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# INFLUENCE OF NISIN AND LAURYL ARGININE ESTER AGAINST SOME FOODBORNE PATHOGENS IN RECOMBINED FETA AND PROCESSED SPREAD CHEESE

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## ABSTRACT

The aim of this study was to evaluate the impact of nisin with lauryl arginine ester (LAE) or their combination by 1: 3 (w/w) on cheese preservation. The study was carried out against the most common foodborne pathogens in recombined feta cheese (RFC) and processed cheese spread (PCS). Combination of nisin with LAE has higher synergistic preservative effect on different widespread foodborne pathogens such as *Bacillus subtilis*, *Clostridium sporogenes* and *Escherichia coli* compared with individual one. The most sensitive strain was *E. coli* with an effective minimal inhibition concentration (MIC) of 400 ppm, whereas the former spore-forming bacterial strains were totally inhibited using 700 ppm from the combination, respectively. Synergistic combination blend was added by its recommended MIC (700 ppm) in the manufacture of both RFC and PCS. The results indicated that it is efficient enough to inhibit the growth of the most common foodborne pathogens in cheese after their storage for 7 days (RFC) and 30 days (PCS).

## PRACTICAL APPLICATIONS

Inhibiting the foodborne pathogens by the addition of lauryl arginine ester (LAE) and nisin together by 1: 3 (w/w) increased the cheese quality. However, the possibility of contamination of traditional cheese during the preservation in terms of food safety can be controlled by the addition of the two mentioned additives (i.e., LAE and nisin) and can be manufacture and distribute in the dairy industries.

## INTRODUCTION

In developing countries where warm humid climate and bad storage conditions prevail, large amounts of dairy products are lost every year due to spoilage caused by the growth of several foodborne microorganisms that grow during their storage periods. Milk-borne outbreaks were responsible for 25% of all outbreaks due to contaminated food and water (United States Public Health Service and Food and Drug Administration 2011). Even today, human illnesses related to the consumption of unpasteurized and also pasteurized dairy products remain a public health problem (Anaelom *et al.* 2010; European Food Safety Authority 2012).

Among dairy pathogens, *Bacillus* and *Clostridium* (gram-positive spore-forming thermoduric bacteria) are considered to be the most existing pathogens in various dairy products (Papademas 2015). Some species of *Bacillus* are capable of producing gastroenteritis such as *B. cereus* and *B. coagulans* or producing anthrax such as *B. anthracis*, whereas some species of *Clostridium* are responsible for gastroenteritis (*C. perfringens*) and botulism (*C. botulinum*) (Papademas 2015). Sperber and Doyle (2010) reported that the latter pathogens are responsible for a variety of cheese

defects, such as excessive softening of cheese, splits and cracks, off-flavors and abnormal color. Latter spore-formers produce spores that can survive after pasteurization, causing several microbiological defaults after vegetation (Munsch-Alatossava and Alatossava 2006). According to Sharaf *et al.* (2014), the prevalence rates of *Bacillus* and *Clostridium* spp. were 2 and 1% in recombined feta cheese (RFC) samples, respectively. As for processed cheese spread (PCS), another study reported about the same percentages in the PCS samples (Glass and Doyle 2013).

Some other serotypes of pathogenic *Escherichia coli* are associated frequently with milk spoilage and several publications have been published on their pathogenicity (Oliver *et al.* 2009). Pathogenic *E. coli* pathotypes can be divided into two large groups. The intestinal pathogenic *E. coli* not only colonize human intestine but also cause diseases ranging from mild diarrhea to severe colitis and dysentery as well. The extraintestinal pathogenic *E. coli* reside in the intestine asymptomatically but can cause severe infection upon reaching extraintestinal niches such as the urinary tract or bloodstream (Shpigel *et al.* 2008). About the prevalence of *E. coli* in milk, a recent study (Garnica *et al.* 2013) reported that a total of 752 bulk tank milk samples from 205 dairy sheep flocks belonging to Consortium for Ovine Promotion were collected between January and December 2011. Four samplings were carried out in each flock, one per season, throughout one year. *E. coli* was present in 17.4% of the samples and 50.7% of the flocks throughout the year. It was also reported the prevalence of *E. coli* was at its highest limit in autumn and winter coinciding with a rainy weather. Post-contamination may occur after milk pasteurization and this is the only critical hazard step that could permit *E. coli* to post-contaminate pasteurized milk, RFC and PCS and grows further.

Since food safety is an imperative issue for consumers, food manufacturers and government officials (Hoffmann *et al.* 2012), several methods were reported to preserve food. Among such methods is the use of food-grade natural preservatives to extend the shelf life of food products. Nisin is a well-known broad spectrum Food and Drug Administration (FDA)-approved bacteriocin active against gram-positive pathogens associated with foods (Delves-Broughton 2014). Its use as a food biopreservative is limited by the lack of effect against gram-negative bacteria. Moreover, the development of nisin resistance has been reported in sensitive gram-positive pathogens such as some mutants of *Bacillus* and *Clostridium* (Zhou *et al.* 2014) at normal concentrations. Furthermore, Zapico *et al.* (1999) reported that nisin was absorbed to protein and fat globules in dairy products, thus preventing its activity.

Several studies have shown that the nisin spectrum activity may be extended to gram-negative bacteria such as *E. coli* by using it in combination with other agents (Cutter and Siragusa 1995a). Many reports have been published on the synergistic antimicrobial effects of nisin with sucrose fatty acid esters (Thomas *et al.* 1998), the lactoperoxidase system (Boussouel *et al.* 1999), thymol (Ettayebi *et al.* 2000) and carbon dioxide (Nilsson *et al.* 2000).

Lauryl arginine ester (LAE) is a cationic surfactant, ethyl-Nα-lauroyl-L-arginate- HCl, which has a broad inhibitory spectrum against gram-positive and gram-negative bacteria, yeasts and molds (Bakal and Diaz 2005). It may be demonstrated as ethyl lauroyl arginate which acts as a potential alternative for some of the currently approved preservatives such as sulfites, benzoates and sorbates, which have some inherent limitations (Exposito 2006).

The mode of action of ethyl lauroyl arginate includes surface tension reduction and the formation of ionic aggregates leading to changes in the conductivity and solubility of cell membranes that can lead to permanent alterations in cell permeability and growth inhibition or microorganism inactivation (Rodriguez *et al.* 2004).

Therefore, this study was performed to apply hurdle technology via determining the synergistic effect of nisin and LAE on RFC as well as PCS as models of comment consumed cheese in Egypt. Synergistic combination was also examined for its antimicrobial activity against the most widely spread foodborne spore-formers (*Bacillus subtilis* and *Clostridium sporogenes*) and the common food spoilage microorganism (*E. coli*).

## MATERIALS AND METHODS

### Bacterial Strains and Media

*B. subtilis* DB100 host was obtained from the Department of Food Technology, Faculty of Agriculture, Alexandria University. *C. sporogenes* DSM1446 was provided by Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University. *E. coli* DH5α (wild type) was obtained from Genetic Engineering and Biotechnology Research Institute (GEBRI), City for Scientific Research and Technological Applications (SRTA-City). *B. subtilis* and *E. coli* strains were grown in Luria–Bertani medium (Atlas 2004) at 37C. *C. sporogenes* was grown in the Reinforced Clostridial Medium (RCM) medium (Atlas 2004) at 37C.

### Chemicals

Nisinpro (nisin) was purchased from Zhengzhou ChiHon Biotechnology Co., Ltd. (Chihonbio, China). LAE was obtained from Vedeqsa (Barcelona, Spain). Nisin as well as LAE stock solutions were prepared as 10,000 ppm and were sterilized using 0.45-μm filter paper (Sigma, Munich, Germany) and kept in a refrigerator at 4C until use.

## RFC Processing

According to Robinson and Tamime (1996), a mix preparation in the APV Flex-Mix Instant was prepared. The RFC ingredients were as follows: 20% palm oil, 8% skimmed milk powder, 7.2% milk protein concentrate (70% protein), 2.6% sodium chloride, 2.8% GDL (Gluco Delta Lactone), 0.1% potassium sorbate and the examined preservatives (nisin or LAE or their combinations). The Flex-Mix Instant tank is filled with water. The temperature is dependent on the fat/stabilizer requirement. Melted fat is pumped into the Flex-Mix Instant tank under vacuum and during high shear mixing, forming an oil-in-water emulsion. Functional milk proteins, stabilizers and other dry ingredients are also added to the instant tank under vacuum and during high shear mixing. After mixing, a suitable hydration time is applied. After sufficient hydration, the mix is pasteurized and cooled. Rennet and other acidifying ingredients such as GDL were added. At this stage, nisin (at serial concentrations from 100 to 1,000 ppm) or LAE (at serial concentrations from 100 to 1,000 ppm) or their combinations (from 1:1 to 1:5 and vice versa) were added separately for each preparation and the mix is filled into packs. Finally, salt is added and acidification takes place at mesophilic temperature. For positive controls of RFC, *Bacillus* as well as *Clostridium* cultures were added at ($2 \times 10^4$ cfu/mL) to reconstituted milk before pasteurization step, while *E. coli* culture was added at ($2 \times 10^4$ cfu/mL) after pasteurization step to stimulate that post-contamination has occurred. The RCF and their controls samples were collected after storage for 3 days at 4C for further microbiological analysis since the cheese was spoiled after 7 days of storage period.

## PCS Preparation

PCS was prepared as described by Meyer (1973). PCS was made in pilot processing machine (WOLF Anlagen-Technik GmbH & Co. KG Geisenfeld, Germany) at 1,400 rpm, homogenization at 200 bar, total time of acidification is 2 min and heating was at 85C/6 min. Fifty percent of the total amount of water was added before heat treatment, the rest of the amount of water was added when temperature reached 98C and then the temperature was dropped rapidly to 86C. At this stage, nisin (at serial concentrations from 100 to 1,000 ppm) or LAE (at serial concentrations from 100 to 1,000 ppm) or their combinations (from 1:1 to 1:5 and vice versa) were added separately for each preparation, and then the cheese was filled at 74C.

The major steps in process cheese manufacture can be divided into two stages. The first stage consists of ingredient formulation including selection and grinding of natural cheese (on the basis of age, pH, flavor and intact casein content), selection of appropriate emulsifying salt, and formation and computation of other ingredients (in order to meet the targeted moisture, fat, salt and pH values of the final product as per governmental regulations). The second stage consists of process cheese processing and storage. The ingredient blend is processed using heat and mixing to produce a homogeneous mass, which is packaged and cooled. The minimum cook temperature (indirect heating) and time specified for PCS was used (FDA 2006) to preserve the organoleptic properties of the final cheese. For positive controls of PCS, *Bacillus* as well as *Clostridium* cultures were added at ($2 \times 10^4$ cfu/mL) to reconstituted milk before pasteurization step, while *E. coli* culture was added at ($2 \times 10^4$ cfu/mL) after pasteurization step to stimulate that post-contamination has occurred. The PCS and their controls samples were collected after storage for 3 days for further microbiological analysis and the cheese was left for an extended storage period until its spoilage (after 30 days).

## RCF Cheese Preparation for Microbiological Analysis

According to Kourkoutas *et al.* (2006), representative 10-g portions of duplicate samples and controls taken from the cheese interior were blended with 90 mL of sterilized Ringer solution (1/4 strength) and subjected to serial dilutions.

## PCS Cheese Preparation for Microbiological Analysis

According to Muir *et al.* (1999), representative 10-g portions of duplicate samples and controls taken from the cheese interior were dispersed in 90 mL sterile solution of trisodium citrate (0.07 M/L) and subjected to serial dilutions.

## Inhibitory Effect of Either Nisin or LAE or Their Preferred Combination (1:3, w/w)

According to Mayr-Harting *et al.* (1972), RFC and PCS and their prepared samples for microbiological analysis were subjected to serial dilutions. Each dilution was spread over a Petri dish containing nutrient agar (Difco, NJ, USA). Colonies were counted after overnight incubation of inoculated Petri dishes at the optimum growth conditions (37C, aerobically for the detection of each *E. coli* and *Bacillus*, and 37C anaerobically for the detection of *Clostridium*).

# RESULTS

## Antimicrobial Activity of Nisin

After 24 h of incubation, it was observed that nisin has an ascending antimicrobial activity against *B. subtilis* DB100 host and *C. sporogenes* DSM1446 with its gradual increase

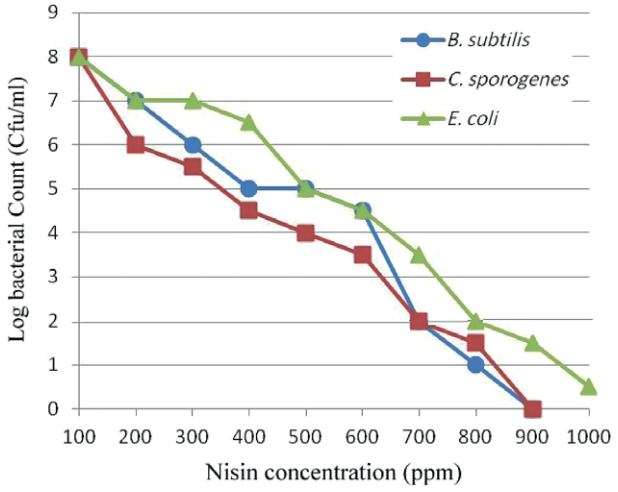


**FIG. 1.** ANTIMICROBIAL ACTIVITY OF NISIN AGAINST *Bacillus subtilis* DB100 HOST (●), *Clostridium sporogenes* DSM1446 (■) AND *Escherichia coli* DH5A (WILD TYPE) (▲)

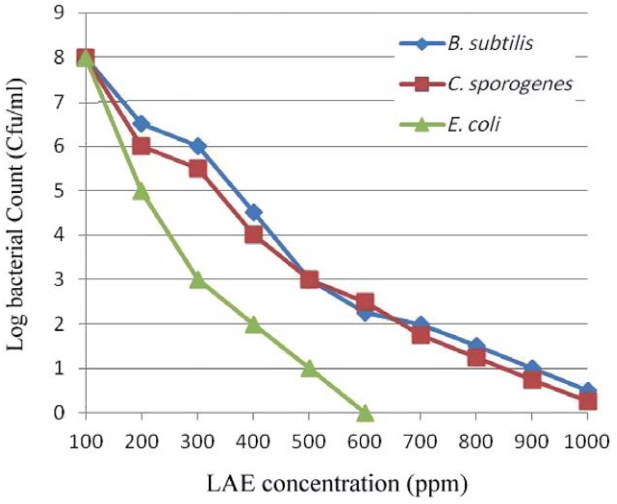


**FIG. 3.** ANTIMICROBIAL ACTIVITY OF LAE AGAINST *Bacillus subtilis* DB100 HOST (◆), *Clostridium sporogenes* DSM1446 (■) AND *Escherichia coli* DH5A (WILD TYPE) (▲)

in concentration. However, it has a weak antimicrobial activity against *E. coli* DH5α (wild type) (Fig. 1). The nisin minimal inhibition concentration (MIC) was 900 ppm for *B. subtilis* DB100 host and *C. sporogenes* DSM1446 and 1,000 ppm for *E. coli* DH5α. Even after using 1,000 ppm nisin, *E. coli* DH5α formed very tiny colonies, indicating its bacteriostatic rather than bactericidal activity. Figure 2 shows that the nisin MIC that inhibited both *B. subtilis* DB100 host and *C. sporogenes* DSM1446 did not affect the growth of *E. coli* DH5α completely.

## Antimicrobial Activity of LAE

The antimicrobial activity of LAE was investigated against *B. subtilis* DB100 host, *C. sporogenes* DSM1446 and *E. coli* DH5α (wild type). LAE had a strong activity against *E. coli* DH5α (wild type) after 24 h of incubation (Fig. 3) as it needed only 600 ppm LAE to be completely inhibited. However, it had moderate antimicrobial activity against both *B. subtilis* DB100 host and *C. sporogenes* DSM1446 as

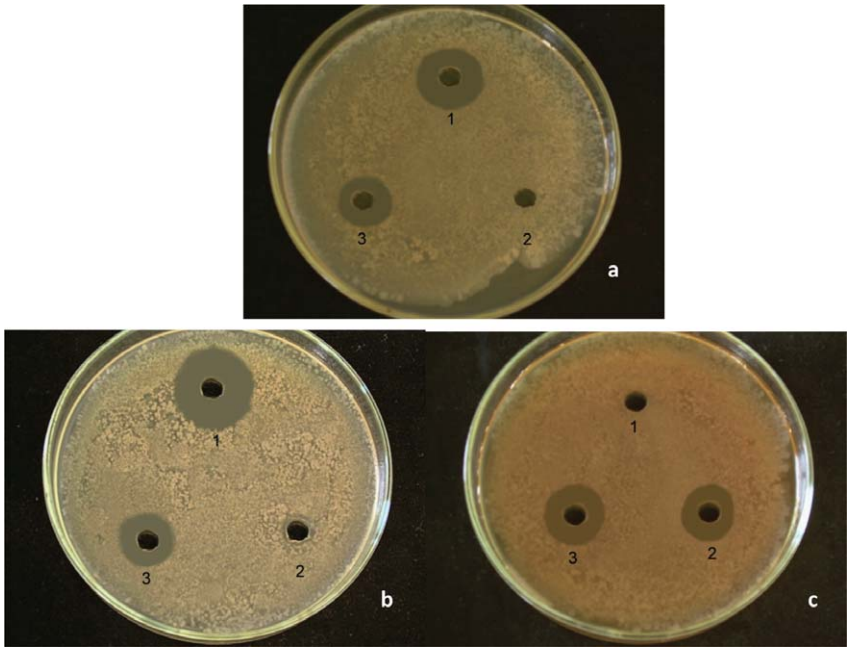


**FIG. 2.** THE EFFECT OF NISIN (1) OR LAE (2) OR THE BLEND (3) ON *Bacillus subtilis* DB100 HOST (A) OR *Clostridium sporogenes* DSM1446 (B) OR *Escherichia coli* DH5A (C)

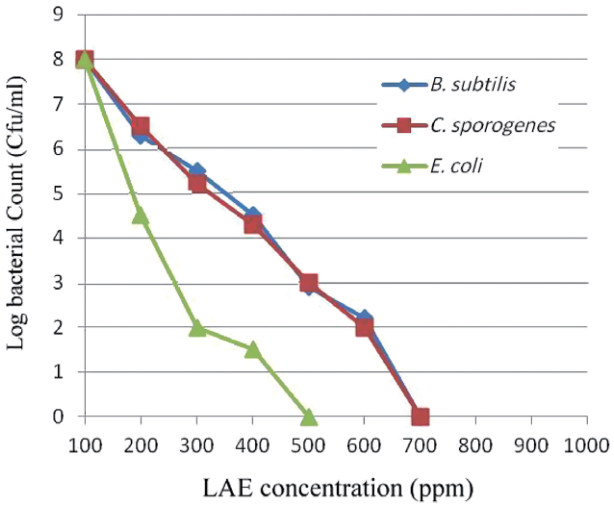


**FIG. 4.** ANTIMICROBIAL ACTIVITY OF SYNERGISTIC COMBINATION AGAINST *Bacillus subtilis* DB100 HOST (◆), *Clostridium sporogenes* DSM1446 (■), AND *Escherichia coli* DH5A (WILD TYPE) (▲)

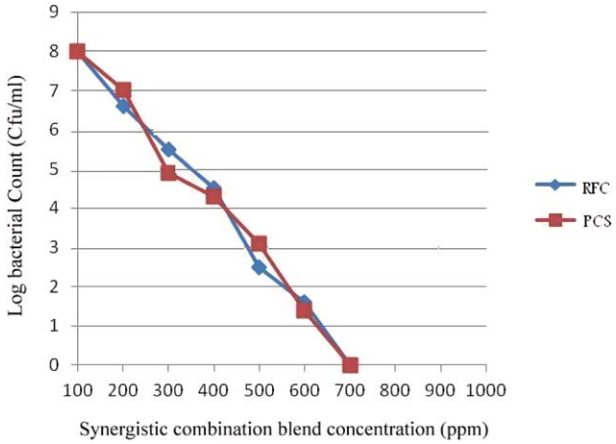


**FIG. 5.** EFFECT OF SYNERGISTIC COMBINATION BLEND (700 PPM) THAT INHIBITS THE GROWTH OF FOODBORNE PATHOGENS IN RECOMBINED FETA CHEESE (RFC) (◆) AND PROCESSED CHEESE SPREAD (PCS) (■)

their LAE MIC was 1,000 ppm. Even after using 1,000 ppm LAE, *B. subtilis* and *C. sporogenes* continued to grow due to the nonspecificity of LAE to influence different gram-positive bacteria. Figure 2 shows that the nisin MIC that inhibited both *B. subtilis* DB100 host and *C. sporogenes* DSM1446 did not affect the growth of *E. coli* DH5α completely.

### Synergistic Effect of Nisin and LAE against Pathogens

It was observed that nisin in combination with different concentrations of LAE (1:2 and 1:3 w/w) had a strong antimicrobial activity with a clear synergistic effect. The synergistic combination minimal antimicrobial concentration (MIC) was 700 ppm for both *B. subtilis* DB100 host and *C. sporogenes* DSM1446 and 400 ppm for *E. coli* DH5α. Such concentrations are significantly low in comparison with that of nisin and LAE separately. Figure 4 indicates the antimicrobial activity of synergistic combination against the above pathogens. From the previous figure, it was indicated that the synergistic combination that composed of nisin : LAE (by 1:3, w/w) is the most efficient blend regarding its strong antimicrobial activity than other blends. Figure 2 shows that the mixture MIC inhibited *B. subtilis* DB100 host, *C. sporogenes* DSM1446 and *E. coli* DH5α completely.

### Synergistic Combination Usage in RFC and PCS Manufacture

Synergistic combination blend (nisin : LAE; 1:3, w/w) was proven to be the most efficient blend added by its recommended MIC (700 ppm) in the manufacture of both RFC and PCS. The results indicated in Fig. 5 showed that such concentration of synergistic combination was efficient enough to inhibit the growth of the most common foodborne pathogens in cheese after their storage for 7 days (RFC) and 30 days (PCS).

## DISCUSSION

The emergence of *B. subtilis*, *C. sporogenes* and *E. coli* as important foodborne pathogens has led to a resurgence of interest in antimicrobials suitable for their control; however, consumer demand for foods that contain fewer artificial preservatives and more natural food stuffs has increased (Gould 1992). To address both these issues, much research has focused on the potential of LAE as a cationic surfactant with a very broad spectrum of activity against different microorganisms for its use in food preservation (Bakal and Diaz 2005). For example, it was used against *Salmonella typhimurium* and *Staphylococcus aureus* (Rodriguez *et al.* 2004) and *Listeria monocytogenes* (Luchansky *et al.* 2005). It is hydrolyzed in the human body and it was regarded "generally recognized as safe" (GRAS) by the FDA (Exposito 2006). However, in the present study, LAE (1,000 ppm) was not enough to inhibit spore-forming bacteria such as *Bacillus* and *Clostridium* completely by its own activity.

On the contrary, nisin is a well-known antimicrobial agent naturally produced by *Lactococcus lactis* ssp. *lactis* and used industrially against different gram-positive bacteria. Recently, some nisin-resistant strains of foodborne gram-positive pathogens have emerged. Furthermore, we have

noticed that nisin has a weak antimicrobial activity against *E. coli* which is one of the most common gram-negative pathogen.

Multiple combination preservation technique, or so-called hurdle technology, has been defined by Leistner (2000) as an intelligent combination of hurdles, which secures the microbial safety and stability as well as the organoleptic and nutritional quality and the economic viability of food products. For example, several antimicrobial compounds could be used together with different combinations to broaden the inhibitory spectrum to a degree that could not be reached by using each compound alone. The antimicrobial combinations should have different mechanisms to attain synergistic activity (Smid and Gorris 1999).

Extending the spectrum of nisin activity to gram-negative bacteria is also being examined. The application of nisin in combination with food-grade chelating agents is reported to increase the inhibitory activity and inhibitory spectrum of nisin (Cutter and Siragusa 1995a; Shefet *et al.* 1995). The use of nisin in combination with other bacteriocins such as Pediocin AcH has been reported to demonstrate greater antibacterial activity against a greater number of gram-positive bacteria (Hanlin *et al.* 1993). Nisin in combination with lactate has been found to reduce the numbers of *S. typhimurium* attached to beef and nisin in combination with ethylenediaminetetraacetic acid (EDTA) has been reported to reduce the numbers of *E. coli* O157:H7 attached to beef (Branen and Davidson 2004). Moreover, Kopermsub *et al.* (2012) tried to encapsulate nisin and EDTA combinations by nanoencapsulation technology in niosomes and investigated their synergistic effect on *S. aureus* and *E. coli*. However, EDTA has its own cautions. For example, it can cause the breathing tubes to narrow in some people with asthma, make heart rhythm problems worse, interfere with blood sugar control because it can interact with insulin, decrease serum calcium, potassium and magnesium levels (making hypocalcemia, hypokalemia and hypomagnesemia worse), make liver and kidney diseases worse, increase the risk of seizure in people with epilepsy or in people who tend to have seizures (Crisponi *et al.* 2014).

Another study noticed the synergistic activity of nisin and monolaurin (LAE is one of its derivatives) on *Bacillus* ssp. vegetative cells in milk (Mansour and Milliere 2001). They reported that the use of these inhibitors separately induced an immediate reduction in the bacterial population level but transient because re-growth appeared and was strain-dependent. However, the use of these inhibitors in combination induced a synergistic bactericidal effect, leading to a total inhibition until day 5, except in the case of *B. cereus* where the monolaurin concentration was doubled until the end of the experiment; consequently, sporulation was absent. However, Mhatre and Singare (2013) reported that the usage of monolaurin is found to be cytotoxic than LAE.

FDA has affirmed that nisin derived from certain strains of *L. lactis* ssp. *lactis* is GRAS for use as an antimicrobial agent in various cheese products such as cheese spreads and processed cheese when used at a level that delivers a maximum of 250 ppm of nisin in the finished product (21 CFR 184.1538). Nisin can also be used in meat and poultry at 600 ppm when the meat is fully cooked and used as a component in sauces. Under these conditions, the meat and poultry cannot be more than 50% of the product. On the other hand, FDA has affirmed that LAE is also GRAS for use as an antimicrobial agent in various cheese and other fermented dairy products when used at a level that delivers a maximum of 3,714 ppm of LAE in the finished product as if we exceed such level the LAE toxicity symptoms will appear (21 CFR 170.36). In our study, we used a combination of nisin : LAE (by 1:3, w/w) at 700 ppm. This means that the total concentration of nisin in our combination is about 234 ppm and the total concentration of LAE in our combination is about 525 ppm and both concentrations are under the permitted levels recommended by the FDA.

In the present study, it was found that LAE with various combinations of nisin has a great influence as an antimicrobial agent with significant synergistic activity against different common foodborne pathogens in RFC and PCS. Such approach could be a potential antimicrobial combination that may be used in dairy industries to prolong the shelf life of different fermented dairy products.

## CONCLUSION

It was found that LAE and nisin synergistically and significantly inhibited the growth of *B. subtilis*, *C. sporogenes* and *E. coli*. Thus, the results of the study indicate that such a combination of synergistic combination blend (nisin : LAE; 1:3, w/w) has a significant value to be applied in milk industries as a potential food preservative.

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