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Inhibitory activity of an emulsifying salt polyphosphate (JOHA HBS®) used in processed cheese: An *in vitro* analysis of its antibacterial potential

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ABSTRACT

In the present study, the inhibitory activity of a commercial polyphosphate (JOHA® HBS) was tested *in vitro* against 21 bacterial strains. Firstly, the antimicrobial activity of JOHA® HBS, at different concentrations (from 0.5 to 2.0%) was evaluated in different agar and broth media (namely, Brain Heart Infusion: BHI; Nutrient: NT; Plate Count: PCA; Trypticase Soy: TSA/B), using streak assay (agar) and culture enumeration (broth). Furthermore, the bacterial inhibition of JOHA® HBS (at different concentrations, from 0.2% to 3.0%) was evaluated both on NT agar and broth medium, by streak assay, agar-spot method, and by spot-on-the-lawn and well-diffusion method. Finally, the JOHA® HBS antimicrobial activity was tested on NT agar at pH 6.3. Results of the streak assay on NT agar showed that 11 out the 21 tested strains were highly inhibited at 0.5% of JOHA® HBS. Interestingly, at adjusted pH levels, JOHA® HBS concentrations of 1.0% (w/v) were able to inhibit the same targets, confirming the antimicrobial effect of JOHA® HBS. This study reveals that NT medium and agar-based tests are required to properly test the inhibitory activity of polyphosphates. Furthermore, results confirmed the antimicrobial activity of JOHA® HBS, at low concentrations, against target bacteria of interest to the dairy industry.

1. Introduction

Emulsifying salts are of major importance to processed cheese production where they promote physico-chemical changes, such as the dispersion of proteins and lipids. Serious technological issues during homogenization of processed cheese mass would occur without the addition of emulsifying salts during processing (Buňka et al., 2014; Fox, Guinee, Cogan, & McSweeney, 2016; Vollmer, Kieferle, Youssef, & Kulozik, 2021). From a technological point of view, processed cheese is a dairy product produced by heating a mixture of natural cheeses, under partial vacuum conditions and constant stirring, in the presence of an emulsifying salt capable of chelating calcium; in order to disrupt the structural network and solubilize casein (Buňka et al., 2014; Kapoor & Metzger, 2008). The ability of emulsifying salts to sequester calcium phosphate is relevant to the characteristics of processed cheese, resulting in the conversion of insoluble calcium paracaseinate into soluble sodium paracaseinate (Hammam, Beckman, Sunkesula, & Metzger, 2022; Kapoor & Metzger, 2008; Salek et al., 2019).

In processed cheese production, monophosphates, polyphosphates and citrates are the emulsifying salts commonly used (Buňka, Černíková, & Salek, 2022). Sodium salts of polyphosphate, a long-chain phosphate, are a class of compounds derived from the condensation of phosphate ions, polyanions forming complexes with metal ions and with positively charged macromolecules, such as proteins (Buňka et al., 2022; Iammarino et al., 2020). These salts can inhibit microbial growth and, consequently, are relevant for assuring the safety of processed cheeses

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(Buňková & Buňka, 2017; Martinez-Rios, Jørgensen, Koukou, Gkogka, & Dalgaard, 2019). The antimicrobial activity of phosphates is mainly described against Gram-positive bacteria, and the inhibitory effect is positively related to chain length and level of condensation (Lee, Hartman, Stahr, Olson, & Williams, 1994; Buňková, Pleva, Buňka, Valášek, & Kráčmar, 2008; Ramos, Alves, Spadoti, Zacarchenco, & da Cruz, 2022).

Management strategies that carry out the microbiological control of processed cheeses are based on conservative agents, biopreservatives, control of extrinsic factors and predictive microbiology (Ramos et al., 2022). Therefore, *in vitro* and *in situ* studies have been carried out to assess the antimicrobial effects of commercial emulsifying salts; in order to determine their application as food additives in processed cheeses (Buňková et al., 2008; Lorencová, Vltavská, Budinský, & Koutný, 2012; Maier, Scherer, & Loessner, 1999; Martinez-Rios et al., 2019; Zaika, Scullen, & Fanelli, 1997). However, no standard method has been properly described for the antimicrobial evaluation of long-chain sodium phosphates, in which the polyphosphate condensation ratio can highly affect solubility (Buňka et al., 2022).

In this regard, the present study aims to evaluate the inhibitory activity of a commercial sodium polyphosphate, JOHA® HBS (ICL Food Specialties, Ladenburg, Germany), against a panel of 21 target bacteria using different culture media, polyphosphate concentration and *in vitro* tests, in order to select the best protocol.

2. Materials and methods

2.1. Bacterial strains and food additives

Twenty-one bacterial strains (n = 21) were included in the present study as targets: *Bacillus cereus* INV 10(3); *Bacillus subtilis* ATCC 19659; *Bacillus thuringiensis* CFBP 3476; *Enterococcus faecalis* FAIR-E 77, FAIR-E 179; *Escherichia coli* ATCC 25922; *Lactiplantibacillus plantarum* ATCC 8014; *Latilactobacillus sakei* ATCC 15521; *Listeria innocua* ATCC 33090; *Lactococcus lactis* subsp. *lactis* ATCC 13675, ATCC 19435; *Listeria monocytogenes* ATCC 7644, ATCC 19112, Scott A; *Pseudomonas fluorescens* ATCC 13525, 07A; *Salmonella enterica* subsp. *enterica* ATCC 13076; *Staphylococcus aureus* ATCC 6538, ATCC 14458, ATCC 43300; *Streptococcus salivarium* 20P3. The strains were stored at −80 °C in Brain Heart Infusion (BHI; Kasvi, São José dos Pinhais, PR, Brazil) broth or in Trypticase Soy broth (TSB; Kasvi), both supplemented with glycerol at 20% (v/v; Exôdo Cientifica, Sumaré, SP, Brazil). For microbiological assays, strains were inoculated in BHI, Nutrient (NT; Kasvi) broth or TSB, incubated overnight at 25, 30 or 37 °C and diluted in NaCl 0.85% (w/v) until a turbidity equivalent to 1 MacFarland Equivalence Turbidity Standard (ref. R20411; Remel™, Lenexa, KS, USA) was achieved; corresponding to approximately 3×10^8 colony forming units per mL (cfu/mL). The cultures were diluted in decimal scale to 3×10^6 cfu/mL, to obtain concentrations previously used by Buňková et al. (2008).

A commercially available blend, food grade, long-chain sodium polyphosphate (JOHA® HBS, poly- and sodium phosphates, E 452 and E 339; 69 ± 1% P_2O_5, white powder, ICL Food Specialties, Ladenburg, Germany) was used for microbiological assays. Nisin (Nisaplin®, 2.5% w/w nisin A, Danisco, Copenhagen, Denmark), a commercial antimicrobial agent commonly used in the production of processed cheese, was used as a control for the inhibitory assays.

2.2. Screening of culture media and plating protocols

Culture media and plating methods were assessed to detect the inhibitory activity of polyphosphate, and to select the best combination for further antimicrobial assays. A protocol based on culture streaking (streak assay) was performed on BHI agar, NT agar, Plate Count agar (PCA; Kasvi) and Trypticase Soy agar (TSA; Kasvi), supplemented with JOHA® HBS (at 0.5, 1.0 and 2.0%, w/v). Each agar medium (supplemented with agar at 1.5%, w/v) was prepared, according to the manufacturer's instructions, autoclaved at 121 °C for 15 min, cooled to 45 °C and then added with JOHA® HBS, at the indicated concentrations. This resulted in the following treatments (for each culture media): A1 (0.5% of HBS); A2 (1.0% of HBS); A3 (2.0% of HBS); and AC (no HBS: control). To simulate processed cheese manufacture (Kapoor & Metzger, 2008), the agar suspensions were homogenized then heated at 85 °C for 15 min in a water bath (MA156/6, Marconi, Piracicaba, SP, Brazil), and then poured into Petri dishes. Seven selected target strains (*E. faecalis* FAIR-E 179, *E. coli* ATCC 25922, *L. plantarum* ATCC 8014, *L. innocua* ATCC 33090, *L. monocytogenes* Scott A, *S. aureus* ATCC 6538, and *S. salivarium* 20P), at a final concentration of 3×10^6 cfu/mL, were then streaked and plates incubated at 37 °C for 48 h; sterile water was used as negative control. Absence of bacterial growth indicated the inhibitory activity of the combined culture medium and JOHA® HBS. The assay was conducted in duplicate and in three independent repetitions.

An additional inhibitory assay, based on broth culturing and then plating (broth assay) was also assessed. Three of the target strains (*E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A, and *S. aureus* ATCC 6538) were inoculated into BHI, NT, TSB and a formulated PCA (no agar added) supplemented with JOHA® HBS (at 0.5, 1.0 and 2.0%, w/v), resulting in the following treatments for each culture medium: A1 (0.5% of HBS); A2 (1.0% of HBS); A3 (2.0% of HBS); and AC (no HBS: control). Inoculated tubes were incubated at 37 °C for 24 h, ten-fold diluted in NaCl 0.85% (w/v; Exôdo Cientifica) and drop plated in PCA, and incubated at 37 °C for 48 h. Growth colonies were counted and results expressed as log cfu/mL. This assay was conducted in duplicate and in three independent repetitions.

2.3. Determination of JOHA® HBS inhibitory activity

Based on the previous results, NT was selected as the culture medium for the evaluation of JOHA® HBS inhibitory activity. For this, all 21 selected strains were considered.

The streak assay, as described above, was designed considering NT as the culture medium, supplemented with different concentrations of JOHA® HBS; from 0.2 to 3.0% (w/v), resulting in 8 treatments: T1 (0.2%); T2 (0.4%); T3 (0.6%); T4 (0.8%); T5 (1.0%); T6 (1.5%); T7 (2.0%); T8 (3.0%). In addition, NT supplemented with commercial nisin (Nisaplin®) at 0.0012% (w/v) (Brasil, 1996) and NT (without HBS or nisin) were included as positive (TNis) and negative controls (target growth control, TG), respectively. The same procedures described above were carried out for the 21 selected target strains. This assay was conducted in duplicate and in three independent repetitions.

JOHA® HBS inhibitory activity was also assessed utilizing three diffusion assay-based protocols: spot-on-the-lawn (adapted from Lewus & Montville, 1991), agar-spot (adapted from Moon, Park, & Lee, 2011) and well-diffusion (adapted from Lewus & Montville, 1991). For these assays, JOHA® HBS and nisin were added to sterile distilled water, heated at 85 °C for 15 min, cooled to room temperature, and diluted up to the previously described concentrations (JOHA® HBS from 0.2 to 3.0%, w/v, and nisin at 0.0012%, w/v); sterile distilled water was used as negative control.

For the spot-on-the-lawn assay, 20 μL aliquots of JOHA® HBS prepared solutions and controls were spotted onto plates containing 15 mL of semi-solid NT (0.8 g/100 mL of bacteriologic agar). After absorption (4 °C for 2 h), plates were covered with an overlay of semi-solid NT (8 mL, 0.8 g/100 mL of bacteriologic agar) inoculated with 10^6 cfu/mL of each target strain (n = 21) and incubated at 25, 30 or 37 °C for 48 h. Inhibition zones with diameters higher than 1 mm were considered as indicative of inhibitory activity (Lewus & Montville, 1991).

For the agar-spot assay, JOHA® HBS and controls were added to semi-solid NT (0.8 g/100 mL of bacteriologic agar) at the above reported concentrations, treated at 85 °C for 15 min, and poured into Petri dishes. The plates were spotted with 10 μL of each target bacterial suspension (n = 21) at 3×10^6 cfu/mL and incubated at 25, 30 or 37 °C for 48 h. The absence of microbial growth was considered as indicative of inhibitory activity (Moon et al., 2011).

For the well-diffusion assay, plates containing 20 mL of semi-solid NT (0.8 g/100 mL of bacteriologic agar) previously inoculated with 3 × 10^6 cfu/mL of each target strain (n = 21) were prepared. Wells with a diameter of 3 mm were cut on the plates, and 50 μL aliquots of JOHA® HBS and controls solutions were used at the described concentrations. After absorption (4 °C for 2 h), the plates were incubated at 25, 30 or 37 °C for 48 h. Inhibition zones with diameters higher than 1 mm were considered as indicative of inhibitory activity (Lewus & Montville, 1991).

Finally, the selected strains (n = 21) were subjected to a broth assay to measure the effects of JOHA® HBS on them at specific conditions. Bacterial cultures were diluted up to approximately 3 × 10^6 cfu/mL, ten-fold diluted in NaCl 0.85% (w/v) and drop-plated into plates containing NT supplemented with agar (1.5%, w/v) and JOHA® HBS at different concentrations, as indicated above (from 0.2 to 3.0%, w/v); nisin (0.0012%, w/v) and sterile distilled water as positive and negative control, respectively. Agar NT supplemented with JOHA® HBS and controls were prepared and pH was corrected to 6.3 with 1M NaOH (Exôdo Cientifica). Plates were incubated at 25, 30 or 37 °C for 24 h (based on selected strain), and growth colonies enumerated. Results were expressed as log cfu/mL.

2.4. Statistical analysis

Counts obtained in broth assay for the screening of culture media were compared based on treatments through ANOVA and Tukey's honest significant difference (HSD) test ($p < 0.05$). Counts obtained in broth assay for the determination of JOHA® HBS inhibitory activity were subjected to Normality tests (Shapiro-Wilk, Anderson-Darling, Lilliefors and Jarque-Bera) and then to a nonparametric comparison based on Kruskal-Wallis and Dunn, after Bonferroni correction ($p < 0.005$). All statistics were conducted using the software XLSTAT software (Addinsoft, New York, NY, USA).

3. Results and discussion

Although heat treatment is commonly applied (at temperature between 75 °C and 95 °C for standard processes, and from 110 °C to 140 °C for UHT processes) in processed cheese manufacture (Fox et al., 2016; Kapoor & Metzger, 2008), microbial contamination can still occur, resulting in faster product deterioration, health risks for consumers and shortened shelf life (Ramos et al., 2022). Therefore, the use of emulsifying salts, such as polyphosphates, with antimicrobial activity represents a convenient strategy in processed cheese manufacturing. Thus, the first aim of the present study was to assess the inhibitory effect of the commercial polyphosphate salt, JOHA® HBS, in different culture media and through different assays against a panel of selected target strains.

Overall, when JOHA® HBS was added to BHI and TSA medium, lower bacterial inhibition was recorded, compared to results obtained from NT or PCA through the streak assay protocol (Table 1 and Fig. 1). Although sensitive strains, such as *L. plantarum* ATCC 8014 and *S. aureus* ATCC 6538, were inhibited under all evaluated conditions; *E. faecalis* FAIR-E 179, *E. coli* ATCC 25922, and *S. salivarium* 20P3 were inhibited exclusively by NT and PCA medium at the highest tested salt concentration (as 2.0% w/v), as shown in Table 1. Focusing on medium composition for all tested conditions and for all tested strains, it is interesting to note that results were comparable between BHI and TSA, and between NT and PCA; except for *L. innocua* ATCC 33090 and *L. monocytogenes* Scott A strains, for which a lower minimum salt concentration was determined in NT (1.0% and 0.5% w/v, respectively) when compared to the 2.0% and 1.0% w/v in PCA, respectively (Table 1 and Fig. 1).

Results of the enumeration assay, performed for *E. faecalis* FAIR-E 179, *L. monocytogenes* Scott A and *S. aureus* ATCC 6538, were in accordance with those obtained by streak assay, highlighting high levels of inhibition in PCA (formulated) and in NT broth, as reported in Table 2. For *E. faecalis* FAIR-E 179, the different concentrations of JOHA® HBS significantly affected the growth counts in both PCA and NT media, whereas no significant differences were observed among all tested concentrations in BHI and TSB media ($p > 0.05$). This highlights that the polyphosphate did not affect the growth of this strain. It is also interesting to note that no significant differences between the control (0%) and JOHA® HBS at 0.5% (w/v) were observed for all tested culture media and that the lowest *E. faecalis* FAIR-E 179 cell density was detected in PCA with the highest salt concentration (2% w/v); as shown in Table 2. Focusing on L. *monocytogenes* Scott A, the data indicates that the salt inhibited bacterial growth, with the lowest viable counts detected for all tested media, at a concentration of 2% (w/v).

Table 1
Evaluation of the influence of the culture medium in the inhibitory activity of JOHA® HBS polyphosphate against selected target strains (n = 7).

Agar medium treatment	*E. faecalis* FAIR-E 179	*E. coli* ATCC 25922	*L. plantarum* ATCC 8014	*L. innocua* ATCC 33090	*L. monocytogenes* Scott A	*S. aureus* ATCC 6538	*S. salivarium* 20P3
BHI							
AC, control, 0% HBS	-	-	-	-	-	-	-
A1, 0.5% HBS	-	-	+	-	-	+	-
A2, 1% HBS	-	-	+	-	-	+	-
A3, 2% HBS	-	-	+	-	+	+	-
NT							
AC, control, 0% HBS	-	-	-	-	-	-	-
A1, 0.5% HBS	-	-	+	-	+	+	-
A2, 1% HBS	-	-	+	+	+	+	-
A3, 2% HBS	+	+	+	+	+	+	+
PCA							
AC, control, 0% HBS	-	-	-	-	-	-	-
A1, 0.5% HBS	-	-	+	-	-	+	-
A2, 1% HBS	-	-	+	-	+	+	-
A3, 2% HBS	+	+	+	+	+	+	+
TSA							
AC, control, 0% HBS	-	-	-	-	-	-	-
A1, 0.5% HBS	-	-	+	-	-	+	-
A2, 1% HBS	-	-	+	-	-	+	-
A3, 2% HBS	-	-	+	-	-	+	-

Results: (+) presence of inhibitory activity without bacterial growth; (−) absence of inhibitory activity with bacterial growth.

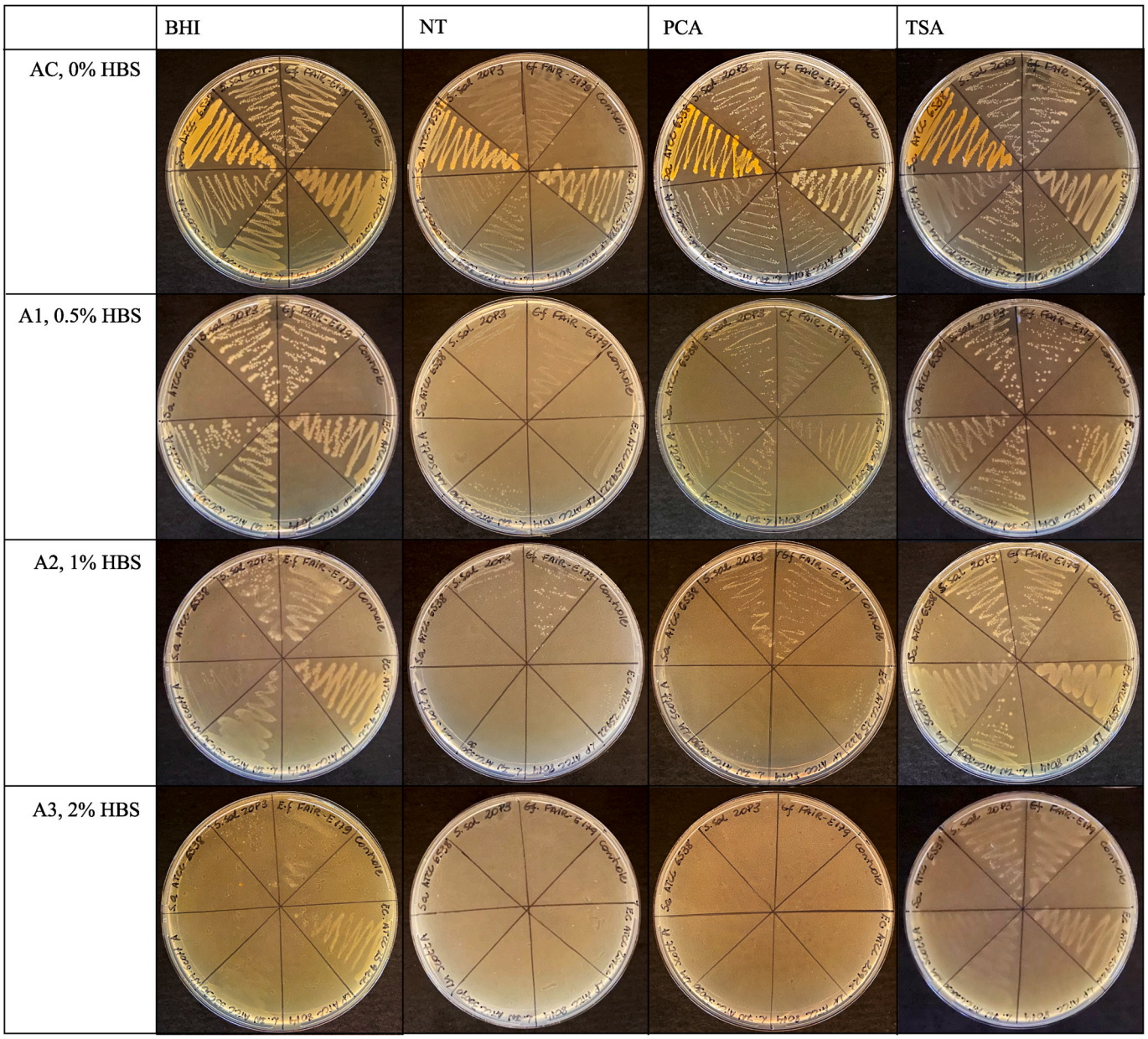

Fig. 1. Comparison of the inhibitory activity of JOHA® HBS added to four different culture media through the streak assay against selected strains (n = 7).

Furthermore, the data highlighted that in BHI medium, a significant difference was observed only at the 2% (w/v) salt concentration. Among the tested culture media, a significant difference was detected for NT medium, where the lowest cell densities were revealed for all tested salt concentrations (Table 2). These results support that *L. monocytogenes* Scott A growth is influenced by both culture medium and JOHA® HBS addition. Results from *S. aureus* ATCC 6538 confirmed that the presence of JOHA® HBS influenced growth, with the highest inhibitory activity detected at the highest salt concentration (2.0% w/v); whereas at 0.5% and 1.0% (w/v) concentrations, no significant differences were revealed for any tested media ($p > 0.05$). In addition, no differences in any media, between the control and the lowest salt concentration (0.5% w/v), was observed; however, at 1.0% (w/v) salt concentrations, differences among media were revealed, with the highest cell densities detected in PCA (Table 2). At 2% (w/v) salt concentration, viable counts were significantly different only in BHI, where the highest count of *S. aureus* ATCC 6538 was enumerated (Table 2). Based on the enumeration assay (in broth), it was confirmed that *E. faecalis* FAIR-E 179 and *S. aureus* ATCC 6538 were not inhibited by the highest concentration of JOHA® HBS (2% w/v); whereas *L. monocytogenes* Scott A was completely inhibited in NT broth, starting from 1.0% (w/v) of JOHA® HBS. Finally, comparing the two protocols, the agar streak assay revealed the highest inhibitory activity.

According to López-Malo, Mani-López, Davidson, and Palou (2020), several factors can influence antimicrobial tests, such as interaction between the antimicrobial compounds, pH and components present in medium substrate. Furthermore, combinations of different effects can cause an apparent increase or decrease of susceptibility, and the inhibitory activity of polyphosphates can be reduced by the presence of multivalent metal ions in culture medium (Zaika et al., 1997; Lee, Hartman, Stahr, et al., 1994; Maier et al., 1999; Buňková et al., 2008). Another important point concerns the heat treatment to which the polyphosphates underwent with the possibility of hydrolysis and loss of antimicrobial potential. JOHA® HBS is a long-chain phosphate and susceptible to hydrolysis. However, hydrolysis of polyphosphates is negligible in water at neutral or slightly acidic pH (5.6) at temperatures

Table 2
Evaluation of the inhibitory activity of JOHA® HBS added to different culture media against selected bacterial targets (n = 3).

Target strain and culture medium	Treatment			
	A1, 0.5% HBS	A2, 1% HBS	A3, 2% HBS	AC, 0% HBS
E. faecalis FAIR-E 179				
BHI	$8.73 \pm 0.014^{bc, A}$	$8.69 \pm 0.28^{b, A}$	$8.86 \pm 0.021^{b, A}$	$8.71 \pm 0.12^{ab, A}$
NT	$8.18 \pm 0.19^{ab, A}$	$6.77 \pm 0.10^{a, B}$	$5.46 \pm 0.042^{a, C}$	$8.11 \pm 0.035^{a, A}$
PCA	$7.96 \pm 0.24^{a, A}$	$6.48 \pm 0.011^{a, B}$	$5.03 \pm 0.05^{a, C}$	$8.13 \pm 0.25^{a, A}$
TSB	$8.89 \pm 0.12^{c, A}$	$9.07 \pm 0.28^{b, A}$	$8.97 \pm 0.12^{b, A}$	$8.97 \pm 0.28^{b, A}$
L. monocytogenes Scott A				
BHI	$9.05 \pm 0.020^{c, A}$	$9.02 \pm 0.065^{c, A}$	$4.71 \pm 0.12^{c, B}$	$9.39 \pm 0.22^{b, A}$
NT	$4.84 \pm 0.011^{b, B}$	N.D.	N.D.	$8.55 \pm 0.010^{a, A}$
PCA	$6.88 \pm 0.091^{a, B}$	$3.91 \pm 0.048^{a, C}$	$3.46 \pm 0.070^{a, D}$	$8.51 \pm 0.14^{a, A}$
TSB	$8.6 \pm 0.058^{d, A}$	$6.41 \pm 0.12^{d, B}$	$5.94 \pm 0.089^{d, C}$	$8.97 \pm 0.15^{c, A}$
S. aureus ATCC 6538				
BHI	$5.08 \pm 0.18^{a, B}$	$5.05 \pm 0.021^{bc, B}$	$5.13 \pm 0.085^{b, B}$	$9.37 \pm 0.043^{a, A}$
NT	$4.99 \pm 0.20^{a, B}$	$4.78 \pm 0.26^{ab, B}$	$4.02 \pm 0.14^{a, C}$	$8.96 \pm 0.12^{a, A}$
PCA	$5.06 \pm 0.11^{a, B}$	$5.4 \pm 0.028^{c, B}$	$4.05 \pm 0.22^{a, C}$	$9.25 \pm 0.072^{a, A}$
TSB	$4.89 \pm 0.06^{a, B}$	$4.42 \pm 0.053^{a, BC}$	$4.27 \pm 0.045^{a, C}$	$9.01 \pm 0.16^{a, A}$

N.D: data not definable, below the detection limit of the method. Capital letters: comparison between JOHA® HBS percentages. Lowercase: comparison between the culture medium tested.

below 100 °C and only in the presence of calcium and temperatures above 120 °C its composition is significantly affected (Rulliere, Perenes, Senocq, Dodi, & Marchesseau, 2012).

Results of the qualitative evaluation of inhibitory effects of JOHA® HBS at the ten different concentrations (from 0.2 to 3.0% w/v) against the 21 selected target bacteria, through streak assay, spot-on-the-lawn, agar spot and well-diffusion assays, are shown in Table 3. In detail, the inhibitory effect of spot-on-the lawn assay was detected only for the two strains of *L. lactis* subsp. *lactis*, only when 3% (w/v) of JOHA® HBS was considered. The well-diffusion assay inhibitory effects were detected for the two *S. aureus* strains, starting from 0.8% (w/v) of salt concentration (Table 3). However, for *L. plantarum* ATCC 8014, *L. monocytogenes* Scott A, and *S. aureus* ATCC 43300 the inhibitory effect was revealed starting from 1.0% (w/v); and for *B. cereus* INV 10(3), *B. subtilis* ATCC 19659, *B. thuringiensis* CFBP 3476, *L. sakei* ATCC 15521, *L. lactis* subsp. *lactis* ATCC 13675, *L. monocytogenes* ATCC 7644 and ATCC 19112 starting from 1.5% (w/v) salt concentration (Table 3). It should be noted that in the well-diffusion assay, a higher volume of treatment solution was used (50 μL), which can have an effect, increasing the sensitivity of the assay. Overall, the inhibition activity was higher in assays performed in agar culture medium, confirming the results obtained in the first step. In spot-on-the-lawn and well-diffusion assays, performed in aqueous solutions, the lower inhibitory rate observed can be related to the low solubility of polyphosphates. These protocols, based on the diffusion of antimicrobial substances in culture media (Moraes et al., 2010), are commonly used for the preliminary screening of bacteriogenic strains for assessing the inhibition spectrum of bacteriocins, medicinal herbal extracts, and plant extracts (Balouiri, Sadiki, & Ibnsouda, 2016; Moraes et al., 2010; Ullah et al., 2016). Although the two assays failed in revealing the inhibitory effect of JOHA® HBS, the well-diffusion assay proved to be more effective than the spot-on-the-lawn assay.

In the present work, streak assay and agar spot assays showed better efficacy in revealing inhibitory effects and the two methods gave similar results (Table 3). By these assays, inhibition was confirmed in at least two treatments (at 2.0 and 3.0% w/v of salt) against all 21 bacterial targets (Table 3). Low concentrations of JOHA® HBS, up to 0.4% (w/v), were able to inhibit 11 out of the 21 targets, namely *B. cereus* INV 10(3), *B. thuringiensis* CFBP 3476, *L. plantarum* ATCC 8014, *L. sakei* ATCC 15521, *L. lactis* subsp. lactis ATCC 13675 and ATCC 19435, *L. monocytogenes* ATCC 19112 and Scott A, *S. aureus* ATCC 6538, ATCC 14458 and ATCC 43300 (Table 3). These results corroborate with those reported by Buňková et al. (2008), who performed an *in vitro* study on the antimicrobial effects of three commercial phosphates (JOHA® HBS, JOHA® S9 and CFB 690) against reference microorganisms and bacteria isolated from long-stored processed cheeses. In this study, Buňková et al. (2008) reported the positive inhibitory activity of JOHA® HBS at 0.3% (w/v) against *B. cereus* CCM 3953, *B. subtilis* CCM 2216 and *S. aureus* CCM 3953; at 0.5% of JOHA® HBS (maximum tested concentration); no inhibitory activity was verified against *E. coli* CCM 180.

Although the inhibitory effects of phosphates on Gram-negative bacteria are rarely reported (Buňková et al., 2008; Buňková & Buňka, 2017); in the present work, JOHA® HBS was found to inhibit, even if at higher tested concentrations, *P. fluorescens* ATCC 13525 and 07A, *E. coli* ATCC 25922, and *S. enterica* subsp. *enterica* ATCC 13076 (at 1.0, 1.5 and 2.0% w/v, respectively; Table 3). Compared to Gram-negative, Gram--positive bacteria highlighted higher sensitivity to polyphosphates and these results could be related to the cell-wall structure and, in particular, to the presence of teichoic acids (Buňková et al., 2008; Wang et al., 2021). Among the Gram-positive target strains, *S. salivarium* 20P3, *E. faecalis* FAIR-E 77, and *E. faecalis* FAIR-E 179 were inhibited at higher concentrations (at 1.5, 1.5 and 2.0% w/v, respectively; Table 3). Lorencová et al. (2012) carried out a study on the antibacterial effect of seven phosphates at different chain lengths (different phosphates compared to those used in this study) and less effective at the suppression of bacterial growth than salts obtained from JOHA® HBS. Against *E. faecalis* CCM 4224, a positive inhibition at 2% (w/v) of HEXA68 polyphosphate and >2% (w/v) of HEXA70 polyphosphate was reported. However, a minimum concentration of 2% (w/v) of HEXA68 and HEXA70 polyphosphate was required to inhibit *B. subtilis* subsp. *subtilis* CCM 2216 and *E. coli* CCM 3954 (Lorencová et al., 2012); while a lower concentration of JOHA® HBS inhibited *B. subtilis* ATCC 19659 (0.6%; Table 3) and *E. coli* ATCC 25922 (1.5% w/v; Table 3).

These differences might be related to the divergence in the structure of polyphosphates, as the inhibitory effect of phosphates is dependent on the length of their chains. Long-chain phosphates, as in the case of JOHA® HBS, revealed a higher inhibitory effect compared to short-chain phosphates (Buňková & Buňka, 2017; Lee, Hartman, Olson, & Williams, 1994; Lee, Hartman, Stahr, et al., 1994). In the study performed by Lorencová et al. (2012) with phosphates at different chain lengths, the authors pointed out that the inhibitory effect can be affected by intrinsic factors, such as number of phosphorus atoms and acid basic properties of phosphates in aqueous solutions. In addition, the inhibitory mechanisms of long-chain phosphates are mainly based on the chelation of divalent metal ions (Ca^{2+} and Mg^{2+}), which are involved in the transverse bridges among teichoic acids in the Gram-positive cell wall (Lee, Hartman, Olson, & Williams, 1994; Lee, Hartman, Stahr, et al., 1994). Chelation of divalent ions can also affect some essential physiological growth processes (Maier et al., 1999).

Comparing the effect of JOHA® HBS with those of commercial nisin (Table 3), JOHA® HBS was more effective against Gram-positive bacteria. As expected, nisin did not inhibit Gram-negative bacteria, where their outer membrane acts as a protective barrier against nisin (Małaczewska & Kaczorek-Łukowska, 2021). Nisin is a natural antimicrobial peptide produced by *L. lactis* subs. *lactis*, has been approved for use in over 50 countries and was granted generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA) in 1988 (Ibarra-Sánchez, El-Haddad, Mahmoud, Miller, & Karam, 2020). In our tests,

Table 3
Evaluation of the inhibitory activity of JOHA® HBS against twenty-one target strains (n = 23) through four different qualitative protocols.

Target strains and protocols	Treatments									
	T1	T2	T3	T4	T5	T6	T7	T8	TNis	TG
B. cereus INV 10(3)										
streak assay	+	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	+	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
B. subtilis ATCC 19659										
streak assay	-	-	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
B. thuringiensis CFBP 3476										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
E. faecalis FAIR-E 77										
streak assay	-	-	-	-	-	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-
E. faecalis FAIR-E 179										
streak assay	-	-	-	-	-	-	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	-	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-
E. coli ATCC 25922										
streak assay	-	-	-	-	-	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-
L. plantarum ATCC 8014										
streak assay	-	+	+	+	+	+	+	+	+	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	+	-
well-diffusion assay	-	-	-	-	+	+	+	+	+	-
L. sakei ATCC 15521										
streak assay	-	+	+	+	+	+	+	+	+	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	+	-
well-diffusion assay	-	-	-	-	-	+	+	+	+	-
L. innocua ATCC 33090										
streak assay	-	-	-	-	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	+	+	-	-
L. lactis subsp. *lactis* ATCC 13675										
streak assay	+	+	+	+	+	+	+	+	+	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	+	+	-
agar spot assay	+	+	+	+	+	+	+	+	+	-
well-diffusion assay	-	-	-	-	-	+	+	+	+	-
L. lactis subsp. *lactis* ATCC 19435										
streak assay	-	+	+	+	+	+	+	+	+	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	+	+	-
agar spot assay	-	+	+	+	+	+	+	+	+	-
well-diffusion assay	-	-	-	-	-	+	+	+	+	-
L. monocytogenes ATCC 7644										
streak assay	-	-	-	-	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	+	-
L. monocytogenes ATCC 19112										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
L. monocytogenes Scott A										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	+	+	+	+	+	-
P. fluorescens ATCC 13525										
streak assay	-	-	-	-	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-

(*continued on next page*)

Table 3 (*continued*)

Target strains and protocols	Treatments									
	T1	T2	T3	T4	T5	T6	T7	T8	TNis	TG
agar spot assay	-	-	-	-	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	+	+	-	-
P. fluorescens 07A										
streak assay	-	-	-	-	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	+	+	+	-	-
S. enterica subsp. *enterica* ATCC 13076										
streak assay	-	-	-	-	-	-	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	-	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-
S. aureus ATCC 6538										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	+	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	+	+	+	+	+	+	-
S. aureus ATCC 14458										
streak assay	-	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	+	-
agar spot assay	-	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	+	+	+	+	+	+	-
S. aureus ATCC 43300										
streak assay	+	+	+	+	+	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	+	-
agar spot assay	+	+	+	+	+	+	+	+	-	-
well-diffusion assay	-	-	-	-	+	+	+	+	+	-
S. salivarium 20P3										
streak assay	-	-	-	-	-	+	+	+	-	-
spot-on-the-lawn assay	-	-	-	-	-	-	-	-	-	-
agar spot assay	-	-	-	-	-	+	+	+	-	-
well-diffusion assay	-	-	-	-	-	-	-	-	-	-

Treatments: (T1) 0.2% of HBS; (T2) 0.4% of HBS; (T3) 0.6% of HBS; (T4) 0.8% of HBS; (T5) 1% of HBS; (T6) 1.5% of HBS; (T7) 2% of HBS; (T8) 3% of HBS; (TNis) 0.0012% of nisin, and (TG) without HBS and nisin. Streak assay and agar spot assay: (+) presence of inhibitory activity without bacterial growth; (−) absence of inhibitory activity with bacterial growth. Spot-on-the-lawn assay and well-diffusion assay: (+) presence of inhibitory activity with inhibition zones; (−) absence of inhibitory activity without inhibition zones.

we used the maximum allowable concentration of nisin in processed cheese. According to the Codex Committee on milk and milk products from Food and Agriculture Organization and World Health Organization (FAO/WHO), nisin can be used as a food additive for processed cheese at a concentration of 12.5 mg/kg product (Ibarra-Sánchez et al., 2020). In Brazil, nisin is used as a preservative in the manufacture of pasteurized cheese, processed cheese and "requeijão", with a maximum limit of 12.5 mg/kg (Brasil, 1996).

Results obtained in broth assay on NT agar with different concentrations of JOHA® HBS (from 0.2 to 3.0% w/v), and with adjusted pH against the bacterial panel (n = 21) are shown in Fig. 2. In detail, prior to adjustment, the pH values of the substrates were: T1: 6.05 (0.2% of HBS); T2: 5.51 (0.4% of HBS); T3: 5.49 (0.6% of HBS); T4: 5.26 (0.8% of HBS); T5: 5.18 (1.0% of HBS); T6: 4.87 (1.5% of HBS); T7: 4.53 (2.0% of HBS); T8: 4.04 (3.0% of HBS); TNis: 6.68 (0.0012% of nisin); and TG: 6.67 (without HBS and nisin). Counts of the selected strains did not follow a normal distribution ($p < 0.005$ in Normality tests), leading to a nonparametric analysis based on Kruskal-Wallis and Dunn. Results indicate that *L. plantarum* ATCC 8014 and *L. lactis* subsp. *lactis* ATCC 19435, highlighted similar inhibitory profiles in both conditions tested; the same occurred for *L. lactis* subsp. *lactis* ATCC 13675 and *S. aureus* ATCC 43300 in treatments with the addition of JOHA® HBS (Fig. 2). The inhibitory activity of JOHA® HBS at the adjusted pH (6.3) was revealed at a higher concentration when compared to treatments without pH adjustment; for *B. cereus* INV 10(3), *B. thuringiensis* CFBP 3476, *L. sakei* ATCC 15521, *L. monocytogenes* ATCC 19112, Scott A, and *S. aureus* ATCC 14458. At high pH values, a higher dissociation of the phosphate molecules provides better ion exchange, corroborating the hypothesis that inhibitory effects may be caused by a combination of the pH value of the growth medium (by the tolerance of the tested targets to neutral pH) and the sequestration effect (Lorencová et al., 2012). Moreover, data regarding the enumeration assay confirmed the results (Fig. 2) obtained in the qualitative assay (Table 3) for bacterial targets that have shown greater resistance to JOHA® HBS; highlighting that these bacteria were mostly influenced by the pH adjustment. In particular, concentrations above 1% (w/v) of JOHA® HBS at pH 6.3 were not able to inhibit *E. faecalis* FAIR-E 77, FAIR-E 179, *E. coli* ATCC 25922, *L. innocua* ATCC 33090, *L. monocytogenes* ATCC 7644, *P. fluorescens* ATCC 13525, 07A, *S. enterica* subsp. *enterica* ATCC 13076, or *S. salivarium* 20P3. This result could be related to the fact that most bacteria grow better at neutral pH values and only a few species are able to grow at values below 4.0; where pH influences the microbial respiratory enzymes, disturbing the transport of nutrients into the cell (Buňková & Buňka, 2017). Treatments without pH adjustment showed high levels of inhibition. Therefore, for some targets, inhibition occurs in relation to pH. On the other hand, for some targets such *B. subtilis* ATCC 19659, *L. plantarum* ATCC 8014, *L. lactis* subsp. *lactis* ATCC 13675, *L. lactis* subsp. *lactis* ATCC 19435, *S. aureus* ATCC 6538 and *S. aureus* ATCC 43300, pH adjustment did not influence inhibitory activity, confirming the antimicrobial potential of JOHA® HBS. In addition, the results of quantitative protocol are similar to the results of qualitative agar protocols (streak assay and agar spot; Table 3). In addition to pH value of the environment (higher sensitivity at pH > 7.4), the antimicrobial effect is known to be influenced by temperature, initial microbial population or addition of metal ions (Buňková & Buňka, 2017; Lee, Hartman, Olson, & Williams, 1994; Lorencová et al., 2012; Maier et al., 1999; Zaika et al., 1997).

Simultaneously, it could be mentioned that the pH of processed cheese ranges between 5.6 and 6.0, and these values are required for protein configuration, solubility, and the extent to which emulsifying salts bind Ca^{2+} (Buňka et al., 2022; Fox et al., 2016). The effect of adding

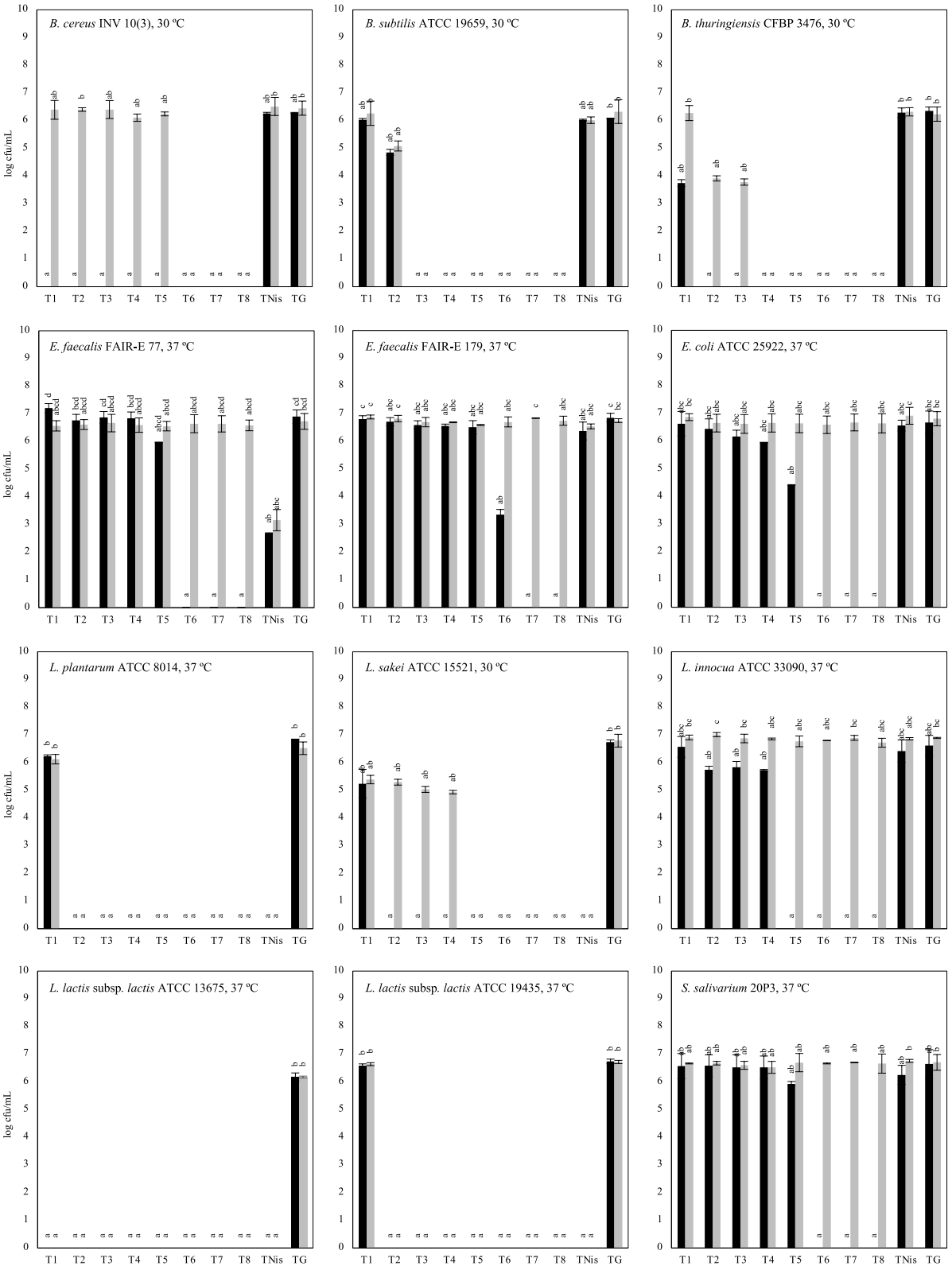

Fig. 2. Mean counts (standard errors) of the selected target strains subjected to a broth assay to measure the effects of JOHA® HBS at specific conditions. Black bars: mean counts without pH correction; gray bars: mean counts with pH correction to 6.3. Treatments: (T1) 0.2% of HBS; (T2) 0.4% of HBS; (T3) 0.6% of HBS; (T4) 0.8% of HBS; (T5) 1% of HBS; (T6) 1.5% of HBS; (T7) 2% of HBS; (T8) 3% of HBS; (TNis) 0.0012% of nisin; and (TG) without HBS and nisin. Incubation temperatures are indicated in each graph, per selected strain. Different letters indicate significant differences of counts distribution per selected strain and treatment, based on Kruskal-Wallis and Dun ($p < 0.05$); mean counts are presented only for illustrative purposes.

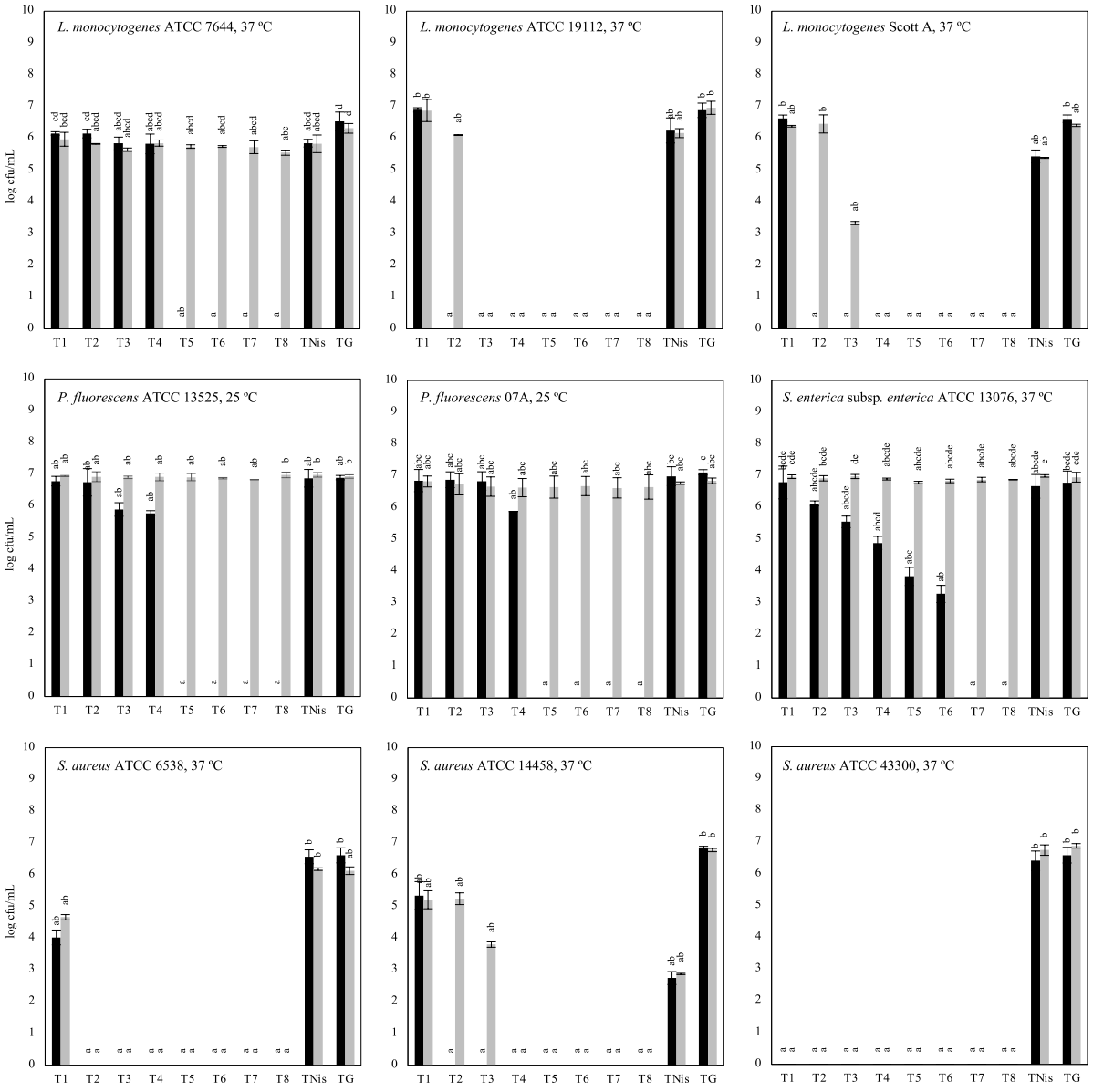

Fig. 2. (*continued*).

emulsifying salts is mainly related to operational conditions at which they can cause a change in pH or ionic strength of the solution (Buňková & Buňka, 2017). Therefore, further research will be carried out *in vitro*, using processed cheese at the corresponding technological conditions.

4. Conclusions

Based on the obtained results, it is possible to rapidly and effectively assess an *in vitro* protocol for screening the inhibitory activity of emulsifying salts with low solubility, such as JOHA® HBS. The obtained concentrations for bacterial inhibition are determinant for future research on *in situ* applications of polyphosphates in processed cheese manufacturing. Processed cheese formulation is critical to the product's stability during its shelf life, and the use of predictive microbiology methods is important for the development of shelf-stable formulations. In this sense, the success of formulations in processed cheese products could be obtained with the addition of emulsifying salts with antimicrobial activity. Accordingly, knowledge of the percentages of polyphosphates to access bacterial inhibition through *in vitro* protocols seems to be possible and promising. Therefore, the results of the present study will allow large-scale analyses on the application of emulsifying salts, which could become an indispensable food additive in the cheese industry or for other food sectors.

CRediT authorship contribution statement

Andressa Fusieger: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Raiane Rodrigues da Silva:** Validation, Formal analysis, Investigation, Visualization. **Sidney Rodrigues de Jesus Silva:** Validation, Formal analysis, Investigation, Visualization. **Jaqueline Aparecida Honorato:** Validation, Formal analysis, Investigation. **Camila Gonçalves Teixeira:** Validation, Formal analysis, Investigation, Visualization. **Luana Virgínia Souza:** Validation, Investigation. **Isabela Natali Silva Magalhães:** Validation, Investigation. **Nayara Aparecida da Silva Costa:** Validation, Investigation. **Alfredo Walter:** Conceptualization, Resources. **Luís Augusto Nero:** Conceptualization, Writing – review &

editing, Visualization, Supervision. **Cinzia Caggia:** Conceptualization, Writing – review & editing, Visualization. **Antonio Fernandes de Carvalho:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, All authors read and approved the final manuscript.

Declaration of competing interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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References

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis, 6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Brasil, Ministério da Agricultura, & e Abastecimento, Pecuária (1996). *Portaria n° 146, de 07 de março de 1996. Aprova os Regulamentos Técnicos de Identidade e Qualidade de Produtos Lácteos. Diário Oficial Da União, 11 de março*. Brasília.

Buňka, F., Černíková, M., & Salek, R. N. (2022). *Chapter 6 - Functionality of salts used in processed cheese manufacture*. Processed Cheese Science and Technology. In M. El-Bakry, & B. M. Mehta (Eds.). Sawston, United Kingdom: Woodhead Publishing. https://doi.org/10.1016/B978-0-12-821445-9.00011-X.

Buňka, F., Doudová, L., Weiserová, E., Černíková, M., Kuchař, D., Slavíková, Š., et al. (2014). The effect of concentration and composition of ternary emulsifying salts on the textural properties of processed cheese spreads. *LWT - Food Science and Technology, 58*(1), 247–255. https://doi.org/10.1016/j.lwt.2014.02.040

Buňková, L., & Buňka, F. (2017). Microflora of processed cheese and the factors affecting it. *Critical Reviews in Food Science and Nutrition, 57*(11), 2392–2403. https://doi.org/10.1080/10408398.2015.1060939

Buňková, L., Pleva, P., Buňka, F., Valášek, P., & Kráčmar, S. (2008). Antibacterial effects of commercially available phosphates on selected microorganisms. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 56*(5), 19–24. https://doi.org/10.11118/actaun200856050019

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2016). *Processed cheese and substitute/imitation cheese products*. Fundamentals of Cheese Science. In P. F. Fox, T. P. Guinee, T. M. Cogan, & P. L. H. McSweeney (Eds.). Boston, MA, USA: Springer. https://doi.org/10.1007/978-1-4899-7681-9_17.

Hammam, A. R. A., Beckman, S. L., Sunkesula, V., & Metzger, L. E. (2022). Highly concentrated micellar casein: Impact of its storage stability on the functional characteristics of process cheese products. *LWT - Food Science and Technology, 161*, Article 113384. https://doi.org/10.1016/j.lwt.2022.113384

Iammarino, M., Haouet, N., Di Taranto, A., Berardi, G., Benedetti, F., Di Bella, S., et al. (2020). The analytical determination of polyphosphates in food: A point-to-point comparison between direct ion chromatography and indirect photometry. *Food Chemistry, 325*, Article 126937. https://doi.org/10.1016/j.foodchem.2020.126937

Ibarra-Sánchez, L. A., El-Haddad, N., Mahmoud, D., Miller, M. J., & Karam, L. (2020). Invited review: Advances in nisin use for preservation of dairy products. *Journal of Dairy Science, 103*(3), 2041–2052. https://doi.org/10.3168/jds.2019-17498

Kapoor, R., & Metzger, L. E. (2008). Process cheese: Scientific and technological aspects - a review. *Comprehensive Reviews in Food Science and Food Safety, 7*(2), 194–214. https://doi.org/10.1111/j.1541-4337.2008.00040.x

Lee, R. M., Hartman, P. A., Olson, D. G., & Williams, F. D. (1994). Bactericidal and bacteriolytic effects of selected food-grade phosphates, using *Staphylococcus aureus* as a model system. *Journal of Food Protection, 57*(4), 276–283. https://doi.org/10.4315/0362-028X-57.4.276

Lee, R. M., Hartman, P. A., Stahr, H. M., Olson, D. G., & Williams, F. D. (1994). Antibacterial mechanism of long-chain polyphosphates in *Staphylococcus aureus*. *Journal of Food Protection, 57*(4), 289–294. https://doi.org/10.4315/0362-028X-57.4.289

Lewus, C. B., & Montville, T. J. (1991). Detection of bacteriocins produced by lactic acid bacteria. *Journal of Microbiological Methods, 13*(2), 145–150. https://doi.org/10.1016/0167-7012(91)90014-H

López-Malo, A., Mani-López, E., Davidson, P. M., & Palou, E. (2020). Methods for activity assay and evaluation of results. In P. M. Davidson, T. M. Taylor, & J. R. D. David (Eds.), *Antimicrobials in food* (4th ed., pp. 659–680). Boca Raton, FL, USA: CRC Press. https://doi.org/10.1201/9780429058196.

Lorencová, E., Vltavská, P., Budinský, P., & Koutný, M. (2012). Antibacterial effect of phosphates and polyphosphates with different chain length. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, 47*(14), 2241–2245. https://doi.org/10.1080/10934529.2012.707544

Maier, S. K., Scherer, S., & Loessner, M. J. (1999). Long-chain polyphosphate causes cell lysis and inhibits *Bacillus cereus* septum formation, which is dependent on divalent cations. *Applied and Environmental Microbiology, 65*(9), 3942–3949. https://doi.org/10.1128/aem.65.9.3942-3949.1999

Małaczewska, J., & Kaczorek-Łukowska, E. (2021). Nisin - a lantibiotic with immunomodulatory properties: A review. *Peptides, 137*, Article 170479. https://doi.org/10.1016/j.peptides.2020.170479

Martinez-Rios, V., Jørgensen, M.Ø., Koukou, I., Gkogka, E., & Dalgaard, P. (2019). Growth and growth boundary model with terms for melting salts to predict growth responses of *Listeria monocytogenes* in spreadable processed cheese. *Food Microbiology, 84*, Article 103255. https://doi.org/10.1016/j.fm.2019.103255

Moon, J. H., Park, J. H., & Lee, J. Y. (2011). Antibacterial action of polyphosphate on *Porphyromonas gingivalis*. *Antimicrobial Agents and Chemotherapy, 55*(2), 806–812. https://doi.org/10.1128/AAC.01014-10

Moraes, P. M., Perin, L. M., Tassinari Ortolani, M. B., Yamazi, A. K., Viçosa, G. N., & Nero, L. A. (2010). Protocols for the isolation and detection of lactic acid bacteria with bacteriocinogenic potential. *LWT - Food Science and Technology, 43*(9), 1320–1324. https://doi.org/10.1016/j.lwt.2010.05.005

Ramos, G. L. P. A., Alves, A. T. S., Spadoti, L. M., Zacarchenco, P. B., & da Cruz, A. G. (2022). Chapter 14 - microbiology of processed cheese. In M. El-Bakry, & B. M. Mehta (Eds.), *Processed cheese science and Technology* (pp. 427–449). Sawston, United Kingdom: Woodhead Publishing. https://doi.org/10.1016/B978-0-12-821445-9.00016-9.

Rulliere, C., Perenes, L., Senocq, D., Dodi, A., & Marchesseau, S. (2012). Heat treatment effect on polyphosphate chain length in aqueous and calcium solutions. *Food Chemistry, 134*(2), 712–716. https://doi.org/10.1016/j.foodchem.2012.02.164

Salek, R. N., Vašina, M., Lapčík, L., Černíková, M., Lorencová, E., Li, P., et al. (2019). Evaluation of various emulsifying salts addition on selected properties of processed cheese sauce with the use of mechanical vibration damping and rheological methods. *LWT - Food Science and Technology, 107*, 178–184. https://doi.org/10.1016/j.lwt.2019.03.022

Ullah, N., Parveen, A., Bano, R., Zulfiqar, I., Maryam, M., Jabeen, S., et al. (2016). *In vitro* and *in vivo* protocols of antimicrobial bioassay of medicinal herbal extracts: A review. *Asian Pacific Journal of Tropical Disease, 6*(8), 660–667. https://doi.org/10.1016/S2222-1808(16)61106-4

Vollmer, A. H., Kieferle, I., Youssef, N. N., & Kulozik, U. (2021). Mechanisms of structure formation underlying the creaming reaction in a processed cheese model system as revealed by light and transmission electron microscopy. *Journal of Dairy Science, 104*(9), 9505–9520. https://doi.org/10.3168/jds.2020-20080

Wang, M., Li, Z., Zhang, Y., Li, Y., Li, N., Huang, D., et al. (2021). Interaction with teichoic acids contributes to highly effective antibacterial activity of graphene oxide on Gram-positive bacteria. *Journal of Hazardous Materials, 412*, Article 125333. https://doi.org/10.1016/j.jhazmat.2021.125333

Zaika, L. L., Scullen, O. J., & Fanelli, J. S. (1997). Growth inhibition of *Listeria monocytogenes* by sodium polyphosphate as affected by polyvalent metal ions. *Journal of Food Science, 62*(4), 867–872. https://doi.org/10.1111/j.1365-2621.1997.tb15474.x