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Antimicrobial effect of nisin electrospun amaranth: pullulan nanofibers in apple juice and fresh cheese

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ABSTRACT

Nisin-loaded amaranth protein isolate:pullulan (API:PUL) nanofibers were prepared by the electrospinning method. The nisin release kinetic was evaluated at pH 3.4 and 6.1 and the antimicrobial effectiveness of the electrospun mats was evaluated in apple juice and fresh cheese. The nisin API:PUL fibers with 120 nm average diameter reached 81.49% and 43.85% nisin release after 12 h at pH 3.4 and 6.1, respectively. The encapsulation of nisin in electrospun fibers allowed complete bactericidal activity against *Salmonella* Typhimurium, *L. monocytogenes* and *L. mesenteroides* inoculated in apple juice after 48, 20 and 48 h, respectively. When nisin API:PUL fibers were applied to fresh cheese, microorganism inactivation was complete after 142, 120 and 170 h, respectively. The results demonstrated that nisin API:PUL electrospun fibers significantly reduce the bacterial population and can be used in food products for microbiological safety.

1. Introduction

Nisin is a bacteriocin that decreases its antimicrobial activity when added to food and therefore there is a need to understand the factors that affect its activity so that its antimicrobial performance can be protected. Several studies have shown that the antimicrobial activity of nisin is affected by factors such as pH, temperature, composition, structure, and natural microbiota of food. The interaction of nisin with food components such as lipids, proteins and enzymes, can reduce its efficacy and often large amounts of nisin are required to gain significant reductions in the product pathogen load (Zhou et al., 2014, Pinilla and Brandelli, 2016; Senan et al., 2016). Proteolysis in cheese-making process also affect the activity of nisin limiting its antimicrobial efficacy; Cleveland et al. (2001), reported loss of nisin activity (10–32%) in Ricotta cheese after 10 weeks of storage. Aasen et al., 2003, studied the recovery of nisin after its incorporation in different food matrices like salmon, chicken and oils, and they observed a 70% lose of nisin concentration after 5 h which influenced the antilisterial activity of the bacteriocin, mainly in contact with chicken. In this context, systems to release and deliver nisin with low cost and effectiveness are in demand. Examples of reported nisin carriers are liposomes (Laridi et al., 2003), nano-emulsions (Imran et al., 2012), solid lipid nanoparticles (Prombutara et al., 2012) and capsules (Xiao et al., 2011); all of these systems protect nisin from environment containing agents, but low encapsulation load, low stability and short shelf lives, are some important disadvantages. Nanofibers, ultrathin structures with average diameters lower than 100 nm, seem to be a good alternative due to their large surface area and porosity, capability to carry heat sensitive compounds, high encapsulation load, and sustained release of the encapsulated active (Blanco-Padilla et al., 2014; Khan and Deog-Hwan, 2016).

In a previous report, nisin was loaded into electrospun nanofibers produced with amaranth protein isolate (API) and pullulan (PUL). The resulting nanofibers showed diameters between 124 and 173 nm and demonstrated antimicrobial activity against *L. mesenteroides* (Soto et al., 2016). To further evaluate the use of the nisin-API-PUL electrospun fibers as a food preservation strategy, the antimicrobial performance of the nisin encapsulated system in two food matrices: apple juice and fresh Panela cheese was addressed.

Fruit juice is a popular beverage being a source of bioactive compounds including vitamins and phenolic compounds; however, it contains high levels of sugars and other nutrients that support microbial growth (Gouma et al., 2015; Sung et al., 2014). Fruit juices are believed to be free from foodborne pathogens due to their relatively low pH but particularly susceptible to fungal spoilage even at refrigeration temperatures (Maftei et al., 2014); however, there have been several

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outbreaks of foodborne illnesses associated to consumption of fruit juices containing acid resistant pathogens such as *Escherichia coli* O157:H7 and *Salmonella* spp. due to the lack or inadequate pasteurization (Oladunjoye et al., 2016; Sung et al., 2014). On the other hand, fresh cheeses in Mexico are the most consumed type of cheese. This type of cheese is characterized by a short lifespan, due to the high level of moisture, low concentration of salt and neutral pH (Torres-Vitela et al., 2012). One of the most important representatives of this group of cheese is the Panela, which is a warm and humid cheese with a sweet taste (Jiménez-Guzmán et al., 2009). Panela cheese is usually prepared with unpasteurized milk, and then this product is related to various outbreaks of foodborne diseases. According to Centers for Disease Control and Prevention (CDC), 29% of foodborne illness is related to the consumption of cheese (Saxer et al., 2013). Microbial contamination can occur at any stage of cheese production, with the most frequent pathogens *E. coli*, *Campylobacter*, *Salmonella* and *Listeria* (Guzman-Hernandez et al., 2016).

2. Materials and methods

2.1. Materials

The commercial amaranth protein concentrate (*Amaranthus hypochondriacus L.* Revancha variety) was supplied by Nutrisol (Hidalgo, Mexico), and the amaranth protein isolate (API) was prepared based on a methodology previously reported (Aceituno-Medina et al., 2013). Hydrochloric acid (HCl), sodium hydroxide (NaOH), pullulan (PUL), Tween 80, formic acid and nisin from *Lactococcus lactis* and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis MO, USA). De Man, Rogosa and Sharpe (MRS) agar and tryptic soy (TS) agar and broth were obtained from Becton Dickinson (New Jersey, USA). Apple juice was obtained from Gerber brand and fresh cheese was purchased in a local market of Queretaro, Mexico. All the other chemicals were purchased from Sigma- Aldrich Co., unless otherwise specified.

2.2. Effect of pH on nisin release kinetics from electrospun API:PUL fibers

Fibers containing a concentration of 20 mg nisin/mL of polymeric solution were prepared and Scanning Electron Microscopy (SEM) was used to determine morphology as described by Soto et al. (2016). 10 mg of nanofibers loaded with nisin were placed in either 2 mL of 0.1 M acetate buffer solution at pH 3.4 or 0.1 M phosphate buffer solution at pH 6.1. The suspension was stirred at room temperature and then sonicated for 2 min and maintained at room temperature under mild shaking. At scheduled times (0, 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h) the pH was measured; the buffer solution was filter and replaced with fresh buffer to continue shaking. The buffer solution was centrifuged at 4470*g* for 5 min, the supernatant was collected, filtered through a 0.2 mm filter membrane and 20 µL were injected in triplicate into a reversed phase column (Poroshell 120, EC-C18 column, Agilent technologies, 2.7 µm, 4.6 mm × 50 mm), using an Agilent HPLC system (Agilent Technologies, Palo Alto, CA, USA). Chromatographic conditions: λ_{max} 220 nm; gradient mobile system consisting of H_2O/0.05% trifluoacetic acid (TFA), eluent A, and acetonitrile/0.05% TFA (eluent B) at a flow rate of 0.9 mL/min. The gradient was programmed as follows: 0–5 min, 20% eluent B; 5–20 min from 20% eluent B to 80% eluent B. The nisin concentration was calculated on the basis of the peak area using a standard curve obtained with nisin solutions in the range of 0.01–5 mg/mL.

2.3. Release mechanism

In order to examine the mass transport mechanism of the loaded nisin nanofibers, the release profile of nisin from API-PUL fibers at pH 3.4 and 6.1 was fitted to Ritger and Peppas model:

$$M_t/M_\infty = kt^n \quad (1)$$

where Mt. is the quantity of nisin released at any time (t); M∞ corresponds to 19.09 mg considering the 95.45% nisin API-PUL fiber loading efficiency value previously reported (Soto et al., 2016); k is the release rate constant, and n indicates the release exponent. By fitting the 65% and 48% of nisin release data at pH 3.4 and 6.1, n was determined by minimizing the difference between Eq. (1) and a logarithmic plot of the experimental curves using the solver tool in Microsoft Excel. In addition, four more kinetic release models were used to further analyze the release profile by fitting the experimental data of the 80% and 48% of nisin release at pH 3.4 and 6.1.

$$\text{Zero} - \text{order model: } M_t/M_\infty = kt \quad (2)$$

$$\text{First} - \text{order model: } \ln(1 - M_t/M_\infty) = -kt \quad (3)$$

$$\text{Higuchi model: } M_t/M_\infty = kt^{1/2} \quad (4)$$

$$\text{Hixson Crowell model: } (1 - M_t/M_\infty)^{1/3} = -kt \quad (5)$$

2.4. Antimicrobial activity of nisin-API:PUL fibers in apple juice

Pasteurized apple juice without additives was bought at a local self-service store and sterilized by filtration with a 0.45 µm polytetrafluoroethylene (PTFE) membrane filter. Filtered apple juice was subjected to microbiological analysis by counting aerobic mesophilic bacteria, total coliforms and lactic acid bacteria. Nisin (20 mg), nisin-API:PUL nanofibers (20 mg of nisin encapsulated into the fibers) and API-PUL nanofibers were separately added to 15 mL of apple juice. Filter sterilized apple juice was used as negative control. Then, the samples were inoculated with a suspension of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) (ATCC 14028), *Listeria monocytogenes* (ATCC 19115) and *Leuconostoc mesenteroides* (ATCC8923) which previously were activated for 24 h at 35 °C in TS broth to obtain a final concentration of ≈6 Log CFU/mL. Aliquots were taken at 0, 24, 48, 72 and 96 h to count *Salmonella* and *L. monocytogenes* in TS agar and *L. mesenteroides* in MRS agar by the spread-plate method. Non-inoculated sterile apple juice samples were used as a control.

2.5. Antimicrobial activity in of nisin-API:PUL fibers in fresh cheese

Fresh Panela cheese purchased on the market was aseptically cut into cubes with 1 cm^2 of surface and average weight of 0.89 g ± 0.1 g. A surface of the cube was inoculated with a concentration of 10^5 CFU/mL of *L. mesenteroides*, *L. monocytogenes* and *S.* Typhimurium, separately. The inoculum was allowed to dry in a laminar flow hood and subsequently the samples were divided into four treatments: 1) Panela cheese (negative control), 2) Panela cheese with nisin (20 mg of nisin were spread on the surface), 3) Panela cheese with nisin-API:PUL fibers (20 mg of encapsulated nisin), and 4) Panela cheese with API:PUL fibers. For the treatments involving fibers, the fiber mat was located on the surface of the inoculated cheese cubes and subsequently stored in hermetic bags at refrigeration temperature (4 °C). Microbial counts were carried out at times 0, 4, 8, 12, 24, 48, 72, 120, 144 and 168 h. For microbial counting, 9 mL of peptone diluent were added to the cheese cubes and homogenized in a stomacher (Stomacher 400, Seward, London, England), then an aliquot of 1 mL was taken and dilutions were made to further count the microorganisms by the pouring technique plate on TS agar; plates were incubated for 24 h at 30 °C for *L. mesenteroides* and 35 °C for *L. monocytogenes* and *S.* Typhimurium. Subsequently, the CFU/g count was performed.

2.6. Statistical analysis

All experiments were conducted in triplicate and expressed as

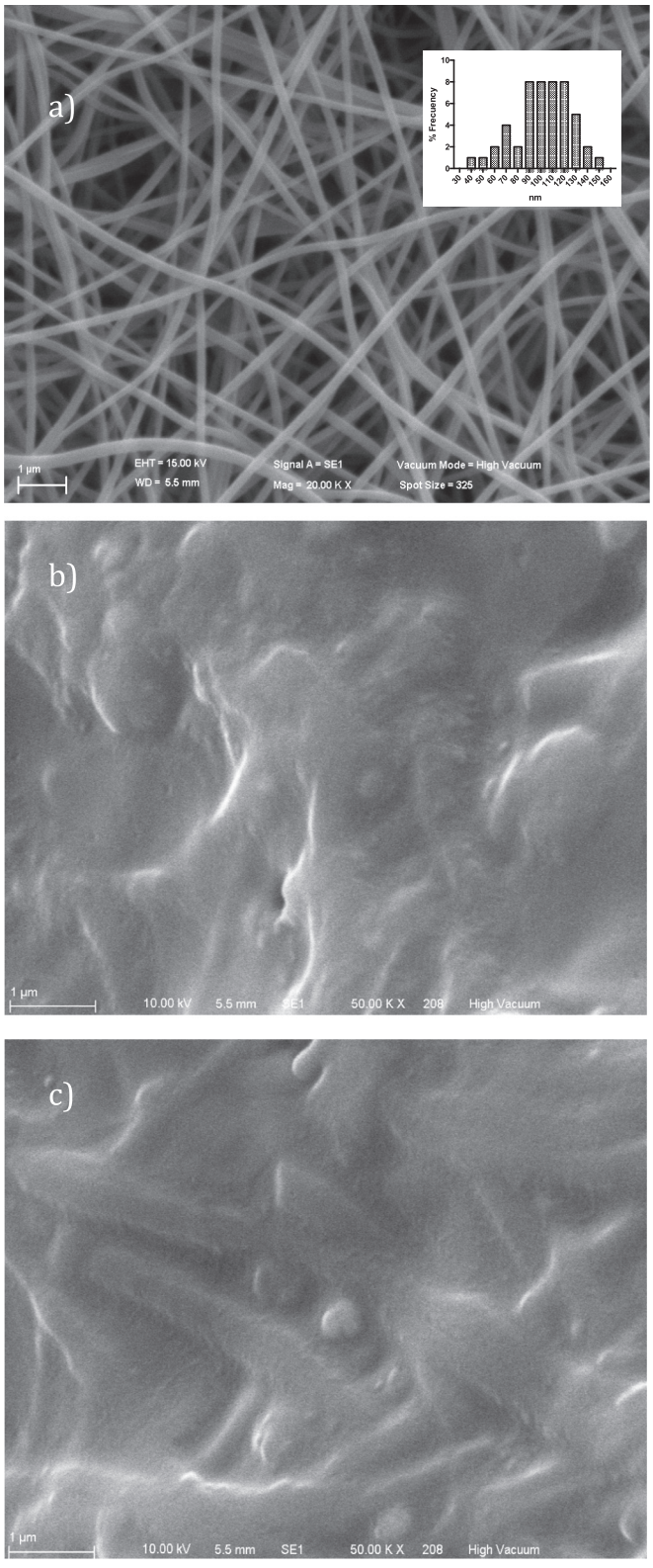


Fig. 1. SEM image of a) nisin-API:PUL electrospun nanofibers and after 8 h of exposition into b) Apple juice and c) Fresh cheese.

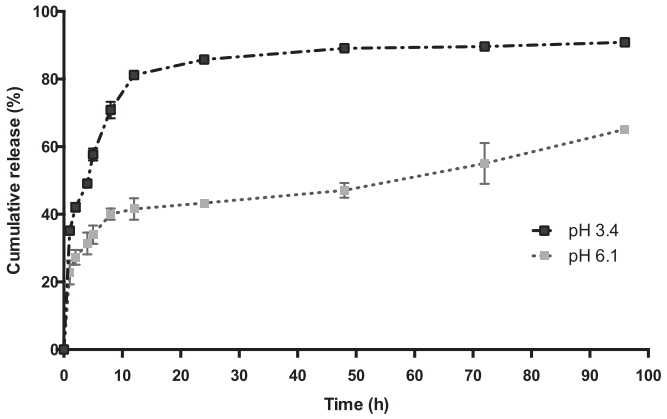


Fig. 2. *In vitro* release profiles of nisin-API:PUL electrospun nanofibers in acetate buffer pH 3.4 and phosphate buffer pH 6.1 at room temperature.

means ± SE of at least three independent experiments. Comparisons between samples were evaluated using the Tukey test ($\alpha = 0.05$).

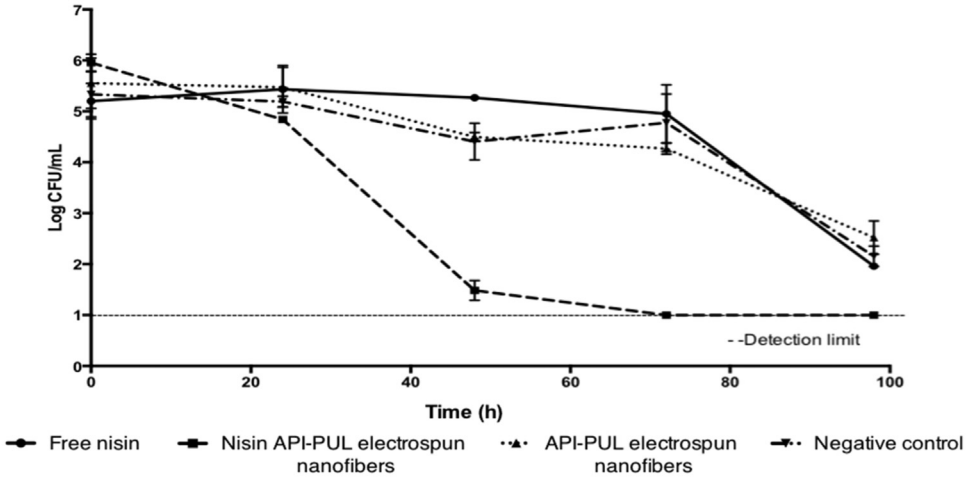
3. Results and discussion

3.1. Release profiles and mechanism of nisin from API:PUL electrospun nanofibers

API:PUL nanofibers loaded with nisin were obtained as previously reported, 20 mg of nisin/mL of polymeric solution was used considering that in the previous work this amount was the highest concentration of nisin that API:PUL electrospun fibers can afford (Soto et al., 2016). As expected, fibers with smooth surface, lacking of bead defects and with an average diameter of 120 nm were produced (Fig. 1a). Smooth fibers with diameters in the nanometer scale lead to large surface areas with porous interconnected architecture, which allowed a sustained release of the encapsulated active agent, contrary to the quick release associated with other encapsulation systems such as nanocapsules (Pillay et al., 2013). Then, the release profile of nisin from API:PUL electrospun fibers was measured in pH 3.4 acetate buffer and 6.1 phosphate buffer to simulate the pH that apple juice and fresh Panela cheese usually have. Fig. 2 illustrates the release of nisin at different pH values as a function of time; the nisin release performance was characterized by a gradual but rapid release in the first 12 h in both pH media (reaching 81.49% and 43.85% nisin release at pH 3.4 and 6.1) followed by a slower sustained release phase until the nisin content reach a constant cumulative value. Higher release of nisin is observed when the fiber was placed in an acid medium reaching up to 92.77% release at 100 h, while at pH 6.1 a percentage of 65.12% was observed. This difference can be explained by the isoelectric point of the proteins that influence the charge of proteins in the solution. In this way, the isoelectric point of amaranth protein and nisin are 5.0 and 8.8, respectively (Shevkani et al., 2014; Wang et al., 2015); then at pH 3.4 both proteins are positively charged and the electronic repulsion among the fiber structure facilitates the diffusion of nisin from nanofibers to the solution. When the pH is 6.1 on the other hand, electrostatic interactions between amaranth protein and nisin become attractive, since nisin is positively charged and amaranth proteins are negatively charged. Therefore electrostatic attraction, coupled to hydrophobic interactions, may have caused less release of nisin at pH 6.1. It is also noteworthy that the rapid release of nisin observed during the first stage is ascribed to the water solubility of the pullulan as demonstrated by Aceituno-Medina et al. (2013).

The study of the release mechanism of nisin from the fibers is an important issue to determine its possible applications in the food industry, as well as the improvements that the carrier system may need. Mathematical models provide information on the transport of the active

a)



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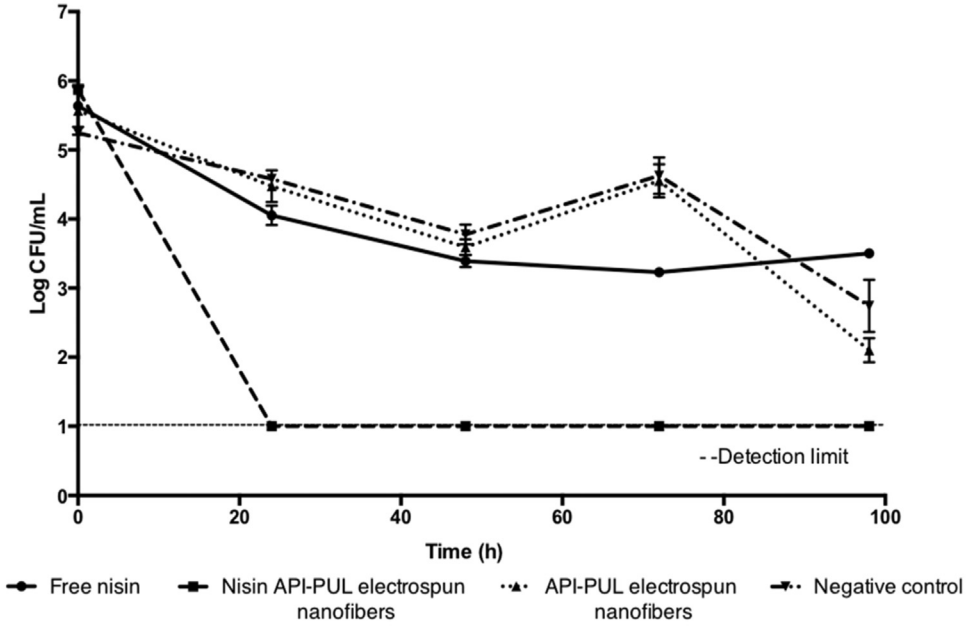

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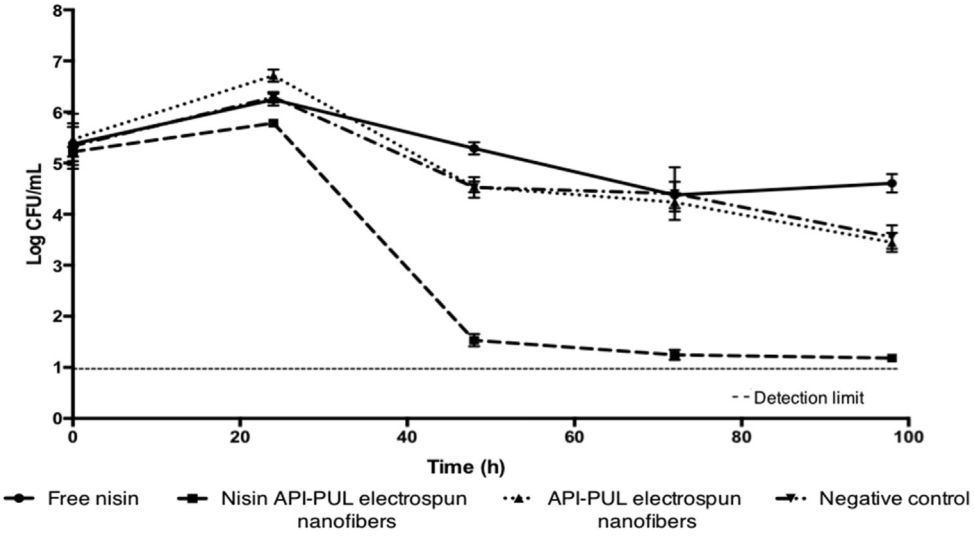

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Fig. 3. Antimicrobial activity of nisin, nisin-API:PUL and API:PUL electrospun fibers in apple juice against a) *S*. Typhimurium b) *L. monocytogenes* and c) *L. mesenteroides*.

compound from fiber mats, which is critical to the development of sustained-controlled delivery systems (Neo et al., 2013). The process of releasing an active compound embedded in an encapsulation system can be carried out by various mechanisms such as diffusion, relaxation or swelling of the polymer chains. For Ritger-Peppas model; the release exponent "n" and regression coefficients (r^2) values were calculated. For pH 3.4 and 6.1 "n" and r^2 values were 0.48, 0.935 and 0. 38, 0.932, respectively. The obtained "n" values are characteristic of a pseudo Fickian diffusion (Singhvi and Singh, 2011; Radisavljevic et al., 2018). The higher r^2 values were obtained with the Higuchi model (pH 3.4, 0.968 and pH 6.1, 0.824) which describes a diffusion controlled release from porous matrices that follows a Fickian type mechanism, an observation that is consistent with the obtained "n" value (MacRi et al., 2012; Yao and Weiyuan, 2010).

In this way, the fastest nisin release during the first 2 h (43.1% for pH 3.4 and 28.3% for pH 6.1) is due to the diffusion of the nisin molecules bonded to the surface of the API:PUL fibers. Later, while the nisin release observed until 12 h (81.5% for pH 3.4 and 46.8% for pH 6.1) can be explained on the basis of the swelling and the erosion of the nanofibers, the slow nisin release observed at longer times is associated to the fact that there is an increase of the path length across the porous fiber. Similar release behavior was observed by Sun et al. (2013) in electrospun nanofibers elaborated with Polyvinyl alcohol (PVA) loaded with curcumin.

3.2. Antimicrobial activity in apple juice

After filtration, the commercial apple juice was free of aerobic mesophilic bacteria, total coliforms and lactic acid bacteria. The antimicrobial effect of nisin-API:PUL fibers was evaluated against three microorganisms of interest in food items: *S*. Typhimurium, *L. monocytogenes*, and *L. mesenteroides*. Growth dynamics of bacteria in apple juice, apple juice added with nisin-API:PUL or API:PUL fibers (negative control) or free nisin are shown in Fig. 3. For the three tested microorganisms, addition of nisin-API:PUL fibers to the apple juice was the most effective treatment in reducing the microbial population; the reduction ranged from 4 to 6 Log CFU/mL after 48 h of incubation. A complete inactivation of *L. mesenteroides*, *S*. Typhimurium and *L. monocytogenes* was achieved after 96, 72, and 24 h, respectively. For the samples of juice added with API:PUL fibers and free nisin, during the first 24 h no significant decrease of microorganisms was observed, and after 96 h a total reduction from 1 to 3 Log CFU/mL was obtained. This does not mean that fibers or nisin displayed antimicrobial activity, because the same effect was observed for the inoculated juice. The acidity of apple juice has resulted in sublethal microorganism injury, this pH effect have been reported by Yuste and Fung (2004) for *S*. Typhimurium and *E. coli* in apple juice and Mataragas et al. (2004) for *L. mesenteroides* at pH 4.5. Unexpectedly, the addition of nisin did not contribute to significant reduction of microbial populations. Nisin antimicrobial activity is strongly related to its interactions with other molecules, then the juice carbohydrate content (0.06 g/mL) may interact with nisin diminishing its activity. But most of all, the antimicrobial activity of nisin is fully pH dependent due to its secondary structure conformation. Modugno et al. (2018) for instance have demonstrated that the highest antimicrobial activity of nisin is observed at pH 5.5 where there is an optimum co-existence of the β-structure and random coiling of the protein. In this way, whereas β-turns configuration in nisin is a key feature to exhibit high antimicrobial activity, at pH 2.8 nisin is randomly coiled with a slight persistence of β–turns and therefore its antimicrobial activity is not important.

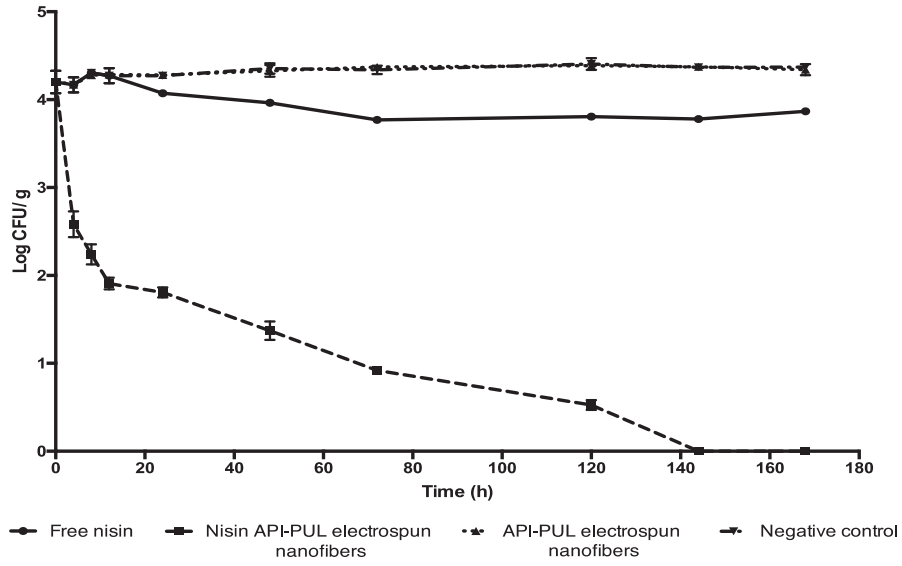
Nisin-API:PUL fibers exhibited an 81.5% release of the antimicrobial agent during the first 12 h (Fig. 2, pH = 3.4). Although further studies of nisin secondary structure conformation after electrospinning are needed, we postulate that nisin molecules are released along with pullulan molecules and during the electrospinning process intermolecular interactions of nisin with the amaranth protein and pullulan may stabilize its β-conformation which enhances the antimicrobial activity.

Although nisin is recognized as active against wide range of Gram-positive bacteria, it can display antimicrobial activity against Gram-negative bacteria when the microorganism has been exposed to stress (Rattanachaikunsopon and Phumkhachorn, 2010). Different authors have studied the antimicrobial effect of nisin on Gram-negative bacteria when combined with essential oils, chelating agents or changes in pH values (Stevens et al., 1992; Zhang et al., 2014; Zou et al., 2013). In this study, the observed nisin antimicrobial activity of nisin-API:PUL fibers against *S*. Typhimurium could be ascribed to the combined effect of low pH and the secondary structure of nisin. The nisin-API:PUL fibers combined with pasteurization, UV or high pressure can be used as antimicrobial control measurement to satisfy the USFDA standard of reducing pathogen microorganism population by 5-Log CFU/mL in fruit juices.

3.3. Antimicrobial activity in fresh cheese

Fig. 4 shows the inactivation curves of *S*. Typhimurium, *L. monocytogenes and L. mesenteroides* inoculated on the surface of fresh cheese with different treatments. As in the case of apple juice, the most effective treatment to reduce the microorganism population was nisin encapsulated into electrospun fibers. The encapsulated nisin produced the complete inactivation of *S*. Typhimurium, *L. monocytogenes and L. mesenteroides* at 144, 120 and 168 h, respectively. The treatment with free nisin demonstrated a small decrease of the population of microorganisms, which corresponded to 0.38, 0.72 and 0.23 Log CFU/g for *S*. Typhimurium, *L. monocytogenes and L. mesenteroides*, respectively. The pH of the surface of cheese was 6.1 and it was expected that at this pH, nisin molecules acquired the optimum secondary structure conformation to demonstrate high antimicrobial activity; however, not all nisin molecules interact with the cheese surface environment but with the bacteria membrane, furthermore the fat and proteins of cheese may associate with the nisin, changing its conformation and decreasing its activity (Khan and Deog-Hwan, 2016). Regarding the API:PUL nanofibers and the negative control, no decrease in the microbial population was observed; although there is a pH = 6.1 it was not possible to observe an increase in the microorganism population due to refrigeration temperatures. The nisin-API:PUL fibers when exposed to a liquid phase with a pH 6.1 exhibited slower nisin release compared with that observed at pH 3.4. In a solid phase, such as the cheese surface, the release of nisin molecules encapsulated into the core of the fibers may take longer. Then, the observed antimicrobial effect in the cheese is mainly due to the nisin molecules that are located in the surface of the fibers, the anchored nisin molecules may exhibit a secondary structure conformation that favored the antimicrobial activity. To observe complete inactivation of microorganisms for the nisin-API:PUL nanofiber on the fresh cheese, it was required twice as long as for the apple juice. Fig. 1b and c show the morphology of the nisin-API:PUL fibers after 8 h of exposition to the apple juice and fresh cheese and, as expected, higher deformation due to swelling is observed for the fibers in the juice. These results support the use of nisin API:PUL fibers as antimicrobial films to control the microbial growth of *S*. Typhimurium, *L. monocytogenes and L. mesenteroides* in cheese surfaces.

a)



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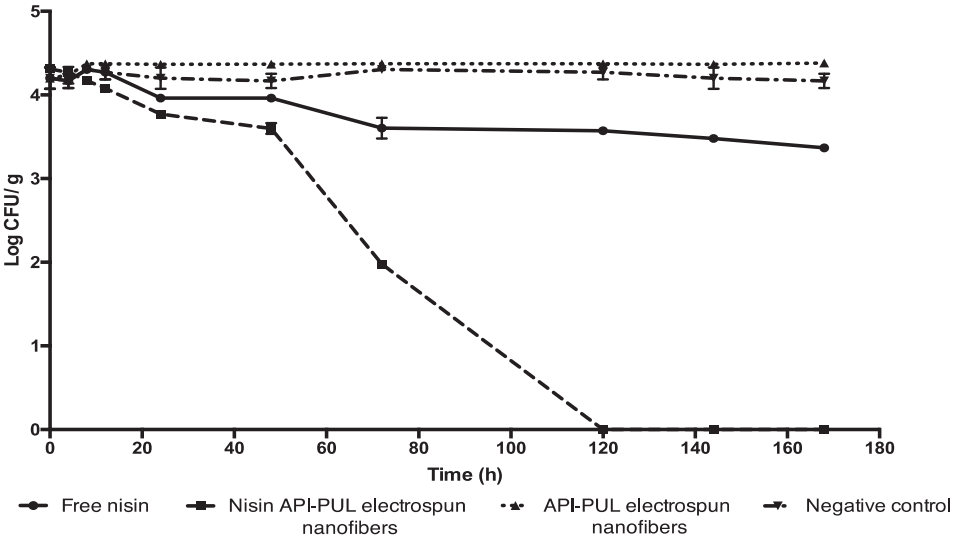

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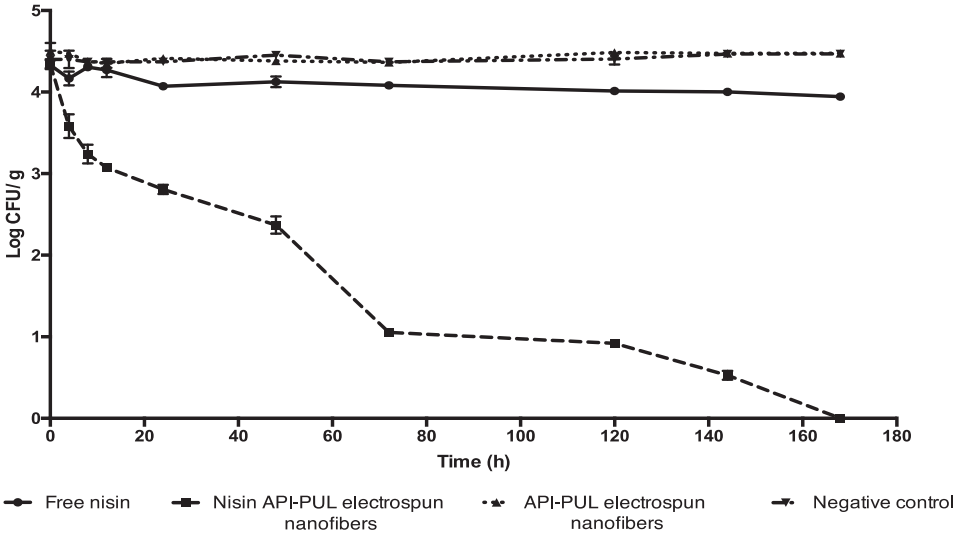

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Fig. 4. Antimicrobial activity of nisin, nisin-API:PUL and API:PUL electrospun fibers in Panela fresh cheese against a) *S*. Typhimurium b) *L. monocytogenes* and c) *L. mesenteroides*.

4. Conclusion

The results of this work, demonstrated that nisin was entrapped in the electrospun nanofibers in an active form. The release behavior of nisin from API-PUL nanofibers at pH 3.4 and 6.1 follows a diffusion-controlled mechanism associated with the solubility of the polysaccharide pullulan reaching 65 and 90%, respectively after 100 h. The nisin molecules attached to the surface of the fibers was released first, then the swelling of the fibers promoted the solubility of the pullulan, generating pores, which favors the release of nisin molecules entrapped into the inner structure of the fiber. In this way nanofibers confer protection to the nisin, decreasing its interaction with food components and maintaining its antimicrobial activity. This activity in API:PUL electrospun fibers against *S*. Typhimurium, *L. monocytogenes and L. mesenteroides* in apple juice and fresh cheese was demonstrated. Nisin-API:PUL nanofibers could also be used to control post-processing contamination of food and beverage products as a food additive, edible film or component of packing material. The nisin active electrospun fibers ensure microbiological safety and therefore, can be seen as a potentially important technology to extend the shelf life of food products. Further studies are also needed to probe that the API:PUL nanofiber promotes optimum ratio of the β-structure and random coiling of the nisin so that high antimicrobial activity can be obtained.

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