	L (+)
SB99	S	S	S	S	L (+)
SB100	S	S	S	S	L (+)
SB112	R	S	S	S	L (+)
C12	S	S	S	S	L (+)
C34	S	S	S	S	L (+)
C54	S	S	S	S	L (+)
CB26	R	S	S	S	L (+)

R (resistant), S (susceptible), LAT (lactic acid type) and DL (racemic mixture).

3.2. Inhibition Testing of Selected Isolates

When the 10 isolates were tested against the pathogenic strains, the most susceptible was *P. vulgaris*, which presented the largest inhibition halos, and the most resistant strains were *S. typhimurium* and *S. marcescens*, which had the smallest inhibition halos. The strains with the highest inhibition were SB112, SB17, and SB5, and those with the lowest inhibition were SB99, C54, and C34; these results are shown in Figure 2. Statistically significant differences were found in the inhibition of the pathogen ($p < 0.0001$) depending on the isolate and the pathogen used.
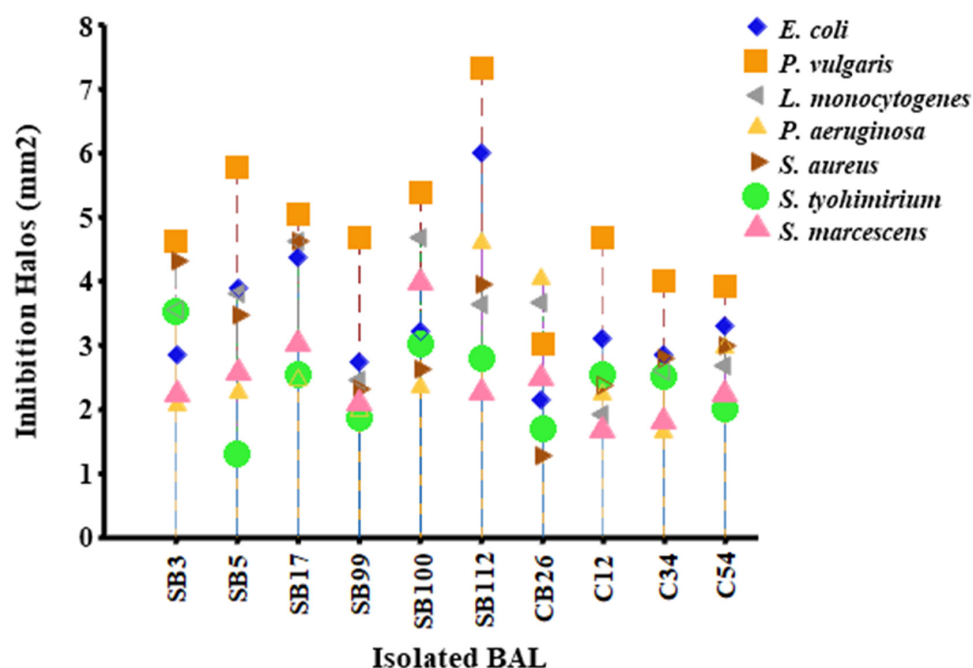

Figure 2. Inhibition halos of pathogenic bacteria vs. native LAB.

3.3. Identification of Inhibitory Substances

3.3.1. Identification of Inhibitory Substances Using the Agar Well Diffusion Method

Fraction C (MRS broth) was negative for inhibiting the three pathogens tested; strain SB99, identified as *Lactococcus lactis*, presented inhibition of *Serratia* and *Listeria* with fraction B. Fraction A was positive for all of the strains, which shows that the inhibition was due to the action of the acids present in the CFE; see Figure 3 and Table 4, which show the pH values of fraction A.
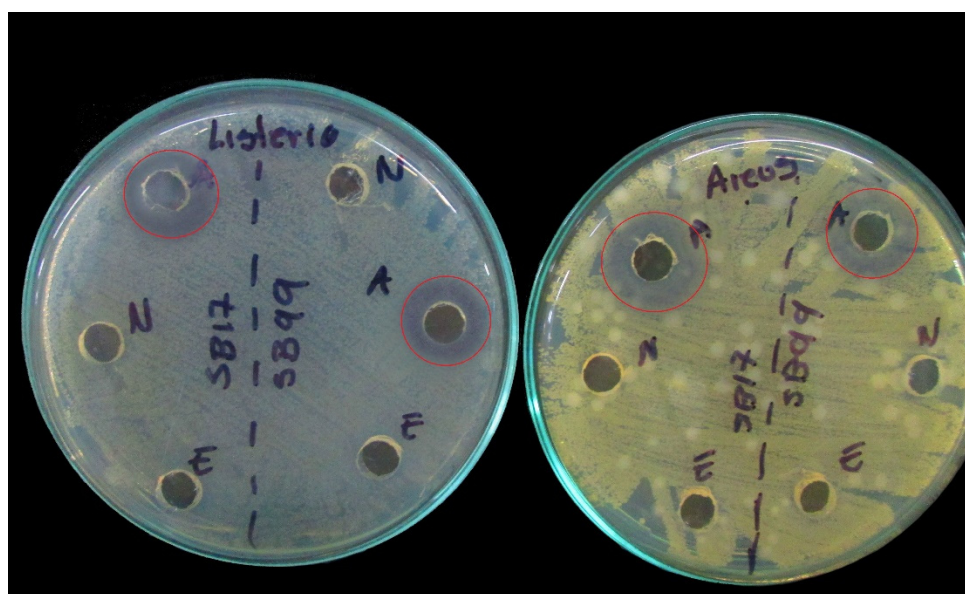
Figure 3. Inhibition results by cell-free extract: the effect of fraction A. Red circles indicate the effect of organic acids on *Listeria monocytogenes* and *Staphylococcus aureus* strains.

Table 4. Inhibition of three pathogens by isolate supernatants.

Strain	Fraction A	Fraction B		
		L. monocytogenes	*S. aureus*	*S. marcescens*
SB112	3.77	−	−	−
C12	4.53	−	−	−
SB17	3.87	−	−	−
SB5	4.29	−	−	−
CB26	4.53	−	−	−
SB99	4.72	+	+	−
C54	4.39	−	−	−
C34	4.48	−	−	−
SB3	4.57	−	−	−
SB100	4.37	−	−	−

Inhibition negative (−) and positive (+) cell-free extract (Fraction A) pH values.

3.3.2. Qualitative and Quantitative Estimation of Organic Acids Produced by the Isolates Detected by HPLC

Table 5 details the concentration of organic acids and shows that lactic acid was the predominant metabolite produced. Its concentration presented high variability, ranging from 1871.67 to 13613.97 mg/L. Succinic acid presented the next highest variability; with values between 100 and 163 mg/L, it showed less variation.

Table 5. Quantification of organic acids produced by LAB isolated from meat products.

| Sample	Citric (mg/L)	Succinic (mg/L)	Lactic (mg/L)	Acetic (mg/L)	Propionic (mg/L)	Ethanol (%)
SB3	ND	124.13 ± 0.03	7801.76 ± 0.01	ND	ND	0.22
SB5	ND	151.47 ± 0.1	7704.21 ± 0.01	ND	ND	0.20
SB17	ND	157.90 ± 0.1	1871.67 ± 0.01	ND	130.35 ± 0.002	0.67
SB99	ND	160.89 ± 0.01	$13{,}613.91 \pm 0.01$	94.36 ± 0.01	ND	0.23
SB100	ND	156.00 ± 0.3	8828.47 ± 0.002	ND	ND	0.64
C12	ND	163.49 ± 0.01	7663.85 ± 0.01	1885.77 ± 0.007	ND	0.49
C34	ND	148.26 ± 0.6	5719.51 ± 0.03	ND	ND	0.22
C54	ND	100.82 ± 0.15	5070.16 ± 0.06	4.71 ± 0.001	ND	0.22
CB26	ND	159.54 ± 0.01	7823.55 ± 0.01	2000.29 ± 0.01	ND	0.57

Not detected (ND).

3.3.3. Purification and Enzyme Assay of Crude Cell Extracts

The activity of the crude extracts of bacterial cells after precipitation with ammonium sulfate was negative for all the bacterial isolates tested. When protein quantification was enhanced, there was a reduction of proteins in the fermented culture medium, indicating consumption of proteins by the isolates tested. The results are shown in Table 6 and Figure 4.

Table 6. Quantification of proteins produced by LAB isolated from meat products.

Strains	Protein Concentration (mg/L)
SB3	152.462
SB5	155.54
SB17	170.15
SB99	142.46
SB100	211.69
CB26	159.89
C12	168.61
C34	157.85
C54	147.85
Control	211.69
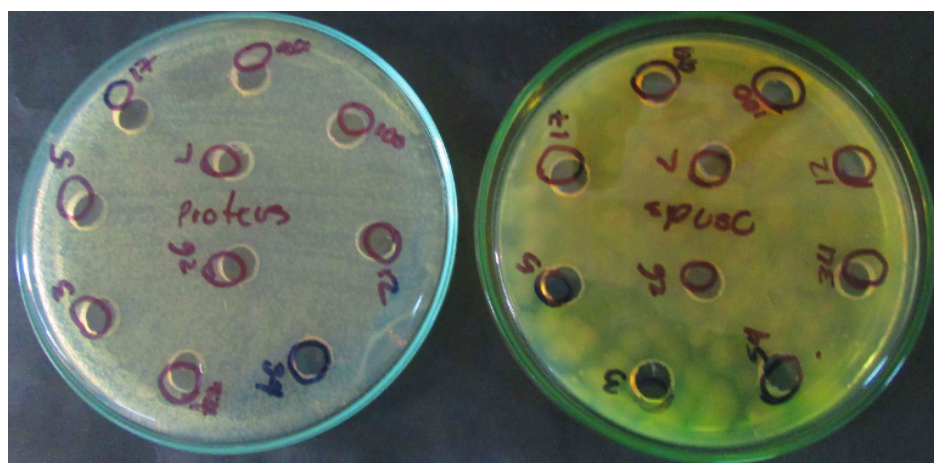
**Figure 4.** Results for isolate supernatants treated with ammonium sulfate.

3.4. Meat Mass Testing

Determination of the Microbiological Characteristics of Chorizo

Mold and yeast counts are shown in Figure 5a. Statistically, there were significant differences between the first three days of measurement and the last two days of measure-

ment, $p < 0.005$; the first days of measurement were zero, but the last two presented values between 5.6–6.5 log CFU/mL. However, no significant differences were found between treatments, $p > 0.282$, even though T2 (strain SB3 + nitrite and nitrate salts) and the control without nitrite salt were already showing growth of molds and yeasts at day 18.

(**a**)　　　　　　　　　　　　　　　　　　　　(**b**)
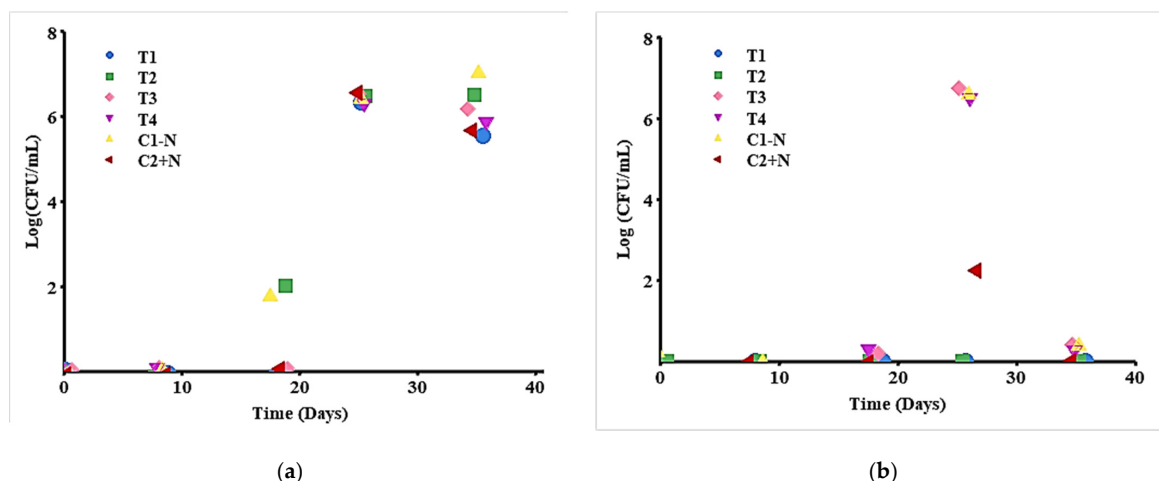
Figure 5. Microbiological characteristics of biopreserved T1–T4 chorizo and controls: (**a**) Microbiological count of molds and yeasts (PDA); (**b**) enteric microbiological count (MacConKey).

The counts of enteric organisms such as *Escherichia coli* and *Staphylococcus* were negative. The colonies that appeared on day 26 were colorless and grew in both the treatments and the control without nitrite salt (T3, T4, and C1 − N) (see Figure 5b) and did not present significant differences ($p > 0.05$). T1 and T2 did not present microbiological growth during the 35-day follow-up, although the control presented growth on day 26. In this case, it was found that day 26 was statistically different ($p < 0.005$) from the other days of measurement, where no microbiological growth was present, $p = 0.999$.

Figure 6a shows the results of LAB monitoring, indicating significant differences ($p = 0.0024$) between T3 on day 18 and the other treatments. It should be noted that T1 presented growth on the same day, but it was not statistically significant. The treatments presented strain SB17 (*Lb. plantarum*) in both the absence and presence of nitrite and nitrate salts. On days 26 and 35, there were no significant differences between the treatments and controls.

(**a**)　　　　　　　　　　　　　　　　　　　　(**b**)
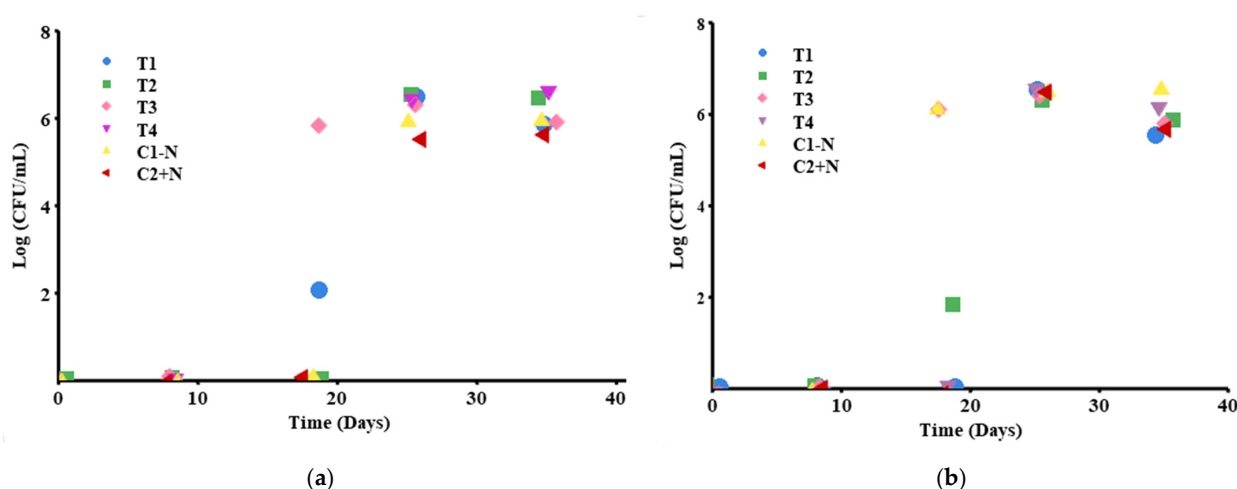
Figure 6. Microbiological characteristics of biopreserved T1–T4 chorizo and controls: (**a**) microbiological count of LAB (MRS); (**b**) microbiological count of mesophiles (PCA).

In terms of time, the measurement taken on the first three days did not differ significantly ($p > 0.005$), but the last two measurements showed significant differences ($p < 0.005$). This is due to the fact that on the first days, there was zero or little growth of LAB, while in the last days, the growth was 5.49 log CFU/mL at minimum.

The presence of mesophilic microorganisms began on day 18, as shown in Figure 6b when treatment T3 (BS17 without nitrite and nitrate salts) and control C1 − N (chorizo without nitrite salts) showed differences from the rest of the treatments ($p < 0.005$) but not from each other. On days 26 and 35, there was no significant difference between the controls and the treatments ($p > 0.005$), which presented counts between 6.45 and 6.5 log CFU/mL.

In terms of time, the measurements taken on day 18 were statistically different from those taken on the other days, $p < 0.005$, which explains the growth of mesophilic organisms. Additionally, the measurements taken on the first two days were significantly different from those taken on the last two days, $p < 0.005$, since on the first days, the growth was zero, and on the last days, it was 6.5 log CFU/mL; however, there was no difference between the first two days and the last two days.

The effects of pH in the controls and treatments are shown in Figure 7. A change was evidenced during storage time, which was associated with the presence of microorganisms: The initial pH value was between 6.25 and 6.37, and the final pH values were between 5.86 and 5.52. Statistically, there was no difference in pH value between treatments ($p = 0.7485$); however, the pH value on day 35 was significantly different from the value on other days, $p < 0.005$.
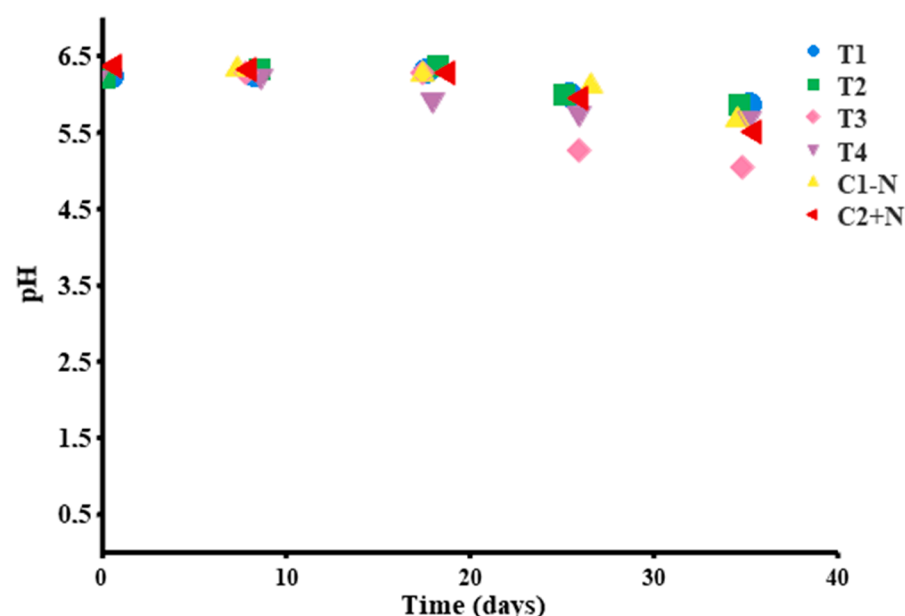

Figure 7. pH monitoring for biopreserved chorizo treatments T1–T4 and controls.

4. Discussion

Ten LAB strains isolated from meat masses were used in this study for the preparation of cooked chorizo and sausages. The LAB were identified as *Lb. sakei* (SB3; SB5), *Lb. plantarum* (SB17), *Lactococcus lactis* (SB99), *Weissella confusa* (SB100; CB26), *Lb. buchneri* (SB112), *Enterococcus faecalis* (C12), *Pediococcus acidilactici* (C34) and *Lactococcus garvieae* (C54). These species have previously been isolated from different types of meats and their derived products [25,26]. Species such as *Lb. Plantarum*, *P. acidilactici*, and *Lb. sakei* are used commercially as starter cultures for the production of meat products since they are competitive and dominate the fermentation process [18,27,28], while *E. faecalis* and *L. garvieae* are found in the first stages of the production of artisanal fermented products [17,18,27], results that coincide with those found in this study.

The selection of LAB as bioprotective cultures should be based on the cultures' ability to survive under stress conditions and their competitive power against indigenous microbiota. To determine this, the strains were subjected to different concentrations of NaCl. All isolates were found to be viable, although there was an interaction effect between the salt concentration and the strain used. Similar results were reported by Castro et al., 2011 [29]; Swetwiwathana and Visessanguan, 2015 [30]; Chavez-Martinez et al., 2016 [31]; Das, Khowala and Biswas, 2016 [32]; Todorov et al., 2017 [33]; and Patil, Disouza and Pawar, 2019 [34] and are due to the characteristics of each bacterial species and their resistance mechanisms.

Nitrite and nitrate salts are used to control *Clostridium botulinum*, and have long been credited with antimicrobial characteristics [10,18,19,35,36]. The results showed that the concentration of the salts did not exert a significantly different effect on the growth of the LAB evaluated; however, a relationship was found between the concentration and the strain evaluated, although it should be noted that the lowest growth was 4.5 log (CFU/mL). Therefore, nitrite and nitrate salts did not exert microbiological control; their use is more likely related to the organoleptic qualities that they contribute to the products [10,18,37].

When pH was evaluated, the strains presented their highest growth at pH values of 6 and 4; pH 3 affected the growth of five strains (Figure 1C). In LAB, a pH < 4 is considered an inhibitory factor that affects viability due to the reduction of intracellular pH, which diffuses through the cell membrane, causing a collapse of the electrochemical gradient that has a bacteriostatic or bactericidal effect [5,30,32]. However, there are LAB that can survive at a pH < 4 and are capable passing through the digestive tract, which is a probiotic characteristic [8,16,30,34,38].

Another important aspect to evaluate is temperature, a factor that affects the viability and survival of bacteria. The isolates were viable at temperatures up to 40 °C; at 50 °C, four isolates did not present growth, and at 60 °C, none of the isolates presented growth. Similar results were reported by Todotov et al., 2017 [33], in which at 40 °C, growth was moderate, and at 50 °C, it was weak or absent. The growth and tolerance to temperature changes shown by six of the isolates were extensive since the optimal growth temperature for LAB is between 35–37 °C [1,39,40].

In the antibiotic sensitivity test, the isolates *W. confusa* (CB26), *Lb. buchneri* (SB112), and *Lb. Sakei* (SB3) were resistant to tetracycline (30 µg), and the remaining eight strains were susceptible to all of the tested antibiotics. Biologically, it has been shown that bacteria transfer their resistance genes to other nearby microorganisms, contributing to a growing problem worldwide [19,24,39,41,42]. Such resistance is of great clinical interest worldwide since it confers to microorganisms the ability to colonize and cause diseases in hospital patients [2,43–45].

Another factor of importance for human health is the type of lactic acid isomer produced by the isolates. In infants, the use of type D (−) lactic acid, which causes acidosis, is rejected [21]. For this reason, the lactic acid produced by the isolates was identified. The two strains identified as *Lb. sakei* (SB3; SB5) presented the racemic mixture (DL), and the remaining ones produced the L(+) type, which has greater inhibitory power than the D type and has not been associated with any pathology [46].

When testing the antimicrobial activity of the 10 isolates against the seven test pathogens, a significant difference was found depending on the strains and the pathogen used, The pathogen with the highest susceptibility was *P. vulgaris*, and the most resistant pathogens were *S. typhimurium* and *S. marcescens*. The effect of LAB against Gram-negative bacteria is mainly attributed to the action of organic acids, which modify pH and hydrogen peroxide activity [19,47]. It has been found that bacteriocins are usually not very effective on this group due to the nature of their outer membrane, which acts as a permeable barrier [6,19,29,48,49].

To identify the inhibitory substances, the agar well diffusion method was performed (see Table 4). The results indicated that the inhibition was mainly due to the action of the acids present in the CFE (fraction A) since in CFE-N (fraction B), only the isolate *Lactococcus*

lactis (SB99) inhibited *S. marcescens* and *L. monocytogenes*. These findings coincide with those reported by Angmo et al., 2016 [22] and Rahmeh et al., 2019 [2] but differ with those of Bungenstock et al., 2020–2021 [26,50] and Yazgan et al., 2021 [28], in which CFE-N presented antimicrobial activity, although not for all of the pathogenic isolates tested, which led them to conclude that crude CFE presents greater antimicrobial activity.

The organic acids produced by LAB were identified (Table 5) as hetero-fermentative due to their production of different acids and ethanol [1,19,51,52], the production of which modifies the pH and has an antimicrobial effect. Lactic acid is considered to enhance the nutritional and sensory characteristics of fermented products [40], and acetic and propionic acid function as strong inhibitory agents by acting on the cell membrane and controlling the growth of molds and yeasts [1,22,53]. Succinic acid exhibits an inhibitory power similar to that of acetic acid, although it usually acts as a buffer solution, keeping the pH stable [2,54–56].

As previously mentioned, the *Lactococcus lactis* strain (SB99) was the only one that showed activity with fraction B. Precipitation was performed with ammonium sulfate, which would allow the identification of the presence of protein compounds such as bacteriocins, but the precipitate did not show antimicrobial activity in any of the isolates. These results differ from those reported by Lü et al., 2014 [57]; Castilho et al., 2019 [58]; Rahmeh et al., 2019 [2], and Bungenstock, Abdulmawjood, and Reich, 2020 [26], in which the precipitates presented inhibitory capacity, but not for all of the evaluated pathogens. The efficiency of the ammonium sulfate precipitation technique depends on the characteristics of the peptide and the amount of antagonistic substances produced [26,59]. It is possible that the activity of SB99 was due to the presence of hydrogen peroxide, another inhibitory substance found in LAB [18,22].

When testing the effects of the isolates *Lb. sakei* (SB3) and *Lb. plantarum* (SB17) on chorizo-type sausage with and without nitrite salt, the microbiological control of molds, yeasts, and enteric organisms (Section 3.4) generally did not differ significantly between the treatments used. which is promising since the microorganisms are giving equal results to the treatments with nitro salts. But there was a significant difference in the presence of microorganisms over time, with the difference being related to packaging—specifically, whether the products were refrigerated or vacuum packed. It has been reported that the loss of vacuum sealing allows the entry of oxygen, which allows the growth of yeasts and enteric bacteria [60–62]; however, in this study, there were no molds, *E. coli* or *Staphylococcus* in the product.

Regarding the monitoring of LAB and mesophilic organisms, there were significant differences between the treatments and the controls. It was expected that the heat treatment of chorizo (80 °C) would eliminate most of the mesophilic organisms, including LAB, but the treatments containing the SB17 strain showed growth in both the mesophilic organisms and LAB count (T3 in both; T1 in LAB). It has been found that the natural flora and starter cultures do not present a uniform distribution but are immobilized in the cavities of the sausage mixture, which act as growth nests, multiplying over time [60]; likewise, emulsion offers protection to the inoculums when they are located in the center and thus allows them to survive the thermal process. Bacteria are also known to temporarily inactivate under unfavorable conditions (latency) [37,52,60,63], which may have occurred in the third measurement performed on day 18 in treatments T3 and T1. Meanwhile, the differences over time (between the first three measurements and the last two) in the mesophilic organism and LAB counts were similar to those found in Laszkiewicz et al., 2021 [52], in which microbiological growth is associated with the deterioration of the packaging and the contamination of the product after cooking.

Finally, the pH of the control and treatments showed a continuous decrease between evaluations over time, with no difference between treatments, which verifies the presence of microorganisms in the products. The T3 treatment, which was inoculated with the *Lb. plantarum* strain (SB17) at pH 5.5; Abhari et al., 2018 [62] point out that cooked products with pH values < 5.8 are expired or damaged, causing organoleptic changes that are not

to the consumer's taste. However, in fermented products, such pH levels are the optimum conditions for avoiding contamination by pathogenic or spoilage organisms [4,37,58].

5. Conclusions

The LAB isolated in this work expressed biopreservative characteristics such as pathogen inhibitory capacity and resistance to different pH, NaCl, nitrate, and nitrite salt concentrations, and temperatures.

It is concluded that the inhibitory activity of the 10 isolates was mainly due to the presence of organic acids, although the strain *Lactococcus lactis* (SB99) presented an inhibitory substance that could not be identified.

The addition of *Lb. plantarum* (SB17) and *Lb. sakei* (SB3) strains to the sausage had similar effects on microbiological control and pH, which is why it is necessary to measure a greater number of variables in the product.

6. Recommendations

For further studies, the performance of encapsulation tests with the isolates is recommended to achieve greater thermostability and guarantee the survival of the inocula.

An additional test of the sausages should be performed to measure variables such as color, texture, water activity, and relative humidity, which are necessary for quality-checking the product. Due to adverse conditions, it was not possible to perform these measurements in the present study.

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