UNIVERSIDADE FEDERAL DE VIÇOSA

TATIANA SANTOS LIMA

EVALUATION OF EMULSIFYING SALT JOHA® HBS AGAINST *Bacillus* spp.

VIÇOSA - MINAS GERAIS
2021

TATIANA SANTOS LIMA

EVALUATION OF EMULSIFYING SALT JOHA® HBS AGAINST *Bacillus* spp.

Dissertation submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

Advisor: Antônio Fernandes de Carvalho

Co-advisors: Evandro Martins
Solimar Gonçalves Machado

VIÇOSA - MINAS GERAIS
2021

TATIANA SANTOS LIMA

EVALUATION OF EMULSIFYING SALT JOHA® HBS AGAINST *Bacillus* spp.

Dissertation submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

APPROVED: February 10, 2021.

____
Tatiana Santos Lima
(Author)

____
Antônio Fernandes de Carvalho
(Advisor)

TATIANA SANTOS LIMA

EVALUATION OF EMULSIFYING SALT JOHA® HBS AGAINST *Bacillus* spp.

Dissertation submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

APPROVED: February 10, 2021.

Dr. Evandro Martins
(UFV)

Dra. Solimar Gonçalves Machado
(UFV)

Dr. Naaman Francisco Nogueira Silva
(UFV)

Dr. Antônio Fernandes de Carvalho
(Advisor)

ACKNOWLEDGEMENT

I thank God and the Holy Mary for the gift of being alive, for all the blessings and for the continued protection each day.

I would like to thank my mom, for being my hero since ever, for teaching me how to be a good human being, and for believing in me in every step of the way. I also thank my family (dad, siblings and nieces) for being my strength and safe haven. I love you immensely!

I also thank my love Hudson, the one and only. Thanks for pushing me forward each and every day, and for taking such a good care of me.

To my Cine Gusta friends: Atalita, Bella, Gusta, Laise, K e Nardy, for being my family. I thank Atalita and Wilton for the support along the way, even from Pernambuco; Bella and Laise for the advices, food and home. You guys played such an important role in the end of the process, and I cannot thank you enough; Gusta for the bad jokes and for choosing great movies for our group movie nights; Nardy for the prayers and comforting words; K for the hair-care advices and for the help with my job search. I thank you all for the laughs and memorable adventures. You guys are the best and made this journey smoother and happier. I love you! I miss you all very much and I cannot wait to see we all reunited.

Thanks to Frida, Jaqueline and Rafaela, for helping me during this work. I am sorry for making you guys stay up until late working, but I really appreciate your help.

To my co-workers that at some point helped me during my Master degree.

To Sci-Hub website, for “removing the barriers in the way of science”.

To my co-advisors Evandro and Solimar that truly taught and helped me in every step of my Master’s degree. Evandro, thank you very much for the laughs, jokes and games! I could not have gotten this done without your help and patience.

To my advisor Prof. Antônio Fernandes de Carvalho for the opportunity to work at the InovaLeite lab, for teaching valuable lessons and for opening my mind towards the food industry. I thank you very my for your comprehension, wisdom and patience.

To the Federal University of Viçosa and its Department of Food Science and Technology, for having given me the opportunity to study.

To the *Conselho Nacional de Desenvolvimento Científico e Tecnológico*, the *Coordenação de Aperfeicoamento Pessoal de nível Superior* and the *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* for financial support for research.

"Challenges are what make life interesting. Overcoming them is what makes life meaningful."

Joshua Marine

ABSTRACT

LIMA, Tatiana Santos, M.Sc., Universidade Federal de Viçosa, February, 2021. **Evaluation of emulsifying salt JOHA® HBS against *Bacillus* spp.** Advisor: Antônio Fernandes de Carvalho. Co-advisors: Evandro Martins and Solimar Gonçalves Machado.

Processed cheese is the most consumed type of cheese in the world and, in Brazil, "requeijão" is a type of highly consumed processed cheese that, in addition to economic importance, also has cultural importance. In the formulation of "requeijão", as well as in processed cheeses in general, emulsifying salts are added to give the product improved properties, such as melting, texture and different flavors, in addition to being able to present bacteriostatic activity. Thus, the aim of this study was to compare the action of the emulsifying salt JOHA® HBS and preservatives commonly used in the production of cheese processed on *Bacillus* spp. In order to analyze the *in situ* effect of the JOHA® HBS salt and the preservatives nisin+potassium sorbate against strains of *Bacillus* spp., a dairy matrix was produced in three treatments: addition of 0.5% JOHA® HBS salt; addition of 0.0012% nisin and 0.1% potassium sorbate combined; and control without the addition of preservatives and/or emulsifying salt. The physical-chemical characterization of the dairy matrix was performed to determine its composition. An *in vitro* evaluation of the antimicrobial activity of the JOHA® HBS salt and preservatives was also carried out to compare with the results obtained in the *in situ* assessments. The minimum inhibitory concentration (MIC) of the JOHA® HBS salt was performed in *in vitro* and *in situ* tests by spectrophotometry and plate counting, respectively. The physical-chemical parameters of the dairy matrix showed a significant difference only for protein and moisture content. The emulsifying salt JOHA® HBS had a similar effect to preservatives in relation to the increase in cell count (CFU/mL) against the microorganisms tested, however, microbial growth could be observed throughout the period of storage of the dairy matrix for all treatments. In *in vitro* tests, the emulsifying salt did not exhibit similar behavior to preservatives, being ineffective against the tested strains. On the other hand, treatment with nisin+sorbate showed positive results in *in vitro* tests, suggesting that the dairy matrix may have interfered with the antimicrobial action of these preservatives in *in situ* tests. The MIC found for the microorganisms evaluated is above 2.86% (w/v) and exceeds the maximum amount of phosphates allowed by Brazilian legislation, in addition to being technologically not viable. The controversial results of this study may be related to microbial resistance and/or the influence of divalent ions in the medium..

RESUMO

LIMA, Tatiana Santos, M.Sc., Universidade Federal de Viçosa, fevereiro, 2021. **Avaliação da ação de sal fundente JOHA® HBS contra *Bacillus* spp.** Orientador: Antônio Fernandes de Carvalho. Coorientadores: Evandro Martins e Solimar Gonçalves Machado.

Queijo processado é o tipo de queijo mais consumido no mundo e, no Brasil, o requeijão é um tipo de queijo processado muito consumido que, além de importância econômica, tem também importância cultural. Na formulação do requeijão, assim como nos queijos processados em geral, são adicionados sais emulsificantes para conferir ao produto propriedades aprimoradas, como derretimento, textura e diferentes sabores, além de também poderem apresentar atividade bacteriostática. Assim, o objetivo deste estudo foi comparar a ação do sal emulsificante JOHA® HBS e de conservantes comumente usados na produção de queijos processados sobre *Bacillus* spp. Para analisar o efeito *in situ* do sal JOHA® HBS e dos conservantes nisina+sorbato de potássio contra estirpes de *Bacillus* spp., matriz láctea foi produzida em três tratamentos: adição de 0,5% sal JOHA® HBS; adição de 0,0012% de nisina e 0,1% de sorbato de potássio combinados; e o controle sem adição de conservantes e/ou sal emulsificante. Foi realizada a caracterização físico-química da matriz láctea para determinação de sua composição. Uma avaliação *in vitro* da atividade antimicrobiana do sal JOHA® HBS e conservantes também foi realizada para comparar com os resultados obtidos nos experimentos *in situ*. A concentração inibitória mínima do sal JOHA® HBS foi realizada em testes *in vitro* e *in situ* por espectrofotometria e por contagem em placas, respectivamente. Os parâmetros físico-químicos da matriz láctea apresentaram diferença significativa apenas para os teores de proteína e umidade. O sal emulsificante JOHA® HBS teve efeito semelhante aos conservantes em relação ao aumento da contagem de células (UFC/mL) contra os microrganismos testados, no entanto, pôde-se observar crescimento microbiano ao longo do período de estocagem da matriz láctea em todos os tratamentos. Nos testes *in vitro*, o sal não apresentou comportamento semelhante aos conservantes, sendo não efetivo contra as estirpes testadas. Por outro lado, o tratamento com nisina+sorbato apresentou resultados positivos nos testes *in vitro*, sugerindo que a matriz láctea possa ter interferido na ação antimicrobiana destes conservantes nos testes *in situ*. A concentração inibitória mínima (MIC) encontrada para os microrganismos avaliados se encontra acima de 2,86% (m/v) e supera a quantidade máxima de fosfatos permitida pela legislação brasileira, além de serem tecnologicamente não viável. Os resultados controversos deste estudo podem estar relacionados com resistência microbiana e/ou com influência de íon divalentes no meio.

LIST OF FIGURES

Figure 1. Processed cheese manufacture flow chart. 17

Figure 2. Structure of nisin (Gross; Morell, 1971). 20

Figure 3. Results for average counting (log CFU/mL) of isolates I-01, I-02 and I-03 in the dairy matrices (T1, T2 and T3) during 60 days stored at 5 °C. 31

Figure 4. *In vitro* antimicrobial activity, by means of optical density (600 nm), of treatment control (T1), 0.5% JOHA® HBS salt (T2), and preservatives nisin+potassium sorbate (0,0012% and 0,1%, respectively) (T3) in BHI broth. 34

Figure 5. MIC of JOHA® HBS salt against Bacillus spp. isolates and reference strain B. thuringiensis CFBP 3476 in BHI broth by means of optical density. 36

LIST OF TABLES

Table 1. Dairy matrices formulation. 24

Table 2. Average increase in counting (log CFU/mL) of isolates I-01, I-02 and I-03 in the dairy matrices (T1, T2 and T3) during storage time at 5 °C. 32

Table 3. Physical-chemical parameters of dairy matrices of each treatment (T1, T2 and T3). 33

SUMMARY

1. INTRODUCTION 13

2. LITERATURE REVIEW 14

2.1. HISTORY AND DEFINITION OF PROCESSED CHEESE 14

2.2. PROCESSING TECHNOLOGY OF PROCESSED CHEESE 15

2.3. MICROBIOLOGY OF PROCESSED CHEESE 18

2.4. RESEARCH QUESTIONS 21

3. OBJECTIVES 21

3.1. MAIN GOAL 21

3.2. SPECIFIC OBJECTIVES 22

4. MATERIAL AND METHODS 22

4.1. MICROORGANISMS AND INOCULUM 22

4.2. DAIRY MATRIX INGREDIENTS 23

4.3. DAIRY MATRIX MANUFACTURE 23

4.4. EVALUATION OF THE EFFECT OF JOHA® HBS SALT AND PRESERVATIVES IN DAIRY MATRIX 24

4.5. PHYSICAL-CHEMICAL ANALYSIS OF THE DAIRY MATRIX 25

4.5.1. Moisture content 25

4.5.2. Fat content 26

4.5.3. Protein content 26

4.5.4. pH 27

4.5.5. Acidity 27

4.5.6. Water activity 28

4.6. IN VITRO EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF SALT AND PRESERVATIVES 28

4.7. IN SITU MINIMUM INHIBITORY CONCENTRATION OF JOHA® HBS SALT BY PLATE COUNTING 28

4.8. *IN VITRO* MINIMUM INHIBITORY CONCENTRATION OF JOHA® HBS SALT BY SPECTROMETRY 29

4.9. STATISTICAL ANALYSES 29

5. RESULTS AND DISCUSSION 29

6. CONCLUSION 39

7. PERSPECTIVES 39

8. REFERENCES 39

1. INTRODUCTION

Processed cheeses are one of the most important products in the dairy industry and can have several applications for consumption, explaining its popularity (OLIVEIRA et al., 2016). The main idea that motivated the creation of processed cheeses was to increase the shelf life of natural cheeses, in addition to finding alternatives for unsold natural cheeses (KAPOOR; METZGER, 2008). Nowadays, there are different types of processed cheeses, including a very popular one in Brazil, called “requeijão”, with hard and creamy varieties. “Requeijão cremoso”, which is a creamy variety, is a spreadable processed cheese, manufactured by mixing the washed curd with fats, such as anhydrous fat and/or butter (OLIVEIRA et al., 2018).

Data show that the consumption of these processed cheeses is quite expressive. Cheese consumption per capita in the United States increased by 16% between 2010 and 2018, while the increase was 48% for processed cheeses in the same period (USDA, 2019). Cheese consumption in Brazil increased by 68.8% from 2009 to 2014, going from 3.5 to 5.9 kg/inhabitant, lagging behind the United States, but exceeding consumption in Mexico (BRASIL DAIRY TRENDS, 2020). According to Brasil Dairy Trends (2020), in 2012, requeijão became the most consumed cheese in Brazil, surpassing mozzarella. In 2012, the amount of requeijão produced was 265 thousand tons, while the amount of mozzarella that same year was 263 thousand tons.

Among the advantages that processed cheeses bring, there are: different flavors from natural cheeses, changes in texture properties, such as spreadability and sliceability, and in cooking properties, such as meltability (FOX et al., 2017). Therefore, each ingredient used in processed cheese formulation is important to confer these properties and also microbiological quality (OLIVEIRA et al., 2016). The microbiological quality achieved through measures of good production, handling and manufacturing practices and quality raw materials are essential for the safety of processed cheeses, since even spores-forming bacteria can be found in requeijão (OLIVEIRA et al., 2018; OLIVEIRA et al., 2016; ÁVILA et al., 2014).

In order to better understand the manufacture of processed cheese, this study will discuss about the history of such product, technology parameters and safety the role of each ingredient used to manufacture processed cheese. Further it will be discussed the effect of emulsifying salt JOHA® HBS on selected strains of *Bacillus* spp. in *in vitro* and *in situ* assays, also compare the effect of such salt with common preservatives used in cheese production.

2. LITERATURE REVIEW

2.1. History and definition of processed cheese

Historically, the use of heat to stop enzymatic and microbial deterioration in cheese was first documented in the end of XIX century, in Europe, method that was advantageous to transport cheese to long distances. Nevertheless, the first processed cheese produced for commercialization is dated from 1911, when the Swiss cheese makers Fritz Stettler and Walter Gerber developed a processed cheese made from Emmental cheese with sodium citrate. Later in 1916, James Kraft patented in the United States a processed cheese made from Cheddar cheese, produced under heat and constant mixing. From then on, processed cheese manufacture grew up all over the world, with consequent development of its processing technology (KAPOOR; METZGER, 2008; VAN DENDER; ZACARCHENCO, 2014).

Standardization of food products and of its processing is essential for international trading and food safety; however, when the matter is processed cheese, we find no standards in the Codex Alimentarius, which is an internationally recognized collection of guidelines for food products. The Codex started working on the development of specifications for processed cheese back in 1994, but the discussion was discontinued in 2017, as, over the years, the Committee had been facing an absence of agreement among the delegations about the product standards (FAO, 2017). As a result, each country is now responsible for the development of its own standards for such product.

Despite each country has autonomy to set its own specific regulation, the definition of processed cheese is similar among many. As example, the Brazilian and the American regulatory agencies characterize processed cheese as being, in sum, the product resulted from the comminuting and mixing of one or more cheeses, with aid of heat and emulsifying agents, with possibility to add other ingredients such as water, salt, cream, spices, harmless artificial coloring and defined preservatives (BRAZIL, 2017; FDA, 2019).

As there are many possible formulations as well as processing technology to produce processed cheese, several varieties of the product are found on the market. Not only differences in composition, processed cheeses vary in their presentation, which can be individual slices or portions, rectangular blocks, tubes or cylinders. One example of processed cheese that can be mentioned is American cheese, which is widely known and appreciated. Another example of such product is “requeijão”, a typical Brazilian processed cheese made from fresh curd. Due to

diversity in is production technology, there are many types of requeijão in Brazil nowadays, with differences in texture, color, flavor and physical-chemical parameters. The same assumption can be made for other types of processed cheese (BRAZIL, 1997b; VAN DENDER; ZACARCHENCO, 2014).

2.2. Processing technology of processed cheese

The manufacture of processed cheese starts with the selection of the raw material that will be used. Choosing the cheese (or cheeses) that will be the basis for processed cheese manufacture depends on the type of processed cheese to be produced and the desired characteristics of it. Varying the type and ripening degree of the cheeses that will be used to fabricate the processed cheese results in a product with different physical-chemical features and sensory attributes. It is important to emphasize that the cheeses used as raw material must be in satisfactory quality conditions in order to obtain a processed cheese safe for consumption and with sensorial quality. Particularly for requeijão, the raw material is not cheese, but the fresh curd (BRAZIL, 1997b). Aside from the cheese (or curd) – which is the main ingredient to manufacture processed cheese, other ingredients such as fat, water, sodium chloride and emulsifying agents play an important role in processed cheese production.

In order to enhance physical-chemical and sensory characteristics of processed cheeses, the above-mentioned ingredients are added during manufacture of such product. As example, meltability, viscosity and texture aspects of the final product are improved with addition of fat. Fat also contributes to the achievement of desired fat content in the final product. Thus, the amount of fat to be added depends on the fat-in-dry matter content desired on the final product and the fat content and dry matter of the cheeses used as raw material (GUINEE, 2009; VAN DENDER et al., 2014).

Water and sodium chloride are as well relevant ingredients to produce processed cheese. While sodium chloride is added to enhance flavor, water has an important role as it facilitates the thermal energy transfer, dissolves salts, and disperses casein (VAN DENDER et al., 2014). Water is also added to the blend to achieve the moisture content required for processed cheese (GOUDA; ABOU EL-NOUR, 2003). Processed cheese softness is another attribute that is achieved with aid of water that is added to the blend (GLIGUEM et al., 2009). It is of relevance to highlight that the amount of water to be added needs to be related to the protein and fat

content and with the percentage of emulsifying salt of the blend, in order to obtain a homogeneous mass (VAN DENDER et al., 2014).

In addition to homogeneous, processed cheese must be a stable mass. Albeit casein from the cheese used as raw material has emulsifying characteristics, the stability required for processed cheese would not be reached relying on casein only (TAMINE, 2011). That is why it is necessary to use emulsifying agents to manufacture processed cheese. Emulsifying salts act removing the calcium from the protein system, peptizing and dispersing of protein, hydration, pH stabilization, and new protein system formation (GOUDA; ABOU EL-NOUR, 2003; VAN DENDER et al., 2014). In addition to the emulsifying role, the presence of such salts in processed cheese results in improvement of the texture, enhancement of flavor and extend of shelf life (AHMAD et al., 2016) resulted from the improved stability of the cheese.

The processing technology applied also influences the final product. The fundamental steps to produce processed cheese are represented in Figure 1. The selection of the natural cheeses (or curd), as stated earlier in this paper, is based on the type of final product that is desired. It can be used two or more types of cheeses or a blend of one type of cheese with different levels of ripening (BRAZIL, 1997a; FDA, 2019). Once the cheeses have been selected and their proportions are established, they follow to the grinding process, which increases the contact surface and facilitates the action of emulsifying salts. After that, emulsifying salt and optional ingredients, such as dairy protein ingredients, dairy fat ingredients, preservatives, coloring agents, flavoring agents, water, spices, sodium chloride and acidulants are added and blended (KAPOOR; METZGER, 2008).

Cooking process is then started, which comprehends of melting the blend under heat and constant stirring. The agitation during the cooking process is of importance as it favors the emulsification of the blend (GOUDA; ABOU EL-NOUR, 2003). It was found that agitation speed influences viscosity right after manufacture, firmness, flow properties and meltability of pasteurized processed cheese food, and that at higher stirring speed, fat globules present themselves in a higher amount per 100 μm^2, due to smaller diameter (PURNA; POLLARD; METZGER, 2006). Thus, the mixing speed is defined according to the desired characteristics of the final product, as example, for processed cheese of creamy consistency, a lower agitation speed is recommended at the beginning of the cooking, with gradual increase of speed during the processing (VAN DENDER et al., 2014).

Another parameter during cooking that is important as well is time-temperature of the process. The Brazilian legislation establishes that processed cheese must undergo a minimum heat treatment of 80 °C for 15 s or equivalent heat treatment, while American legislation requires a minimum of 65.5 °C for 30 s (BRAZIL, 1997a; FDA, 2019). Time-temperature is essential to the manufacture of processed cheese, as it affects the firmness and meltability of the final product, as well as the strength of the processed cheese emulsion (SHIRASHOJI; JAEGGI; LUCEY, 2006; VAN DENDER et al., 2014). There is no defined best time-temperature to manufacture processed cheese because this parameter depends on the features desired on the final product. Also, as there are many types of cookers with various designs and conditions of operation, time-temperature of cooking will vary. In spite of it, temperature of 72 °C or higher should be used in order to guarantee proper pasteurization of the product (GOUDA; ABOU EL-NOUR, 2003; TAMINE, 2011; VAN DENDER et al., 2014).

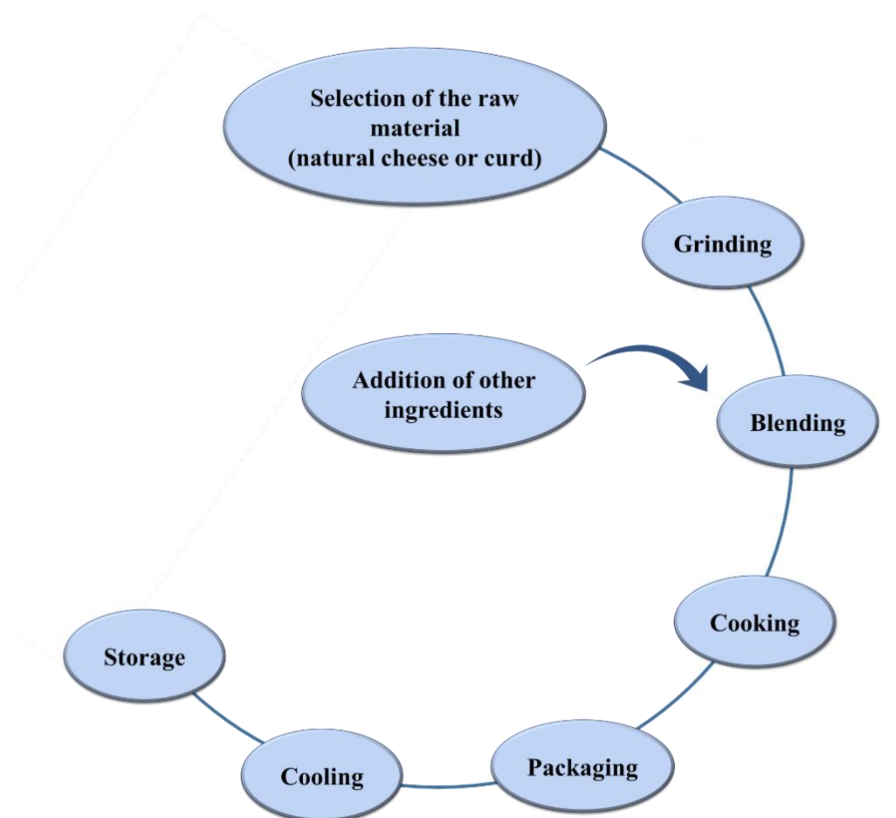

Figure 1. Processed cheese manufacture flow chart.

Subsequently, hermetic packaging is performed with the product still hot, since the processed cheese is fluid, facilitating the packaging, especially for processed cheese with creamy consistency. There upon, the packed product is cooled and stored. Cooling speed will depend on the type of processed cheese being manufactured. For processed cheese spread, for

example, the cooling should be quick in order to prevent separation of water and fat, and below temperature of 10 °C. On the other hand, for block processed cheese, the cooling needs to be slow, although it may result in browning (GOUDA; ABOU EL-NOUR, 2003; VAN DENDER et al., 2014).

2.3. Microbiology of processed cheese

The processing technology is not the only matter that should be brought to attention. Cheese safety is as important as well and affects directly the final product, the consumers and the brand's reputation. As for all food products, microbiological quality and safety of processed cheeses is associated and depends on several factors such as the quality of the raw material, the intensity and effectiveness of the heat treatment, aseptic filling conditions, sterility of packaging and storage conditions for the final product (GLASS; DOYLE, 2005; OLIVEIRA et al., 2016), besides the hygiene quality of the processing environment, utensils and equipment and the hygiene of food handlers.

In order to avoid contamination by microorganisms, the milk production chain and its processing for the manufacture of processed cheeses have several key points that need to be observed. Contamination of raw milk can occur from dairy farm environments, where ruminants are present (MOHAMMED et al., 2009), during milking by unclean equipment, during milk transport or during milk storage in bulk tanks (MELO; ANDREW; FALEIRO, 2015; ALMEIDA et al., 2013).

Good manufacturing practices are also essential to be followed within the production environment, since when applied correctly they can reduce the risk of contamination (DIAS et al., 2012). These measures can be applied to soil, waste and silage, in addition to animal health care and careful handling (OLIVEIRA et al., 2016). The presence of spore-forming bacteria in processed cheeses, for example, can be associated with contamination of milk while still on the farm by microorganisms present in the soil (OLIVEIRA et al., 2016; HEYNDRICKX, 2011). Also, the entire industrial installation must receive attention, as pathogenic microorganisms, such as *Listeria monocytogenes*, can be isolated from floors, drains, standing water and refrigeration equipment (MELO; ANDREW; FALEIRO, 2015; BARANCELLI et al., 2014; DALMASSO; JORDAN, 2014).

The heat treatment applied to processed cheeses is able to inactivate vegetative cells of pathogenic and deteriorating microorganisms (ZWIETERING; DE WIT; NOTERMANS, 1996), thus, pasteurization is an effective treatment method against those type of microbes. Despite this, processed cheeses can be contaminated by sporulating bacteria, since they are able to withstand the processing temperature of this product (OLIVEIRA et al., 2016), therefore representing a challenge for the quality and safety of processed cheeses (OLIVEIRA et al., 2016).

Spore-forming microorganisms are very common in dairy industries and can cause numerous problems. Among the most found spore-forming bacteria, it can be cited the genera *Bacillus* and *Clostridium* (LOPEZ-BREA; GÓMEZ-TORRES; ARRIBAS, 2017, OLIVEIRA et al., 2016), which, in addition to compromising the quality of the product, are also associated with foodborne diseases (LÜCKING et al., 2013; ZEINAB et al., 2015). After germinating these spores, species of the genus *Bacillus* are capable of producing thermotolerant lipolytic and proteolytic enzymes that are responsible for the degradation of the sensory quality of the product throughout its shelf life (DAELMAN et al., 2013; LÜCKING et al., 2013). In turn, *Clostridium* species are responsible for releasing off favors and gases (responsible for the formation of holes in cheese) produced during lactate fermentation (GOMEZ-TORRES et al., 2015; MORANDI et al., 2015). Other non-sporulating microorganisms, albeit to a lesser extent, are also likely to be present in the final product through contamination after heat treatment.

Oliveira et al. (2016) highlights that the ingredients and packaging material used in the process are sources of contamination, if not properly analyzed, handled and stored. On the other hand, some ingredient and additives used for manufacture of processed cheese can help improving the microbiological quality and product safety. Oliveira et al. (2016), citing Cunha, Dias and Viotto (2010), states that each ingredient of the processed cheese "requeijão" formulation influences not only the physical-chemical, sensory and functional properties, but also the microbiological quality and safety of the product. Ingredients can contribute to microbiological quality of processed cheese products by lowering water activity, increasing osmotic pressure or changing the product pH.

Other strategy used to avoid microbial contamination is the use of preservatives, which are capable to stop or avoid microorganism growth in the food product. Potassium sorbate and nisin are among the preservatives cited by Fox et al. (2017) as being commonly used in processed cheese production. Potassium sorbate is one of the water-soluble salts of sorbic acid.

Its molecular formula is $C_6H_7O_2K$, and it has been widely used as food preservative. The mechanism of action of sorbates in general has been studied by many researchers over the years. Alterations in cell membrane (SOFOS et al., 1989), influence on transport of substrate and electron (FREESE; SHEU; GALLIERS, 1973), inhibition of metabolic activity through effect on enzymes (SOFOS et al., 1989) and excessive energy consumption due to stress response (BRACEY; HOLYOAK; COOTE, 1998) are some suggestions of the action mechanism of sorbates against microorganisms.

Nisin is a polypeptide produced by *Lactococcus lactis* subsp. *Lactis* bacteria, and since 1969 it has been recognized as safe to use as biological food preservative (PUNYAUPPA-PATH; PHUMKHACHORN; RATTANACHAIKUNSOPON, 2015). Nisin is used worldwide, and studies about its mechanism of action and target microorganisms have been performed over the years. Nisin amino acid structure representation was elucidated by Gross and Morell (1971) and it is presented in Figure 2. Studies have stated that nisin mechanism of action is through electrostatic interactions with components of the cytoplasmatic membrane of vegetative cells (HENNING; METZ; HAMMES, 1986). It interacts with lipid II of the cell membrane and penetrate the cell wall. Then, four complexes nisin-lipid II assemble and form a stable pore, through which internal components leave the cell, resulting in cell death (MORALES et al., 2019; WILLIAMS; DELVES-BROUGHTON et al., 2003).

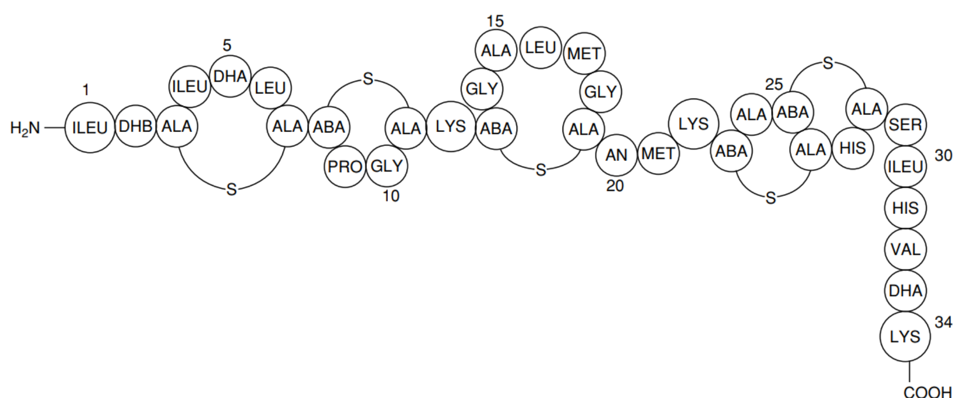

Figure 2. Structure of nisin (Gross; Morell, 1971).

Although preservatives are often added to the product, their activity spectrum may not effectively cover certain microbial groups that may be present in processed cheese. As example, nisin alone is not effective against gram-negative microorganisms due to their outer membrane, which does not allow the permeability of the bacteriocin (MÁRQUEZ et al., 2019). Thus, the

study of alternatives to prevent the multiplication of microorganisms in the final product is important. Researchers around the world have investigated the efficacy of emulsifying salts as antimicrobial agents and favorable results have been reported (OBRITSCH et al., 2008; ZAIKA; SCULLEN; FANELLI, 1997; ZESSIN; SHELEF, 1988).

As stated previously in this work, emulsifying salts act removing calcium from the protein system, meaning that they are chelating agents. Thus, some researchers who studied the antimicrobial effect of this salts, have suggested that they act in the same way on the bacterial cell, removing divalent cations from cell wall, thus destabilizing it. (LEE et al., 1994; MAIER; SCHERER; LOESSNER, 1999). In addition to its chelating effect, the inhibition of synthesis and/or activity of some important enzymes for microbial cell (WAGNER; BUSTA, 1985, 1986), and even changes in water activity in the media (SNYDER; MAXCY, 1979) have been reported as likely to be polyphosphate mechanisms of action against bacteria.

Many are the studies providing evidence that emulsifying salts have antimicrobial effect against certain strains, but not all use bacterial strains isolated from the dairy industry, and less than that studied the effect of emulsifying salts against bacterial strains on a dairy matrix. Thus, it is necessary to further research the efficiency of these polyphosphates *in situ*, and investigate whether they can, in fact, be considered as alternative preservatives in dairy industry.

2.4. Research questions

There are a few questions that the present thesis aims to answer: Does emulsifying salt JOHA® HBS, in concentration commonly used in dairy industry, have *in vitro* antimicrobial activity on selected strains of *Bacillus* spp.? Is its antimicrobial effect better, worse or equivalent to preservatives generally used in dairy industry? What is the minimum concentration of emulsifying salt JOHA® HBS that has antimicrobial effect against strains of *Bacillus* spp.? Does dairy matrix interfere on emulsifying salt JOHA® HBS antimicrobial activity against selected strains of *Bacillus* spp.?

3. OBJECTIVES

3.1. Main Goal

Investigating the antimicrobial effect of commercially available emulsifying salt on *Bacillus* spp.

3.2. Specific objectives

- Comparing the antimicrobial effect of emulsifying salt JOHA® HBS to preservatives commonly used in processed cheese manufacture against selected strains of *Bacillus* spp.

- Determining the minimum inhibitory concentration of JOHA® HBS salt against selected strains of *Bacillus* spp.

- Evaluate whether the dairy matrix interferes on the antimicrobial effect of the studied emulsifying salt.

4. MATERIAL AND METHODS

4.1. Microorganisms and inoculum

Four strains isolated from UHT milk and belonging to the InovaLeite culture collection were used in this study. The isolates were sequenced by the 16S gene and correspond to *Bacillus* spp. and are here identified as I-01, I-02, I-03 and I-04. Reference strain of *B. thuringiensis* CFBP 3476 was also used in this study. The strains were activated twice in Brain-Heart-Infusion (BHI) (Kasvi®, Italy) broth prior analyses. For that, 50 µL of stock culture were inoculated in 5 mL of BHI broth and then incubated for 18 h at 32 °C. Then, 100 µL of this first activation were inoculated in 10 mL of BHI broth, followed by incubation for 18 h at 32 °C. Plate counting in BHI (Kasvi®, Italy) agar was performed in order to obtain cell concentration after 18 h incubation, and a cell concentration of 10^7-10^8 CFU/mL was found.

The strains under study were activated in falcon tubes as describe above, followed by centrifugation (Megafuge 8R, Thermo Fisher Scientific, Germany) for 15 minutes at 25 °C, at 3260 x g. The supernatant was discarded and the pellet was washed twice with sterile saline water (0.85%) (Dinâmica, Brazil). After the washing, the supernatant was discarded and the cells were suspended in 10 mL of sterile reconstituted skim milk (Molico®, Nestlé), and this suspended cells, with 10^7-10^8 CFU/mL. Prior the experiment, the suspended cells were diluted via serial dilutions in reconstituted skim milk to reach concentration of 10^4-10^5 CFU/mL. This diluted suspension was used as inoculum for this study.

4.2. Dairy matrix ingredients

Mozzarella cheese (Sérvulo®, Senador Firmino, Minas Gerais, Brazil), containing 24% of protein, 17% of fat and 54% water content was used as basis for the dairy matrix. Homogenized UHT table cream (Piracanjuba®, Bela Vista do Goiás, Goiás, Brazil), containing 30% of fat was also used in this study, as source of milk fat to produce the dairy matrix.

Emulsifying salts JOHA® HBS FG (ICL Food Specialties), lot: 8-42709-56, production date: 03/2018, expiration date 03/2021 and JOHA® S9 FG (ICL Food Specialties), lot: B3180508A, production date: 02/2018, expiration date 02/2021 were the emulsifying agents used in this study. Nisin powder (Globalnisin® – Globalfood), lot: 19C1022, production date: 04/11/2019, expiration date 10/11/2019 and potassium sorbate were also used as preservatives.

4.3. Dairy matrix manufacture

Dairy matrices were produced to perform *in situ* tests. The dairy matrices imitate the processed cheese "requeijão cremoso", with modification, as it was not made from curd, thus not being possible to be called "requeijão" in this study. Three dairy matrices were produced with the formulation presented in Table 1. JOHA® S9 salt was used as emulsifying agent for the production of the dairy matrices in this study, as addition of no emulsifying agent would not result in an emulsified dairy matrix. For the treatment T2, JOHA® S9 salt was added together with JOHA® HBS salt, as the manufacturer instructed that JOHA® HBS should be used together with another emulsifying agent.

The amount of emulsifying salt JOHA® HBS used in this study was based on studies reported in the literature (BUŇKOVÁ et al., 2008; LOESSNER et al., 1997; MAIER; SCHERER; LOESSNER, 1999; NOVÁKOVÁ, 2008). The amount of both JOHA® S9 and JOHA® HBS added to the product are in accordance with the manufacturer's instruction and do not exceed the maximum of g of P_2O_5 per Kg of final product established by Brazilian regulation law for processed cheese (BRAZIL, 1997a) and requeijão (BRAZIL, 1997b). Preservatives nisin and potassium sorbate were added in the maximum amount allowed by Brazilian regulation law for processed cheese (BRAZIL, 1997a) and requeijão (BRAZIL, 1997b), which are 12.5 mg and 1000 mg per Kg of final product, respectively.

The production process of each dairy matrix occurred as follow: all ingredients were placed in Thermomix® (TM6, Vorwerk) utensil. The addition of salt or preservatives occurred

during the production of the dairy matrix to simulate the real making process. The utensil was closed, time and temperature were set to 15 min and 85 °C, respectively, and then the equipment were started by activating the stirring. Stirring speed were gradually turned up every minute until it reached speed 3.5 on the equipment. The equipment remained in this stirring speed until the last 2 min, when the speed was dropped 0.5 on the equipment scale in every 15 s. After that, the stirring remained at initial speed until the processing time finished. Thereupon, the dairy matrix was filled in sterile plastic bags and cooled to approximately 32 °C for inoculation of the microorganisms.

Table 1. Dairy matrices formulation.

Ingredient	**Amount of ingredient (g/100g) per treatment**		
	T1	**T2**	**T3**
Mozzarella cheese	35.00	35.00	35.00
Table cream with 30% fat	50.00	50.00	50.00
Distillated sterile water	13.50	13.00	13.3988
JOHA® S9	1.50	1.50	1.50
JOHA® HBS	0.00	0.50	0.00
Nisin	0.00	0.00	0.0012
Potassium sorbate	0.00	0.00	0.10

4.4. Evaluation of the effect of JOHA® HBS salt and preservatives in dairy matrix

In order to analyze the activity of JOHA® HBS salt and of preservatives against three isolates (I-01, I-02 and I-03) *in situ*, each isolate were separately inoculated in 500 g samples of dairy matrix from each treatment (T1, T2 and T3). For that, an aliquot of 500 µL of the inoculum (4.1) were added to the samples of the dairy matrix of each treatment, thus, reaching the final concentration of approximately 10^2-10^3 CFU/mL. The matrix of each treatment, after inoculation, was homogenized (BagMixer® 400 VW, Interscience, France) for 2 min at speed 9 of the equipment, filled in plastic pots previously sanitized with chlorine solution (200 ppm) and stored under refrigeration at 5 ºC.

The plastic pots remained closed until the date of analyses and after that, the plastic pot was properly discarded. The counts of viable cells of the microorganisms were performed at 0, 15, 30 and 60 days, by spread plate method on MYP (Oxoid®, Basingstoke, Hampshire, England) agar. For that, 25 g of the dairy matrix was diluted in 225 mL of saline solution

(0.85%), homogenized for 2 min at speed 9 (BagMixer® 400 VW, Interscience, France). Then, serial dilutions in saline water were done and an aliquot of 0.1 mL of proper dilution were spread onto MYP agar, with aid of sterile plastic cell spreader. This experiment was carried out in triplicate with two repetitions. The increase or decrease in the counting, in log CFU/mL was calculated, according to Equation 1, where IC, C_t and C_{t-1} stand for increase in average counting, average counting (log CFU/mL) on a specific time, and average of previous counting (log CFU/mL), respectively. Positive value for IC means that there was an increase in counting, while negative value for IC means a decrease in counting.

$$IC = C_t - C_{t-1} \tag{1}$$

4.5. Physical-chemical analysis of the dairy matrix

Samples of non-inoculated dairy matrices (T1, T2 and T3) were used to perform physical-chemical analyses, in order to evaluate whether there were significant differences between the dairy matrices regarding physical-chemical properties. The physical-chemical analyses performed were moisture, fat and protein content, pH, acidity and water activity, and the methodology used for each analysis is described as follow.

4.5.1. Moisture content

Moisture content of the dairy matrix in the three treatments under study was assessed by the gravimetric method, according to Pereira et al. (2001), where approximately 5 g of each sample were weighted in previously weighted Petri dishes containing purified sand. The sample was mixed to the sand with aid of glass stick, and then dried for 3 h at 105 °C. After cooling in desiccator, each sample was weighted and then dried at 105 °C for 30 min. After cooling in desiccator, the samples were separately weighted again. This last procedure was repeated until the difference of two consecutive weighing was less or equal to 0.5 mg.

The moisture content was calculated according to Equation 2, where U stands for water content (%), and W_i, W_f and T stand for initial weight, final weight and weight of Petri dish (with sand and glass stick, without sample), respectively. This experiment was performed in triplicate with two repetitions.

$$U = \left[1 - \frac{(W_f - T)}{(W_i - T)}\right] \times 100$$

(2)

4.5.2. Fat content

Fat content of the dairy matrix under study was assessed by the Van Gulik method, according to Pereira et al. (2001), where 5 mL of distilled water at 60-70 °C were added to a butyrometer (Gerber, Switzerland) containing 3 g of dairy matrix. Then, 10 mL of sulfuric acid (d_{20} = 1.825 g/L, Dinâmica, Brazil) were gently poured to the mixture. The butyrometer was closed and shaken until complete dissolution of proteins. After that, 1 mL of isoamyl alcohol (d_{20} = 811 g/L, Synth, Brazil)) and warm distillated water were added, until reach number 30 on the butyrometer scale. The butyrometers were placed in centrifuge, and centrifugation at 14000 rpm for 5 minutes were performed. The butyrometers were then placed in water bath at 65 °C for 3 minutes, and then the fat content was read on the butyrometer scale. This experiment was performed in triplicate, with two repetitions.

4.5.3. Protein content

Total nitrogen content was determined by the Kjeldahl method, according to Pereira et al. (2001), with modification. Thus, approximately 1 g of each sample was placed in glass tubes, then 1.5 g of potassium sulfate (Synth, Brazil), 0.1 g of copper sulfate (Dinâmica, Brazil), 4 mL of sulfuric acid (Synth, Brazil), and 4 glass pearls were added. The glass tubes were carefully shaken and brought to the fume hood. The temperature was increased by 50 ºC every 30 minutes until it reached 400 ºC. After digestion, 30 mL of distilled water was added to the glass tubes. The solution was placed on the distillate, where and Erlenmeyer glass containing 10 mL of boric acid (Synth, Brazil) 4% (w/v) and 15 mL of sodium hydroxide (Dinâmica, Brazil) 35% (w/v) was coupled to the condenser outlet. When it was obtained approximately 100 mL of distillate, it was titrated with hydrochloric acid solution (Dinâmica, Brazil) 0.05 mol/L. When the color changed, the titration was stopped, and the volume of hydrochloric acid were used to calculate the total nitrogen content, according to Equation 3, where %TN stands for total nitrogen content, and A, B, C_i, f_c and g stand for volume of hydrochloric acid spent to titrate the sample, volume of hydrochloric acid spent to titrate the control (blank), concentration of the hydrochloric acid solution, correction factor for the hydrochloric acid, and weight of the sample, respectively.

$$\%NT = \frac{(A - B) \times C_i \times f_c \times 1.4}{g}$$

(3)

4.5.4. pH

The pH value of the samples was measured in pH meter (Kasvi, Brazil). For that, samples were prepared according to Pereira et al. (2001), where approximately 10 g of each sample were weighted and mixed to 10 mL of distillated water with aid of glass stick. Then, 30 mL of water were added to it, followed by mixing with the glass stick. This mixture was then left resting for 5 minutes. After that, the mixture was filtered through hydrophilic cotton. The cotton was rinsed with approximately 10 mL of distillated water. Thereupon, the electrode bulb was immersed in the filtrate. After stabilization, the pH value was displayed on the equipment. This experiment was performed in duplicate, with two repetitions.

4.5.5. Acidity

Acidity of the samples was determined according to Pereira et al. (2001), where 5 g of teach sample were weighted and added of approximately 10 mL of distillated water at 30-40 °C. This mixture was mixed with aid of a glass stick, and then added of approximately 40 mL of cold distillated water. The mixture was left resting for 5 min, and then filtered through hydrophilic cotton, which were then rinsed with approximately 20 mL of distillated water. Afterwards, four drops of alcoholic solution of phenolphthalein 1% (w/v) (Synth, Brazil) were added to the filtered solution. Subsequently, titration using a sodium hydroxide solution (0.1 mol/L) (Dinâmica, Brazil) were executed until change of color. The volume of sodium hydroxide spent for the titration was noted and the acidity was calculated using the Equation 4. where A, C_i, f_c, V and g stand for acidity (expressed in percentage of acid lactic), concentration of the sodium hydroxide solution (mol/L), correction factor of the sodium hydroxide solution, volume of the sodium hydroxide solution spent on titration and mass of the sample, respectively. This experiment was performed in duplicate, with two repetitions.

$$A = \frac{C_i \times f_c \times 9 \times V}{g}$$

(4)

4.5.6. Water activity

Water activity of samples was assessed by placing an amount of them, separately, in a disposable sample cup, completely covering the bottom of the cup. Each sample cup was then placed in the drawer of the equipment (Aqualab 3TE series, Decagon Devices, United States), which was then turned on. When the water activity measurement was finished, the result was displayed on the display of the equipment. This experiment was performed in triplicate with two repetitions.

4.6. In vitro evaluation of the antimicrobial activity of salt and preservatives

To assess the *in vitro* activity of JOHA® HBS and preservatives, three treatments were carried out, namely T'1, T'2 and T'3, which respectively correspond to treatments control (BHI broth with no addition of salt nor preservatives), BHI broth with 0.5 % of JOHA® HBS, and BHI broth with preservatives nisin and potassium sorbate at concentration levels of 0.0012% and 0.1%, respectively. All treatments were separately inoculated with isolates I-01, I-02 and I-03, at a concentration of approximately 10^3-10^4 CFU/mL. The optical density (600 nm) was verified in a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Finland) for 24 h at 32 °C.

4.7. In situ minimum inhibitory concentration of JOHA® HBS salt by plate counting

Dairy matrix T1 was manufactured according to item 4.2. with modification, as JOHA® S9 salt was not added to this formulation, being replaced by sterile water. JOHA® HBS salt was added during the dairy matrix production at the following concentrations: 2.86%, 1.43%, 0.72%, 0.36% and 0%. Inoculum of isolates I-01, I-02, I-03 and I-04, as well as reference strain *B. thuringiensis* CFBP 3476 was added, separately, to each treatment of the dairy matrix in order to reach a level of approximately 5 log CFU/mL. For that, 30 µL of the inoculum containing 10^7-10^8 CFU/mL (4.1) were added to 30 g of dairy matrix in each salt concentration evaluated. A control treatment was performed, where dairy matrices with the studied salt concentration was not inoculated with any microorganism. The inoculated samples plus the treatment control were separately homogenized (BagMixer® 400 VW, Interscience, France) for 2 minutes at speed 9. After homogenization, they were stored at 32 °C, and viable cell count was performed

at the times 0 h and 24 h, by drop plate method in BHI agar. This experiment was performed in triplicate, with two repetitions.

4.8. *In vitro* minimum inhibitory concentration of JOHA® HBS salt by spectrometry

In vitro MIC was verified for all isolates and for the reference strain under study by means of optical density (600 nm) in spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Finland). For that, BHI broth was prepared with the following salt concentrations: 2.86%, 1.43%, 0.72%, 0.36% and 0%. An aliquot of 20 µL of inoculum containing 10^7-10^8 CFU/mL (4.1) was added to 180 µL of BHI broth, to reach level of approximately 10^5 CFU/mL, then it was placed in the Multiskan GO equipment at 32 °C for 24 h. Optical density (600 nm) was measured every one hour. This experiment was performed in triplicate, with two repetitions.

4.9. Statistical analyses

ANOVA followed by Tukey test ($p<0.05$) analyses were performed to evaluate whether there were significant differences among the dairy matrices (T1, T2 and T3) regarding to physical-chemical parameters. Same statistical approach was used to analyze the average increase in counting (log CFU/mL) for isolates I-01, I-02 and I-03 in the studied dairy matrices during the storage period.

5. RESULTS AND DISCUSSION

Processed cheese is consumed worldwide and in large scale, becoming a valuable product for dairy industries. In Brazil, among the processed chesses produced in the country, requeijão is greatly consumed and has an important cultural value. As processed cheese is a key product for cheese manufacturers, its quality and safety are primordial. New alternatives to assure safety of dairy products are being searched around the globe, and among them, polyphosphates gained attention for being reported to have antimicrobial effect, besides its technological use in the dairy industry.

Given the scarcity of studies about the antimicrobial effect of polyphosphates in a dairy matrix, this study aimed to investigate the *in situ* antimicrobial effect of a commercially

available polyphosphate against certain strains of *Bacillus* spp. in comparison with the antimicrobial effect of two common preservatives used for processed cheese manufacture, which are potassium sorbate and nisin.

The counting (log CFU/mL) results obtained by the *in situ* evaluation of JOHA® HBS and preservatives against *Bacillus* spp. are shown in Figure 3, and the results for the average increase (or decrease) in counting (log CFU/mL) along the storage time for each isolate evaluated are shown in Table 2.

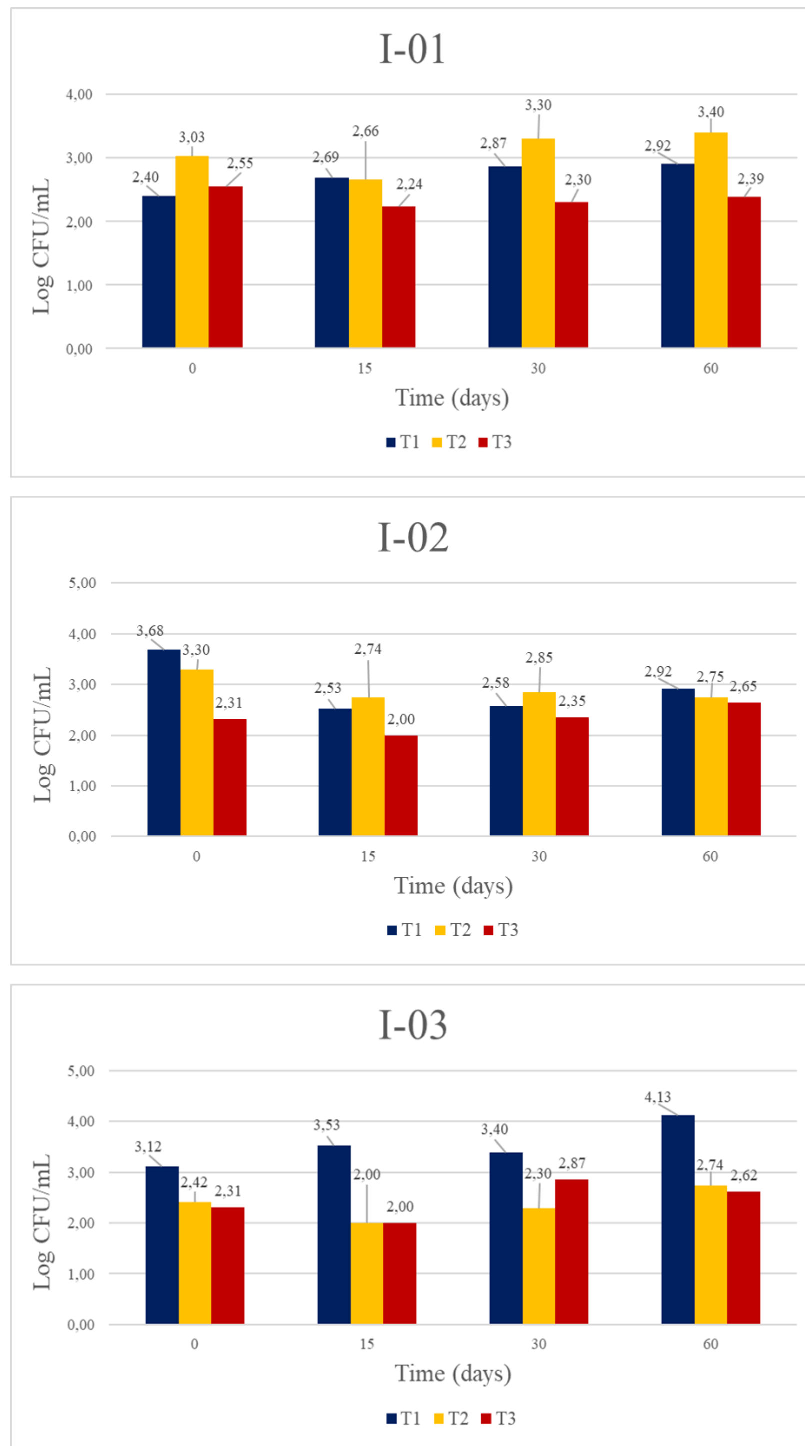

Figure 3. Results for average counting (log CFU/mL) of isolates I-01, I-02 and I-03 in the dairy matrices (T1, T2 and T3) during 60 days stored at 5 °C.

Table 2. Average increase in counting (log CFU/mL) of isolates I-01, I-02 and I-03 in the dairy matrices (T1, T2 and T3) during storage time at 5 °C.

I-01			
	Average increase in counting (log CFU/mL)		
Time	T1	T2	T3
0 to 15 days	0.29^{a}	-0.37^{a}	-0.32^{a}
15 to 30 days	0.18^{a}	0.64^{b}	0.06^{b}
30 to 60 days	0.05^{a}	0.10^{b}	0.09^{b}

I-02			
	Average increase in counting (log CFU/mL)		
Time	T1	T2	T3
0 to 15 days	-1.16^{a}	-0.56^{b}	-0.31^{b}
15 to 30 days	0.06^{a}	0.11^{a}	0.35^{a}
30 to 60 days	0.34^{a}	-0.10^{a}	0.30^{a}

I-03			
	Average increase in counting (log CFU/mL)		
Time	T1	T2	T3
0 to 15 days	0.41^{a}	-0.42^{b}	-0.31^{b}
15 to 30 days	-0.13^{a}	0.30^{b}	0.87^{b}
30 to 60 days	0.74^{a}	0.44^{a}	-0.25^{b}

Means followed by the same letter in each raw do not differ significantly by Tukey test ($p < 0.05$).

From the results above presented, it can be seen that, despite bacterial count (log CFU/mL) started raising after 15 days of storage, the increase in counting (log CFU/mL) caused by JOHA® HBS salt was similar to the increase resulted from addition of preservatives in the dairy matrix, except for isolate I-03 during the last 30 days of storage.

For isolate I-01, the average increase in counting for treatments T2 and T3 was similar to the average increase observed for treatment T1 in the first 15 days of storage. Same behavior was observed for isolate I-02 in the last 30 days of storage. Two hypotheses were raised: JOHA® HBS salt and preservatives did not have any effect on such isolates at these specific times of storage, or there was an influence of the matrix T1 on the isolates. In order to verify whether the dairy matrix influenced the growth of the microorganisms, physical chemical analyses were performed and the results are shown in Table 3. *In vitro* assessment to verify the effect of JOHA® HBS salt and preservatives were also performed, and the results are shown in Figure 4.

Table 3. Physical-chemical parameters of dairy matrices of each treatment (T1, T2 and T3).

Parameter	**Treatments**		
	T1	**T2**	**T3**
Aw	$0.983^{a} \pm 0.001$	$0.980^{a} \pm 0.001$	$0.982^{a} \pm 0.004$
Moisture (%)	$57.99^{a} \pm 0.26$	$59.29^{b} \pm 0.49$	$57.95^{a} \pm 0.10$
pH	$6.21^{a} \pm 0.05$	$6.22^{a} \pm 0.02$	$6.26^{a} \pm 0.04$
Acidity (% lactic acid)	$0.094^{a} \pm 0.007$	$0.097^{a} \pm 0.000$	$0.089^{a} \pm 0.005$
Protein content (%)	$11.93^{a} \pm 0.01$	$11.25^{b} \pm 0.18$	$11.83^{a} \pm 0.11$
Fat content (%)	$24.09^{a} \pm 0.59$	$24.67^{a} \pm 0.23$	$25.25^{a} \pm 0.82$

Means followed by the same letter in each raw do not differ significantly by Tukey test ($p < 0.05$).

According to the data shown on Table 3, only moisture and protein content parameters had significant difference between the treatment with JOHA® HBS salt and the other treatments under study. This slight increase in the moisture content of the sample containing the JOHA® HBS salt may be due to its hygroscopic properties, since one of its effects is the hydration of the caseins (JANA; PADHIYAR; CHAVAN, 2017). The difference between the protein content of the sample containing the JOHA® HBS salt and the others may be due to random errors that may occur in the manufacture of such product or during the analysis.

Results of the *in vitro* assessment mismatch the results of the *in situ* assessment, as the emulsifying salt JOHA® HBS did not present antimicrobial effect of the studied isolates in 24 h. Although microorganism growth did happen, it can be observed that the presence oh the salt in the medium resulted in an extended lag phase for the three isolates and a growth curve remained below the growth curve of the treatment control (no addition of salt nor preservatives) for isolates I-02 and I-03. From results showed on Figure 4, it is observed that dairy matrix possibly interferes with the action of preservatives, as nisin + potassium sorbate completely inhibited the growth of the isolates in the *in vitro* test within 24 h.

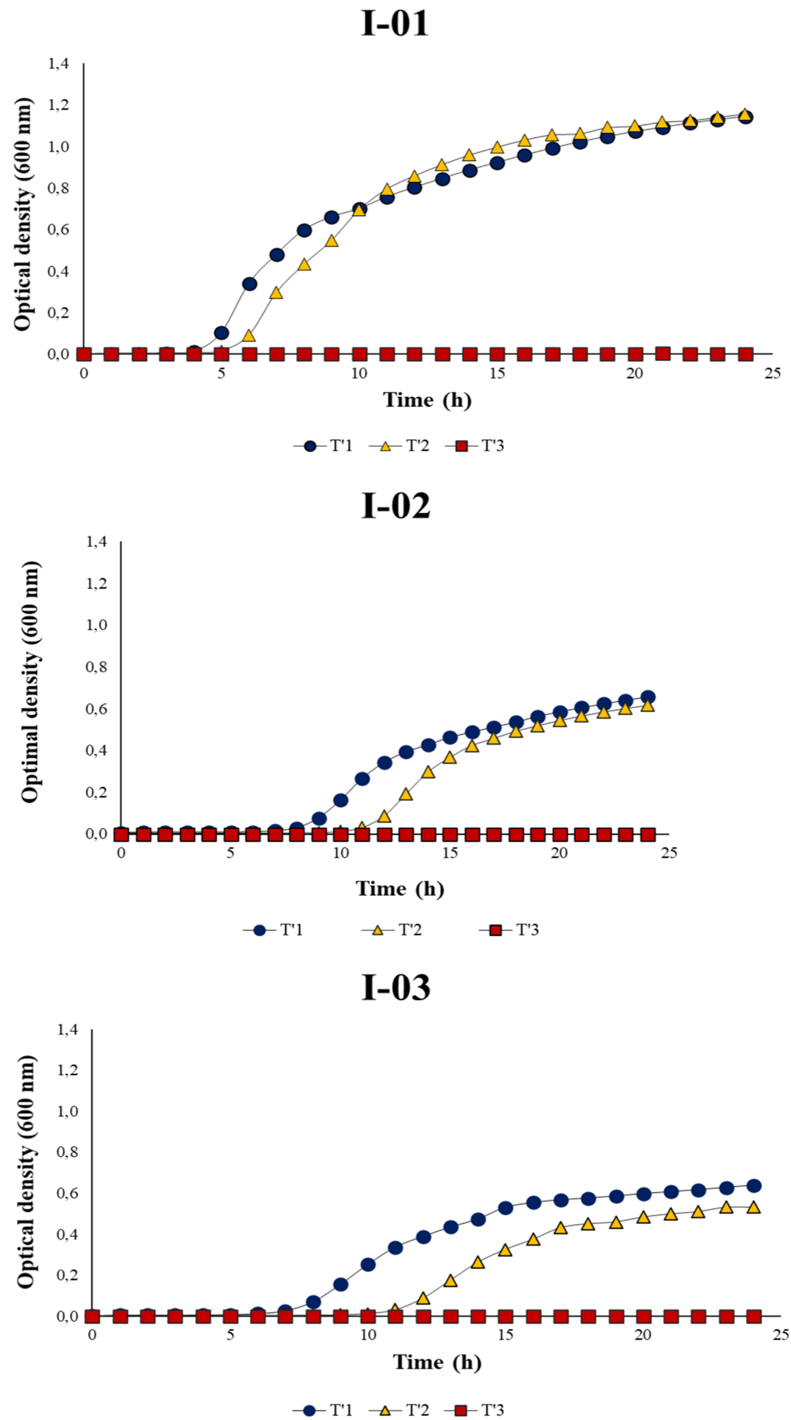

Figure 4. *In vitro* antimicrobial activity, by means of optical density (600 nm), of treatment control (T1), 0.5% JOHA® HBS salt (T2), and preservatives nisin+potassium sorbate (0,0012% and 0,1%, respectively) (T3) in BHI broth.

Unlike the literature (BUŇKOVÁ et al., 2008; LOESSNER et al., 1997; MAIER; SCHERER; LOESSNER, 1999; NOVÁKOVÁ, 2008), the concentration of 0.5% of JOHA® HBS salt had no effect on microorganisms in the in vitro test. In this way, the next step of this work was to determine the minimum inhibitory concentration through *in vitro* and *in situ*

assessments, using salt levels ranging from 0.36% to 2.86%. This concentration range comprehend values lower than the salt concentration used in the dairy matrix (0.5%), but that is still above of values reported to have antimicrobial effect (MAIER; SCHERER; LOESSNER, 1999) and values up to the maximum allowed by the Brazilian legislation regarding to P_2O_5 in final product (BRAZIL, 1997b), considering the technical specification of the salt.

The results from the *in situ* MIC assessment by plate counting showed that at time 0 h the counting ranged from 5 to 6 log CFU/mL for the studied strains at each level of emulsifying salt in the dairy matrix. At time 24 h the counting showed that all treatments presented microorganism growth of 2 log CFU or more, meaning that the different concentrations of salt in the dairy matrix was not sufficient to stop microorganism growth at temperature of 32 °C in 24 hours. The control treatment, where the salt was added to the matrix and no microorganism was inoculated did not present growth of any microorganism at 0 h and 24 h, showing that the growth observed in each treatment corresponds only to the strain inoculated.

From the results of the MIC assessment *in vitro*, showed in Figure 5, it can be observed that the minimum concentration of JOHA® HBS salt to inhibit the multiplication of the studied microorganisms is between 2.86% and 5.71%. However, this value is greater than the maximum that could be added to the product so that the P_2O_5 concentration is within the limit allowed by Brazilian legislation. This value found is also much higher than the maximum limit of JOHA® HBS salt recommended by the manufacturer (1% of the weight of the final product). Also, according to the manufacturer's specifications, the JOHA® HBS salt is used together with other JOHA® emulsifying salts, which would further increase the concentration of P_2O_5 in the final product. Different optical densities (600 nm) at time 0 h that can be observed in Figure 5 may be due to the weak solubility of the salt in the media. Nevertheless, what should be brought to attention is that de optical density (600 nm) keeps raising, suggesting bacterial growth.

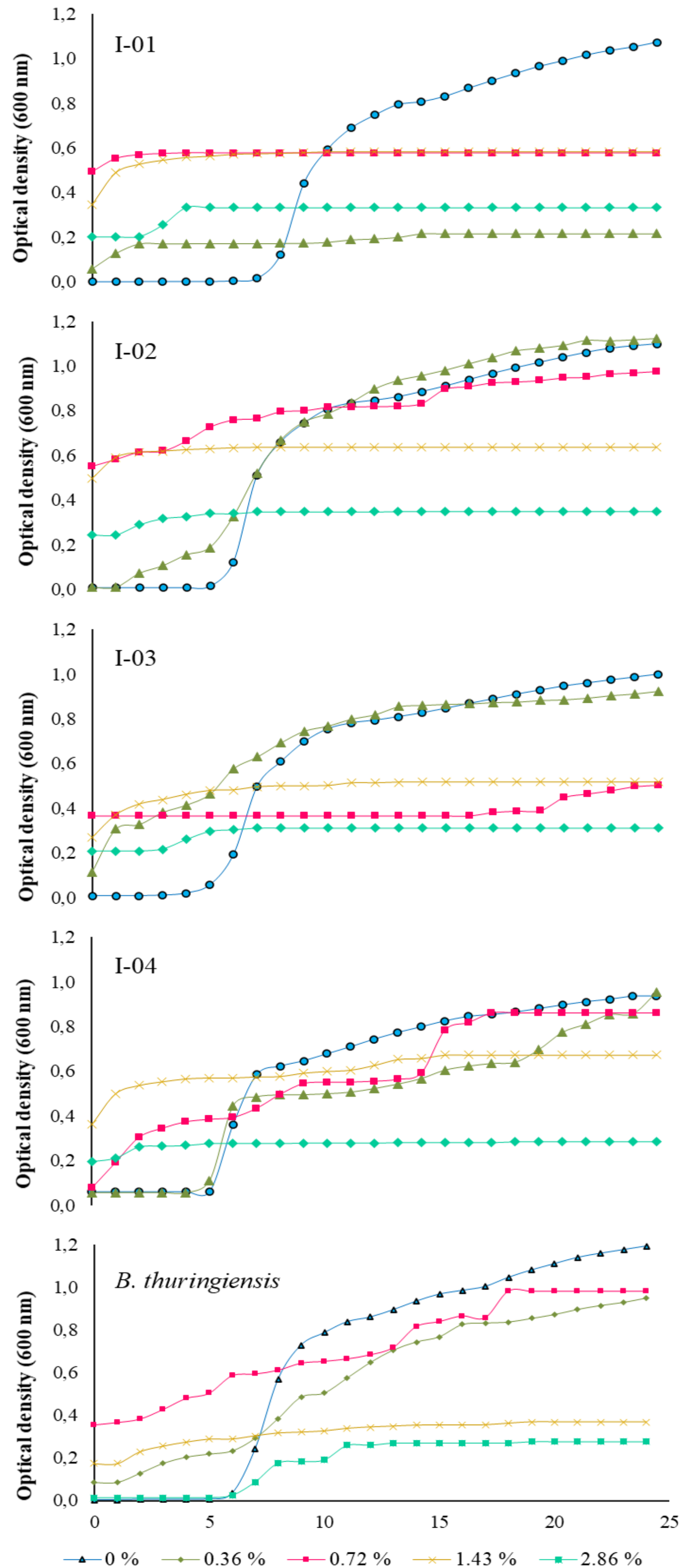

Figure 5. MIC of JOHA® HBS salt against *Bacillus* spp. isolates and reference strain *B. thuringiensis* CFBP 3476 in BHI broth by means of optical density.

Controversial results are found for studies involving the evaluation of microbiological effect of JOHA® HBS. In a study conducted by Buňková et al. (2008), the effects of three commercial emulsifying salts (HBS, S9 and 690) on gram-positive and gram-negative bacteria contaminating food and on bacteria isolated from processed cheeses were evaluated. Among the group of gram-positive contaminating bacteria, sporulated strains of the genus *Bacillus* sp. (*Bacillus cereus* CCM 2010, *B. subtilis* CCM 2216, *B. sphaericus* CCM 1615, *B. brevis* SPSM 4101 and *B. stearothermophilus* SPSM 4103) were studied. The salts were evaluated in different concentrations (0.1; 0.2; 0.3; 0.4 and 0.5% w/v) in nutrient broth, and only the JOHA® HBS salt had a significant inhibitory effect on the strains of gram-positive bacteria, in concentrations ranging from 0.1 to 0.3% (w/v).

In another study conducted by Maier, Scherer and Loessner (1999), it was evaluated the microbial effect of the polyphosphate JOHA® HBS on *Bacillus cereus* vegetative cells and spores in Plate Count Broth, and the results showed that a concentration of 0.01% (w/v) of this salt was capable of lysing 20% of *B. cereus* bacterial cells during the log phase in the 1h period. In turn concentrations of 0.05% and 0.1% (w/v) were able to lysis about 50% and more than 90%, respectively. In addition, they found that the concentrations of 0.05 and 0.1% (w/v) were capable to completely inhibit the growth of *B. cereus* spores.

Nováková (2008) also tested the antimicrobial activity of JOHA® HBS salt on strains of *Bacillus* spp. in Plate Count Agar (PCA) and observed that the concentration of 0.1% (w/v) of the emulsifying salt was capable to decrease approximately one log CFU/mL of strains of *Bacillus cereus* and *B. subtilis.* Also, this concentration was capable to completely inhibit the growth of *B. stearothermophilus*. The authors also showed that at concentration level of 0.5% (w/v) of JOHA® HBS salt the strains of *Bacillus* spp. cited, plus strain of *B. brevis,* showed no growth.

Loessner et al. (1997) evaluated the effect of three emulsifying salts (JOHA® HBS, JOHA® HBS-1 AND JOHA® HBS-9) on *Clostridium tyrobutyricum* cells and spores. The test involving the *in vitro* inhibition of strains, in liquid modified Reinforced Clostridial Medium (RCM) supplemented with varying concentrations (0.01, 0.03, 0.1, 0.3, 0.5 and 1.0% (w/v)) of emulsifying salts showed that the MIC for inhibition was 0.1% (w/v). A slight inhibition was also observed in the concentration of 0.03% (w/v) for the JOHA® HBS salt. In this same work, in situ assessment was performed and the results showed that concentration of 0.5% (w/v) of

polyphosphate was able to delay bacterial growth by 3 weeks and that the concentration of 1% (w/v) completely inhibited cell growth.

Buňková et al. (2008) suggested that the minimum concentration to inhibit the growth of microorganisms in a food matrix is likely to be higher than those determined under laboratory conditions, given that food matrices are complex. The results obtained by MIC assessments in the present study corroborates such statement, as MIC for the *in situ* test was greater than it was in the *in vitro* test. Comparing the results of the present study with the results obtained by Loessner et al. (1997), it is important to note that in their study, they added the emulsifying salt after the production of the dairy matrix and immediately afterwards they added the inoculum of microorganism. In other words, the emulsifying salt did not exercise its technological function, allowing its chelating action to occur on the membrane of the inoculated microorganisms. Contrary, in the present study the emulsifying salt was added during the production of the dairy matrix, which probably meant that its chelating action did not occur on the membrane of the inoculated microorganisms, but in the matrix protein network.

The uncommon results obtained in the present work raised the hypothesis that bacterial resistance could be considered as one reason for the ineffectiveness of the emulsifying salt. Although the literature presents studies in which JOHA® HBS salt had antimicrobial effect on species of *Bacillus* spp., it is important to note that the strains used in the present work are originated from a different environment then those ones used in other researches, thus being likely to have different degree of resistance to the JOHA® HBS salt or other antimicrobial agents.

Together with bacterial resistance, the medium used in the *in vitro* assessments can be another factor to explain the lack of efficiency of JOHA® HBS salt against the studied microorganisms. Studies found in the literature show that the presence of free divalent cations in the medium, such as ions of Ca and Mg interferes with the antimicrobial effect of polyphosphates (MAIER; SCHERER; LOESSNER, 1999). As the medium used in the present study (BHI broth) is a very nutritious media, it is possible that the antimicrobial effect of JOHA® HBS was reverted by cations present in the media. Supplemental information of the study conducted by Damo et al. (2013) supports the suggestion of the presence of cation in BHI broth, as the authors found magnesium and calcium in BHI broth, among other elements.

6. CONCLUSION

The antimicrobial effect of JOHA® HBS salt against strains of *Bacillus* spp. was evaluated and the results showed that the emulsifying salt did not have a satisfactory performance. Although 0.5% of salt showed a similar behavior as preservatives nisin+potassium sorbate in *in situ* assessments regarding to increase/decrease of counting (log CFU/mL) of the bacteria under study during the storage time of the matrices, bacterial growth raised over the time. The results from in vitro assessments showed ineffectiveness of the salt. As the medium used in the *in vitro* assessments could have disrupted the antimicrobial effect of the salt, new assessments must be performed in order to accept or refute such hypothesis. Bacterial resistance to the emulsifying salt also needs to be investigated. In addition, results of the present study suggest that the dairy matrix interferes with the antimicrobial effect of preservatives, nonetheless, more research towards this insight is needed.

7. PERSPECTIVES

Resistance of reference strains of *Bacillus* spp. to emulsifying salt JOHA® HBS should be investigated, as the isolates under study may have greater resistance to such salt. For that, the use of other *in vitro* methodology should be considered. Moreover, analyses with other sporulating microorganisms, as well as their spores should be performed in order to investigate the effect of JOHA® HBS salt on them in dairy matrix, since it is reported in the literature that such salt has effect on sporulation of some bacteria. Evaluation of the effect of other emulsifying salts on the isolates of the present study is important as well, in order to investigate the resistance of such isolates to other emulsifying salts commonly used in processed cheese manufacture. In addition, the same analyses should be performed using other non-sporulating bacteria, as well as mold and yeasts, which are, at some level, likely to be found in processed cheese.

8. REFERENCES

AHMAD, S.; BUTT, M. S.; PASHA, I.; SAMEEN, A. Quality of processed cheddar cheese as a function of emulsifying salt replaced by κ-carrageenan. **International Journal of Food Properties**, v. 19, n. 8, p.1874–1883, 2016.

ALMEIDA, G.; MAGALHÃES, R.; CARNEIRO, L.; SANTOS, I.; SILVA, J.; FERREIRA, V.; HOGG, T.; TEIXEIRA, P. Foci of contamination of Listeria monocytogenes in different cheese processing plants. **International journal of food microbiology**, v. 167, n. 3, p. 303-309, 2013.

ÁVILA, M.; GÓMEZ-TORRES, N.; HERNÁNDEZ, M.; GARDE, S. Inhibitory activity of reuterin, nisin, lysozyme and nitrite against vegetative cells and spores of dairy-related Clostridium species. **International journal of food microbiology**, v. 172, p. 70-75, 2014.

BARANCELLI, G. V.; CAMARGO, T. M.; GAGLIARDI, N. G.; PORTO, E.; SOUZA, R. A.; CAMPIONI, F.; FALCÃO, J. P.; HOFER, E.; CRUZ, A. G.; OLIVEIRA, C. A. Pulsed-Field Gel Electrophoresis characterization of Listeria monocytogenes isolates from cheese manufacturing plants in São Paulo, Brazil. **International Journal of Food Microbiology**, v. 173, p. 21-29, 2014.

BRACEY, D., HOLYOAK, C., COOTE, P. Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? **Journal of Applied Microbiology**, v. 85, p. 1056-1066, 1998.

BRASIL DAIRY TRENDS. ZACARCHENCO, P. B.; VAN DENDER, A. G. F.; REGO, R. A. **Tendências do Mercado de Produtos Lácteos**. 1. ed. – Campinas: ITAL, 2017. Available in: http://www.brasildairytrends.com.br/

BRAZIL, Decreto nº 9.013, de 29 de março de 2017: dispõe sobre o regulamento da inspeção industrial e sanitária de produtos de origem animal, que disciplina a fiscalização e a inspeção industrial e sanitária de produtos de origem animal, instituídas pela Lei nº 1.283, de 18 de dezembro de 1950 , e pela Lei nº 7.889, de 23 de novembro de 1989. **Diário Oficial da União**, Brasília, DF, de 30 março de 2017.

BRAZIL, Resolução da Diretoria Colegiada – RDC nº 359, de 04 de setembro de 1997: Aprova regulamento técnico para fixação de identidade e qualidade de queijo processado ou fundido, processado pasteurizado e processado ou fundido U.H.T. (UAT). **Diário Oficial da União**, Brasília, DF, 08 de setembro de 1997a.

BRAZIL, Resolução da Diretoria Colegiada – RDC nº 356, de 04 de setembro de 1997: Aprova regulamento técnico para fixação de identidade e qualidade de requeijão ou requesión **Diário Oficial da União**, Brasília, DF, 08 de setembro de 1997b.

BUŇKOVÁ, L.; PLEVA, P.; BUŇKA, F.; VALÁŠEK, P.; KRÁČMAR, S. Antibacterial effects of commercially available phosphates on selected microorganisms. **Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis**, v. 56, n. 5, p. 19–24, 2008.

CUNHA, C. R.; DIAS, A. I.; VIOTTO, W. H. Microstructure, texture, colour and sensory evaluation of a spreadable processed cheese analogue made with vegetable fat. **Food Research International**, v. 43, n. 3, p. 723-729, 2010.

DAELMAN, J.; VERMEULEN, A.; WILLEMYNS, T.; ONGENAERT, R.; JACXSENS, L.; UYTTENDAELE, M.; DEVLIEGHERE, F. Growth/no growth models for heat-treated psychrotrophic *Bacillus cereus* spores under cold storage. **International Journal of Food Microbiology**, v. 161, n. 1, p. 7-15, 2013.

DALMASSO, M.; JORDAN, K. Absence of growth of Listeria monocytogenes in naturally contaminated Cheddar cheese. **The Journal of Dairy Research**, v. 81, n. 1, p. 46, 2014.

DAMO, M. S.; KEHL-FIE, T. E.; SUGITANI, N.; HOLT, M. E.; RATHI, S.; MURPHY, W. J.; ZHANG, Y.; BETZ, C.; HENCH, L.; FRITZ, G.; SKAAR, E. P.; CHAZIN, W. J. Molecular basis for manganese sequestration by calprotectin and roles in the innate immune response to invading bacterial pathogens. **Proceedings of the National Academy of Sciences**, v. 110, n. 10, p. 3841-3846, 2013.

DELVES-BROUGHTON, J.; BLACKBURN, P.; EVANS, R. J.; HUGENHOLTZ, J. Applications of the bacteriocin, nisin. **Antonie Van Leeuwenhoek**, v. 69, n. 2, p. 193-202, 1996.

DIAS, M. A. C.; SANT'ANA, A. S.; CRUZ, A. G.; JOSÉ DE ASSIS, F. F.; DE OLIVEIRA, C. A. F.; BONA, E. On the implementation of good manufacturing practices in a small processing unity of mozzarella cheese in Brazil. **Food control**, v. 24, n. 1-2, p. 199-205, 2012.

FAO – FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. Committee on Milk and Milk Products (CCMMP): General standard for processed cheese. **Report of 40th Session of the Codex Alimentarius Commission - REP17/CAC**, p. 15-16, 2017.

FDA – U. S. FOOD AND DRUG ADMINISTRATION - **Code of Federal Regulations Title 21: Part 133** – Cheeses and related cheese products, 2019. Available in https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=133.

FOX, P. F.; GUINEE, T. P.; COGAN, T. M.; MCSWEENEY, P. L. Processed cheese and substitute/imitation cheese products. In: **Fundamentals of cheese science**. Springer, Boston, MA, 2017. p. 589-627.

FREESE, E.; SHEU, C.W.; GALLIERS, E. Function of lipophilic acids as antimicrobial. **Food Additives**, v. 241, n. 5388, p. 321–325, 1973.

GLASS, K., & DOYLE, M. E. Fri food safety review: Safety of processed cheese. Madison**: Fri Briefings.** Retrieved from: http://fri.wisc.edu/docs/pdf/ ProcCheese_may2005_v2.pdf, 2005.

GLIGUEM, H.; GHORBEL, D.; GRABIELLE-MADELMONT, C.; GOLDSCHMIDT, B.; LESIEUR, S.; ATTIA, H.; OLLIVON, M.; LESIEUR, P. Water behaviour in processed cheese spreads. **Journal of Thermal Analysis and Calorimetry**, v. 98, n. 1, p. 73–82, 2009.

GÓMEZ-TORRES, N.; GARDE, S.; PEIROTÉN, Á.; ÁVILA, M. Impact of Clostridium spp. on cheese characteristics: microbiology, color, formation of volatile compounds and off-flavors. **Food Control**, v. 56, p. 186-194, 2015.

GOUDA, A.; ABOU EL-NOUR, A. Cheeses | Processed cheese. *In*: CABALLERO, B.; FINGLAS, P.; TOLDRA, F. **Encyclopedia of food sciences and nutrition.** 2nd Ed. Academic Press, 2003. p.1108–1115.

GROSS, E.; MORELL, J. L. Structure of nisin. **Journal of the American Chemical Society**, v. 93, n. 18, p. 4634–4635, 1971.

GUINEE, T. P. The role of dairy ingredients in processed cheese products. *In:* CORREDIG, M. **Dairy-Derived Ingredients**, Oxford, U K. Ed. Woodhead Publishing, 2009. p. 507–538.

HENNING, S.; METZ, R.; HAMMES, W. P. Studies on the mode of action of nisin. **International Journal of Food Microbiology**, v. 3, n. 3, p. 121–134, 1986.

HEYNDRICKX, M. The importance of endospore-forming bacteria originating from soil for contamination of industrial food processing. **Applied and Environmental Soil Science**, v. 2011, 2011.

JANA, A.; PADHIYAR, D.; CHAVAN, R. S. Role of Emulsifying Salts in Cheese Products. **Dairy Engineering: Advanced Technologies and Their Applications**, p. 53, 2017.

KAPOOR, R.; METZGER, L. E. Process cheese: Scientific and technological aspects—A review. **Comprehensive Reviews in Food Science and Food Safety**, v. 7, n. 2, p. 194-214, 2008.

LEE, R. M.; HARTMAN, P. A.; OLSON, D.G.; WILLIAMS, F. D. Bactericidal and bacteriolytical effects of selected foodgrade phosphates using *Staphylococcus aureus* as a model system. **Journal of Food Protection**, 57: 276-283(1994).

LOESSNER, M. J.; MAIER, S. K.; SCHIWEK, P.; SCHERER S. Long-chain polyphosphates inhibit growth of *Clostridium tyrobutyricum* in processed cheese spreads. **Journal of Food Protection**, v. 60, n. 5, p. 493–498, 1997.

LOPEZ-BREA, S. G.; GÓMEZ-TORRES, N.; ARRIBAS, M. A. 2017. Spore-forming bacteria in dairy products. *In*: POLTRONIERI, P. **Microbiology in Dairy Processing**, West Sussex, UK. FT Press Series, Wiley-Blackwell, 2017. p. 11-36.

LÜCKING, G.; STOECKEL, M.; ATAMER, Z.; HINRICHS, J.; EHLING-SCHULZ, M. Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. **International Journal of Food Microbiology**, v. 166, n. 2, p. 270-279, 2013.

MAIER, S. K.; SCHERER, S.; LOESSNER, M. J. Long-chain polyphosphate causes cell lysis and inhibits *Bacillus cereus* septum formation, which is dependent on divalent cations. **Applied and Environmental Microbiology**, v. 65, n. 9, p. 3942–3949, 1999.

MÁRQUEZ, I. J. G.; MCKAY, B.; WONG, A.; CHEETHAM, J. J.; BEAN, C.; GOLSHANI, A.; SMITH, M. L. Mode of action of nisin on *Escherichia coli*. **Canadian Journal of Microbiology**, v. 66, n. 2, p. 161-168, 2019.

MELO, J.; ANDREW, P. W.; FALEIRO, M. L. *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: The role of stress responses. **Food Research International**, v. 67, p. 75-90, 2015.

MOHAMMED, H. O.; STIPETIC, K.; MCDONOUGH, P. L.; GONZALEZ, R. N.; NYDAM, D. V.; ATWILL, E. R. Identification of potential on-farm sources of Listeria monocytogenes in herds of dairy cattle. **American journal of veterinary research**, v. 70, n. 3, p. 383-388, 2009.

MORALES, M. C. S.; JUÁREZ, A. C.; MONTICH, G. G.; ÁLVAREZ, R. M. S. Interaction of the antibiotic peptide nisin with anionic membranes in different phase-states: a vibrational study. **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,** v. 215, p. 389-397, 2019.

MORANDI, S.; CREMONESI, P.; SILVETTI, T.; CASTIGLIONI, B.; BRASCA, M. Development of a triplex real-time PCR assay for the simultaneous detection of *Clostridium beijerinckii, Clostridium sporogenes* and *Clostridium tyrobutyricum* in milk. **Anaerobe**, v. 34, p. 44-49, 2015.

NOVÁKOVÁ, A. **Antimicrobial Effects of Linear Polyphosphates for Chosen Microorganisms**. Tomas Bata University in Zlín. Faculty of Technology. Diploma Work. 2008. Available in http://dspace.k.utb.cz

OBRITSCH, J. A.; RYU, D.; LAMPILA, L. E.; BULLERMAN, L. B. Antibacterial effects of long-chain polyphosphates on selected spoilage and pathogenic bacteria. **Journal of Food Protection**, v. 71, n. 7, p. 1401–1405, 2008.

OLIVEIRA, R. B.; MARGALHO, L. P.; NASCIMENTO, J. S.; COSTA, L. E.; PORTELA, J. B.; CRUZ, A. G.; SANT'ANA, A. S. Processed cheese contamination by spore-forming bacteria: A review of sources, routes, fate during processing and control. **Trends in Food Science & Technology**, v. 57, p. 11-19, 2016.

OLIVEIRA, R. B.; LOPES, L. S., BAPTISTA, R. C., CHINCHA, A. A., PORTELA, J. B., NASCIMENTO, J. S.; COSTA, L. E. O.; SANT'ANA, A. S. Occurrence, populations, diversity, and growth potential of spore-forming bacteria in "requeijão cremoso". **LWT**, v. 89, p. 24-31, 2018.

PUNYAUPPA-PATH, S.; PHUMKHACHORN, P.; RATTANACHAIKUNSOPON, P. Nisin: production and mechanism of antimicrobial action. **International Journal of Current Research and Review**, v. 7, n. 2, p. 47-53, 2015.

PEREIRA, D. B. C.; SILVA, P. H. F. COSTA JÚNIOR, L. C. G.; OLIVEIRA, L. L.. **Físico-química do leite e derivados - métodos analíticos**. 2. ed. Juiz de Fora: Templo, 2001. v. 1. 234p.

PURNA, S. K. G.; POLLARD, A.; METZGER, L. E. Effect of formulation and manufacturing parameters on process cheese food functionality – I. Trisodium citrate. **Journal of Dairy Science**, v. 89, n. 7, p. 2386–2396, 2006.

SHIRASHOJI, N.; JAEGGI, J. J.; LUCEY, J. A. Effect of trisodium citrate concentration and cooking time on the physicochemical properties of pasteurized process cheese. **Journal of Dairy Science**, v. 89, n. 1, p. 15–28, 2006.

SNYDER, L. D.; MAXCY, R. B. Effect of a_w of meat products on growth of radiation resistant Moraxella-Acinetobacter. **Journal of Food Science,** v. 44, n.1, p. 33–36, 1979.

SOFOS, J. N.; PIERSON, M. D.; BLOCHER, J.C.; BUSTA, F.F. Mode of action of sorbic acid on bacterial cells and spores. **International Journal of Food Microbiology**, v. 3, n.1, p. 1–17, 1986.

TAMINE, A. Y. Processed cheese and analogues: an overview. *In:* ______. (org.). **Processed cheese and analogues**. Chichester: Blackwell Publishing, 2011.

USDA – UNITED STATES DEPARTAMENT OF AGRICULTURE. Dairy Data. **Per capita consumption of selected cheese varieties (Annual).** 2019. Available in: https://www.ers.usda.gov/data-products/dairy-data

VAN DENDER, A. G. F.; MORENO, I.; ALVES, A. T. S.; SPADOTI, L. M. Requeijão e outros queijos fundidos: tecnologia de fabricação e parâmetros de controle de processo. *In:* VAN DENDER, A. G. F. **Requeijão cremoso e outros queijos fundidos**. 2ª Ed. Revisada e ampliada. São Paulo: Setembro Editora, 2014. p.57–90.

VAN DENDER, A. G. F.; ZACARCHENCO, P. B. Requeijão e outros queijos fundidos: história e aspectos gerais. *In:* VAN DENDER, A. G. F. **Requeijão cremoso e outros queijos fundidos**. 2ª Ed. Revisada e ampliada. São Paulo: Setembro Editora, 2014. p.21–28.

WAGNER, M. K.; BUSTA, F. F. Inhibition of *Clostridium botulinum* 52A toxicity and protease activity by sodium acid pyrophosphate in media systems. **Journal of Applied and Environmental Microbiology**, v. 50, n. 1, p. 16-20, 1985.

WAGNER, M. K.; BUSTA, F. F. Association of [^{32}P] with *Clostridium botulinum* 52A vegetative cells following growth in a medium containing sodium dihydrogen [^{32}P]-pyrophosphate. **Journal of Food Protection**, v. 49, n. 5, p. 352–354, 1986.

WILLIAMS, G. C.; DELVES-BROUGHTON, J. Nisin. *In*: CABALLERO, B.; FINGLAS, P.; TOLDRA, F. **Encyclopedia of Food Sciences and Nutrition**, 2nd Ed. Academic Press, 2003, p. 4128-4135.

ZAIKA, L. L.; SCULLEN, O. J.; FANELLI, J. S. Growth inhibition of *Listeria monocytogenes* by sodium polyphosphate as affected by polyvalent metal ions. **Journal of Food Science**, v. 62, n. 4, p. 867–872, 1997.

ZEINAB, S.; REFAAT, B.; ABD EL-SHAKOUR, E. H.; MEHANNA, N. S.; HASSAN, M. S. Potential sources of aerobic and anaerobic spore former bacteria in processed cheese. **Research Journal Of Pharmaceutical, Biological And Chemical Sciences**, v. 6, n. 1, p. 757-769, 2015.

ZESSIN, K. G.; SHELEF, L. A. Sensitivity of *Pseudomonas* strains to polyphosphates in media systems. **Journal of Food Science**, v. 53, n. 2, p. 669–670, 1988.

ZWIETERING, M. H.; DE WIT, J. C.; NOTERMANS, S. Application of predictive microbiology to estimate the number of *Bacillus cereus* in pasteurised milk at the point of consumption. **International Journal of Food Microbiology**, v. 30, n. 1-2, p. 55-70, 1996.