IMPROVING THE EFFICACY OF NISIN TO CONTROL *LISTERIA MONOCYTOGENES* IN QUESO FRESCO

BY

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DISSERTATION

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ABSTRACT

Hispanic-style cheeses (HSC) are a varied group of cheeses that are originally developed and manufactured in Mexico and other Latin American countries. In particular, Queso Fresco (QF) is the most widespread HSC in the United States. Most of the HSC are characterized by being fresh, having a delicate flavor and texture, and a short-shelf life that requires to store them under refrigeration. Different from aged cheeses, fresh cheeses provide conditions for the growth of foodborne pathogens, notably *Listeria monocytogenes*. HSCs have been linked to historical and recent listeriosis outbreaks. Since 2008, about one out of every five listeriosis outbreaks in the U.S. have been associated with the consumption of HSC, this is higher than for other ready-to-eat foods and other types of cheeses, highlighting the health risk associated between HSCs and *L. monocytogenes*.

Similar to other types of cheeses, HSC can be manufactured safely; however, pasteurization is the only step in the process that eliminates *L. monocytogenes*, and post-pasteurization interventions are needed to ensure their safety if contamination occurs. To date, multiple potential interventions to inhibit *L. monocytogenes* in QF have been evaluated, including non-thermal processes such as High Pressure Processing, or the addition of ingredients with antilisterial activity such as organic acids, bacteriocins, fermentates, bacteriophages or antimicrobial packaging. Unfortunately, those treatments have not been effective to eliminate *L. monocytogenes* in QF, and it is necessary to find antimicrobial treatments that can work in QF. Nisin is an antimicrobial peptide produced by certain strains of *Lactococcus* lactis, it is widely used in the food industry and it is able to inhibit *L. monocytogenes*, however it has limited activity in QF. Therefore, the overall goal of this study is to enhance the antilisterial

effectiveness of nisin in QF by exploring its synergy with novel antimicrobials, and the use of microencapsulation technology and bioengineering approaches.

In Chapter 2, the potential synergy of nisin with the *Listeria* phage endolysin PlyP100 against *Listeria* in QF was evaluated. By combining the commercial preparation of nisin with the purified endolysin PlyP100 in QF, the treatment reduced the viable populations of the pathogen to below the detection limit of enumeration, and in some samples, recovery of the pathogen was not possible, indicating elimination of the pathogen in the cheese. The combination of nisin with PlyP100 could be used as a preservative in QF due to the listeriacidal synergy observed both antimicrobials.

In Chapter 3, a droplet-based microfluidic process was used to microencapsulate nisin with zein for antilisterial activity enhancement. Microencapsulation of nisin with zein allowed for sustained release of nisin in solution, and when added into QF, microencapsulated nisin displayed significantly enhanced listeriacidal effect over the first 3 days of storage at 4 ºC. Fabrication of nisin-zein microcapsules could be further optimized for tuning the release rate and microcapsule properties for extended antilisterial effect of nisin in cheese.

In Chapter 4, a series of nisin A derivatives in which the hydrophobic residues of the C-terminal region were replaced with positively charged amino acids was created. All nisin derivatives created displayed altered antilisterial and biochemical properties relative to unmodified nisin, notably reduced antilisterial activity but significantly higher stability at neutral pH and lower absorption to milk fat. Single substituted nisin derivatives were stable enough in QF to exhibit a listeriastic effect at early cold storage of the cheese despite of their reduced antilisterial activity. Bioengineering of nisin can result in the creation of nisin derivatives with higher stability and extended antimicrobial activity in non-acidic and high-fat food matrices.

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CHAPTER 1

HISPANIC-STYLE CHEESES AND THEIR ASSOCIATION WITH *LISTERIA MONOCYTOGENES*

1.1 Abstract

The rise in consumption of Hispanic-style cheeses (**HSCs**), due in large part to the increasing Hispanic population in the US, has not been met with advances in food safety sufficient to prevent the numerous outbreaks and recalls due to *Listeria monocytogenes*. HSCs are typically high moisture, low salt content, and low acidity from being subjected to little to no ripening. These conditions necessitate refrigeration to maintain safety and quality, as the majority of traditional extrinsic preservation methods are either ineffective or disrupt the mild sensory attributes of HSCs. Unfortunately, the cold-growth of *L. monocytogenes* thereby presents significant problems from insufficient pasteurization or post-pasteurization contamination. In this review, we discuss such factors affecting listerial contamination and growth in HSCs, as well as present current knowledge of *L. monocytogenes* incidence in manufacturing settings and commercial prevalence. Furthermore, we differentiate HSC types by processing methods to aid with interpretation of works involving non-standardized varieties and, lastly, summarize research on intervention methods for eliminating listerial contaminants in HSCs.

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1.2 Introduction

Across the world, cheeses are consumed in numerous styles and broadly acknowledged for their nutritional value. About one third of the milk produced in United States is used for cheese production, which is reflected in the broad variety of cheese varieties made in the U.S., including American types, Italian types, Muenster, Swiss and Hispanic-style cheeses (USDA-NASS, 2015). Hispanic-style cheeses (**HSCs**) in particular, a category of cheeses referring to those originally developed and manufactured in Mexico and Latin America (Van Hekken *et al*., 2007), have been increasing in popularity among U.S. consumers over the last two decades (Hnosko *et al*., 2009), which is reflected in both the rise in production and consumption. Total U.S. cheese production in 2015, excluding cottage cheese, was 5.4 million metric tons, however, HSC totaled only 115.3 thousand metric tons (USDA-NASS, 2016). U. S. per capita cheese consumption was 15.5 kg in 2014, from which the HSC per capita consumption was 0.32 kg (USDA-ERS, 2015). However, HSCs have shown an increase of 271% production and 188% per capita consumption compared to 1996, a growth rate at least 3.5 fold greater than observed for Italian type cheeses, the largest cheese group produced and consumed in the U.S. (USDA-NASS, 1997; USDA-ERS, 2015; USDA-NASS, 2016). This trend is likely driven in large part by the growing Hispanic population in the U.S., which between 1996 and 2014 has increased 93.7%, representing an estimated 17 % (55 million persons) of the U.S. population (U.S. Census Bureau, 1997; U.S. Census Bureau, 2015). However, the appreciation for HSCs is not limited to Hispanic or Latin populations; the remarkable potential for its consumption by non-typical and non-Hispanic consumers in the U.S. has been previously suggested (Clark *et al*., 2001).

The rise of both consumption and production of HSCs is expected to continue in the years to come, which has also increased the need for proper awareness of the microbial safety

concerns of this group of cheeses. It is well known that non-ripened fresh cheeses, such as HSCs, are prone to foodborne pathogen contamination, notably with *Listeria monocytogenes*. However, in the U.S. most HSC safety research has focused on Queso Fresco, leaving other commercially available varieties unaddressed. Combined with the lack of identity standards for HSCs in the U.S., this has made it difficult to assess the listeriosis risks of other varieties and there has been no comprehensive assessment of *L. monocytogenes*-associated food safety risk between varieties of HSC.

1.3 Types of Hispanic-style cheeses

Hispanic-style cheeses comprise a large and diverse group, albeit generally unstandardized. At least thirty different varieties are recognized in countries such as Mexico (Villegas de Gante, 2004). For further information on characteristics and production aspects of artisan cheeses most commonly produced in Mexico, see González-Córdoba et al. (2016). Currently there are 63 HSC-producing plants in the U.S. (USDA-NASS, 2015), manufacturing varieties including, but not limited to, Queso Fresco, Cotija, Oaxaca, Panela, Chihuahua, Queso Blanco, Asadero, Añejo, Manchego, Adobera, and Ranchero (CMAB, 2016). HSCs show considerable variation among shape, size, texture, moisture, and flavor due to differences in cheesemaking procedures such as curd setting methods, pressing, and ripening (Villegas de Gante, 2004). Generally, HSCs are characterized by their high moisture and are consumed shortly after manufacture. To help better understand the subtleties differentiating them, Figure 1.1 delineates several major processing steps that result in eleven select HSCs. The diversity among HSCs allows for several ways to classify them, but for practical purposes these cheeses

can be divided into two main groups according to their degree of ripening: fresh and aged cheeses.

1.3.1 Fresh Hispanic-style cheeses.

Most of the HSCs are fresh (unripened), generally characterized by being soft, high moisture, having a mild fresh milk flavor, and are ready for consumption immediately after manufacture (e.g. Queso Fresco, Queso Blanco, Panela and Ranchero). Moreover, pasta filata varieties (e.g. Oaxaca, Asadero and Adobera), which involve kneading or stretching the curds much like mozzarella, are also considered fresh cheeses. As with all cheeses, HSCs undergo varied manufacturing processes that differentiate styles, which may or may not include a light "cook" step, curd milling, kneading, or pressing. Distinctive characteristics and manufacturing properties of these cheeses are summarized in Table 1.1.

1.3.2 Aged Hispanic-style cheeses.

Aged HSCs are hard or semi hard cheeses subjected to ripening, leading some degree of biochemical transformation of the curd, which affects numerous sensory characteristics. The degree of ripening of aged HSCs is usually less than one month, matching the preference of Hispanic consumers. Further description of distinguishing characteristics of prototypical aged HSCs are outlined in Table 1.2. Aging of these cheeses, while less extensive than that of other aged varieties, may contribute to fewer food safety concerns than with fresh HSCs due to decreases in moisture content and pH.

1.4 Food safety

Several steps can influence pathogen contamination, survival or growth during manufacture of HSCs. The source and microbial quality of milk, adjustment of fat content, milk homogenization, pasteurization, use of starter cultures, coagulation, extensive curd manipulation, hand stirring, salting, whey removal, milling, molding, and storage conditions may all contribute to increase the probabilities of cheese contamination (Ryser, 2007; Fernandez Escartin, 2008).

Traditionally, consumer preferences in Hispanic populations have led to a large proportion of HSCs being made from raw milk (Villegas de Gante, 2004, Torres-Vitela *et al*., 2012), which may contain pathogens such as *Salmonella*, *Escherichia coli* and *Listeria monocytogenes* (FDA, 2015). Consumption of raw-milk fresh cheeses constitutes a significant public health threat evidenced by the number of outbreaks and recalls due to foodborne pathogen contamination.

Foodborne outbreaks caused by consumption of various cheeses have been tied to *Salmonella* spp., *Staphylococcus aureus*, *E. coli* O157:H7 and *L. monocytogenes* (Gould *et al*., 2014). It is recognized that HSCs provide favorable conditions to support the growth or survival of several foodborne pathogens, including *Campylobacter* spp., *E. coli* O157:H7, *Salmonella* spp, *L. monocytogenes*, and their consumption has even caused illness due Norovirus infection; but with the exception of *L. monocytogenes*, HSC consumption represents no more than 0.02-0.5% of the outbreaks due to these pathogens (CDC, 2015a). However, about one of every five listeriosis outbreaks have been traced to HSCs (CDC, 2015a), highlighting the fact that such fresh cheeses support *L. monocytogenes* growth to high levels, as refrigeration is generally the only post-manufacturing hurdle to pathogen growth (ILSI, 2005).

1.4.1 Physicochemical characteristics as risk factors for *L. monocytogenes*.

L. monocytogenes is able to survive in or on several food products, and can grow as long as the conditions provided in the food matrix are favorable. Based on published data, fresh HSCs that support the growth of *L. monocytogenes* include Queso Fresco (Genigeorgis *et al*., 1991b; Leggett *et al*., 2012; Van Hekken *et al*., 2012; Leong et *al*., 2014; Van Tassell et al., 2015), Queso Blanco (Uhlich *et al*., 2006; Leong *et al*., 2014), Panela and Ranchero (Genigeorgis *et al*., 1991b). Often some pasta filata cheeses, such as Oaxaca, are considered low risk for pathogen carriage due manipulation in hot water during manufacturing (thermoplastification). However, laboratory studies have shown that thermoplastification in water at 70°C is insufficient to kill food-borne pathogens such as *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* when pasteurized milk inoculated with those pathogens is used for Oaxaca cheese manufacturing (Orozco, 1999; Fernandez Escartin, 2008).

Regarding aged HSCs, Chihuahua and Manchego have been shown to allow survival of *L. monocytogenes* during manufacture and ripening, but they did not support its growth (Solano-López and Hernández-Sánchez, 2000). The pathogen was unable to initiate growth in Cotija cheese in laboratory study (Genigeorgis *et al*., 1991b). However, contrary to genuine Cotija, the minimal ripening subjected to some commercial Cotija (Villegas de Gante, 2004) may increase the risk for survival of *L. monocytogenes*.

The survival or growth of *L. monocytogenes* in or on different fresh or aged HSCs is not surprising considering their gross physicochemical composition. Fresh HSCs are characterized by their high moisture, salt content as low as 1% and not greater than 3%, and near neutral pH (Table 1.3). Conversely, aged HSCs have mid-level moisture, salt content similar to fresh cheeses, or in the case of Cotija cheese slight higher than 4%, and generally have pH no lower

than 5.0 due their short ripening periods. Additionally, both fresh and aged cheeses require storage at refrigeration temperature. *L. monocytogenes* is capable of growth in a wide range of temperatures (1 to 45°C) and pH (4.1 to 9.6), is tolerant of relatively harsh conditions such as high salt concentrations (10%) and in the presence of numerous antimicrobial agents (Adzitey and Huda, 2010). As such, even under refrigeration, physicochemical properties of HSCs contribute to increased *L. monocytogenes*-associated food safety risk.

1.4.2 Prevalence of *L. monocytogenes* in Hispanic-style cheeses and in processing plants

Very limited research has been conducted on the prevalence of *L. monocytogenes* in commercial or handcrafted HSC varieties; moreover, the majority of such work has been conducted in Mexico (Table 1.4) where poor epidemiological surveillance limits comparability with the listerial food safety landscape in the U.S. The presence of the pathogen has been documented in finished products and marketed cheese samples, showing greater prevalence in fresh cheeses, especially in handcrafted cheeses, although in some aged cheeses sampled (Chihuahua and Manchego) *L. monocytogenes* was not recovered (Table 1.4). It is important to highlight that only a small portion of HSC varieties have been investigated in surveys of *L. monocytogenes* in Mexico. In addition, U.S. surveys have focused on detecting the presence of *L. monocytogenes* in Queso Fresco or HSCs as a group, without distinguishing varieties commercially produced, leading to uncertainty whether all HSCs possess similar risk of *L. monocytogenes* contamination.

The presence of *L. monocytogenes* in HSCs at retail level has a clear relevance in the aforementioned published surveys; however, studies that have investigated listerial prevalence in HSC processing facilities are scarce. Results have suggested that *L. monocytogenes*

contamination occurs predominantly through post-processing steps (Table 1.5). One study conducted in New York City, U.S., found that while isolation frequencies varied between three Latin-style fresh-cheese processing plants, *L. monocytogenes* could be isolated from drains, floors and crates in all plants (Kabuki *et al*., 2004), suggesting that the multiple functions of crates (transportation of pasteurized milk and finished product, and to store finished products in cooler rooms) may contribute to spread contaminants from different areas within the plant.

Other studies conducted in Mexico, have tracked the presence of *L. monocytogenes* through the cheese-making process and environment, across milk samples, processing plants, and retail samples, suggesting that food contact surfaces likely constitute recurrent sources of contamination more commonly than raw or improperly pasteurized milk (Moreno-Enriquez *et al*., 2007; Rosas-Barbosa *et al*., 2014). *L. monocytogenes* strains were isolated from milk and curd tanks, utensils, baskets, cheese molds, refrigerators, brooms, and other handling surfaces. In positive cheese samples (Panela, Queso Fresco and Adobera), common outbreak serovars 1/2a, 1/2b and 4b were found, suggesting that the consumption of these cheeses may increase the risk of listeriosis (Rosas-Barbosa *et al*., 2014). These studies illustrate that contamination at the processing level represents an important source of *L. monocytogenes* regardless of the pasteurization status of the milk used for making HSCs.

Indeed, it has become clear that *L. monocytogenes* persistence in food-associated environments contributes significantly to the prevalence of contaminated food products, recalls, and listeriosis outbreaks (Ferreira et al., 2014). Subtyping isolates from the processing environment and beyond via whole-genome sequencing could help differentiate sporadic isolates from persistent strains that may pose reoccurring problems and identify associated process deviations or sources responsible for their introduction to final products (Stasiewicz et al., 2015).

To our knowledge, such an approach has yet to be implemented for the identification of persistent contaminants in HSCs, but could inform intervention strategies for targeting their sources.

Additionally, it is unclear to what extent consumer contamination of HSCs contributes to foodborne illness, however poor food storage and handling, combined with the prevalence of pathogens such as *L. monocytogenes* in home refrigerators, undoubtedly contributes in some capacity (Kilonzo-Nthenge et al., 2008; Macías-Rodríguez et al., 2013). Consumer handling of ready-to-eat foods, particularly improper storage, is a known contributor to elevated risk of foodborne listeriosis (Yang et al., 2006) and susceptibility of HSCs to further listerial growth during storage suggests that additional hurdles should be implemented to account for this risk.

1.4.3 Listeriosis outbreaks associated with Hispanic-style cheese consumption

Each year in the United States, approximately 1600 persons become seriously ill as a result of *L. monocytogenes* infection, and 260 of these persons die due the infection (CDC, 2014a). During the period of 1998-2014, 56 confirmed listeriosis outbreaks have been recorded, resulting in 707 illnesses, 520 hospitalizations, and 116 deaths (CDC, 2015a). From these outbreaks, 11 (19.6%) implicated HSCs, comprising 98 illnesses, 60 hospitalizations and five deaths. These outbreaks illustrate the key paths for introduction of *Listeria* into manufactured dairy.

Insufficient pasteurization was the first main cause of listerial contamination in HSCs manufacturing. A 1985 outbreak of listeriosis from Mexican-style cheese in Los Angeles County, California, resulted in 142 illnesses and three deaths, allegedly from insufficient pasteurization of milk or the introduction of raw milk into pasteurized milk during

manufacturing (Linnan *et al*., 1988). Similarly, between October 2000 and January 2001, a listeriosis outbreak occurred in Winston-Salem, North Carolina, resulting in 13 illnesses, including five stillbirths, three premature deliveries and three infected newborns. Illnesses were traced to the consumption of Mexican-style cheeses made from contaminated raw milk by unlicensed cheesemakers (McDonald *et al*., 2005).

Perhaps more commonly, outbreaks are traced back to the production of fresh cheeses under conditions that allow post-pasteurization contamination from the manufacturing environment, as described above. A 2008-2009 multistate listeriosis outbreak resulting in eight illnesses across five states was associated with a Mexican-style cheese manufacturing facility where multiple cheese varieties tested positive for the outbreak strain, suggesting post-pasteurization contamination of pasteurized milk (Jackson *et al*., 2011). Several other multi-state outbreaks have been linked with HSCs recently, resulting in eight cases and one death across two states in late 2013 (CDC, 2014b), five illnesses and one death across four states through 2014 (CDC, 2014c), and 30 illnesses and three deaths in 10 states ranging from the summer of 2010 to 2014 (CDC, 2015b). Strains of *Listeria* have been tracked back to environmental samples from the manufacturing facilities for each of these outbreaks, connecting insanitary conditions with post-pasteurization contamination of the cheeses.

1.5 Interventions

In practice, milk pasteurization, plant hygiene, and refrigeration alone comprise insufficient preservation for fresh HSCs. As milk can fail to be properly pasteurized and post-pasteurization contaminating *L. monocytogenes* can grow under refrigeration, fresh cheeses continue to pose a food safety risk. While strategies for preventing environmental contamination

can clearly be improved in many situations, additional interventions and additives have been sought for the prevention of listerial growth and survival in fresh cheeses.

1.5.1 Antimicrobial treatments to reduce *L. monocytogenes* in Hispanic-style cheeses.

Relatively few studies have been published on the examination of antimicrobial methods for the preservation of fresh HSCs. To date, almost all studies have been carried out using Queso Fresco as a HSC model with *L. monocytogenes* or *L. innocua*.

High pressure processing (HPP) is often used as a final hurdle to preserve packaged ready-to-eat foods such as sausages, so it was understandably investigated to address pathogens in HSC. While pressures of 500 to 600 MPa are capable of considerable reduction of listerial survival in Queso Fresco (>5 Log CFU/g), HPP cannot prevent regrowth of surviving cells (Tomasula et al., 2014; Hnosko *et al*., 2012) and compromises protein structure and textural properties in the finished products, resulting in negative impacts on consumer preferences (Sandra et al., 2004; Hnosko *et al*., 2012; Van Hekken et al., 2013).

Organic acids, bacteriocins, and other fermentates, as well as combinations thereof, have been assessed as antilisterials for Queso Fresco with limited success. Overcoming the instability and low activity of organic acids near neutral pH in HSCs by combining with the bacteriocin nisin can improve listerial reduction, but are generally bacteriostatic at best (Gadotti et al., 2014; Van Tassell et al., 2015), though may not diminish consumer acceptability. Queso Blanco manufactured with acetic acid and a commercial bacterial fermentate has been shown to also reduce *L. monocytogenes* by 2-3 log CFU/g over a 6-week shelf life, but even at low inoculation levels did not eliminate the pathogen (Glass et al., 1995). Another promising additive used in meat and poultry, lauric arginate has been demonstrated to maintain consumer acceptability

when applied to Queso Fresco and exhibits moderate reductions in listerial survival (Soni et al., 2010), especially when in combination with a listerial bacteriophage or a potassium lactate–sodium diacetate mixture (Soni et al., 2012).

Inhibition of surface contamination via antimicrobial packaging materials may also improve protection of HSCs, as they have shown promise on meat, produce, and other cheeses (Moreira et al., 2011; Irkin and Esmer, 2015). Edible packaging films of chitosan and lactic acid have been shown to reduce *L. monocytogenes* on the surface of Queso Fresco, enhancing pathogen inhibition further with the grafting of other components within the films (Sandoval et al., 2016), however significant additional moisture loss in the cheeses was observed during storage. Impacts on HSC quality by such coatings remain to be seen, as do their effectiveness against non-surface contaminants, such as those introduced during curd manipulation. For further discussion on the potential and difficulties of antimicrobial food packaging see Malhotra et al. (2015).

The quest for antilisterial treatments in HSCs, illustrated by the aforementioned studies, is characterized by two main gaps. First, even though Queso Fresco is the most well known HSC in the U.S., Queso Fresco is not necessarily representative of all HSCs: different manufacturing processes may imply not only different listerial contamination scenarios, but also that antimicrobial treatments may show different efficacy depending on the cheese variety. Additionally, studies have focused primarily on preservation methods less likely to impact the subtle taste and delicate texture of fresh HSCs, however confirmation of sensory impact or consumer acceptability is less commonly reported. A food antimicrobial agent should meet three important aspects in the food product: i) its antimicrobial activity should be selective, ii) it should have negligible impact on texture and sensory properties, and iii) it should be safe for

human consumption (Oliveira et al., 2012). Therefore, evaluating an antimicrobial with potential application to food industry should not be limited only to its antimicrobial properties.

1.5.2 Cheese surrogates

The simplest method for examining the effect of antilisterial treatments for fresh cheeses involves contaminating and treating commercially prepared or otherwise finished cheeses. By taking slices of prepared cheese and applying antimicrobials and *L. monocytogenes* to the surface, several treatments have shown effectiveness at reducing bacterial growth over product shelf life (Soni et al., 2010; Soni et al., 2012; Malheiros et al., 2012). This is a quick and effective method for assessing antimicrobial efficacy on finished cheeses, but may otherwise offer a narrow view of antimicrobial treatment: antimicrobial interactions are confined to an environment with limited impact by the cheese matrix itself. Homogenization of samples confers greater incorporation of the components into the product, but the disrupted microstructure may poorly reflect the interactions taking place between contaminants and antimicrobials within commercial applications. Unless the antimicrobial treatment is intended solely for external application, its design should consider the logistics of cheesemaking. In this case, laboratory-made cheeses should be preferred over pre-prepared samples for studying preservatives, to allow for the cheese model to resemble the most appropriate steps for contamination and antimicrobial addition without altering the manufacturing process.

Incorporation of pathogens at a large scale can be cumbersome for maintaining proper biosafety, so these methods often still rely on homogenization of the finished cheeses prior to inoculation, but modifying cheese manufacturing processes or incorporating novel antimicrobials into pilot-scale cheese production to assess impact on pathogen growth and survival is not

unheard of (Kasrazadeh and Genigeorgis, 1995; Bolton and Frank, 1999). *L. monocytogenes* has been shown to survive and grow when incorporated into the production process for soft cheeses under various conditions (Leggett et al., 2012), even when commercial starter cultures are present (Solano-López and Hernández-Sánchez, 2000; Leuscher and Boughtflower, 2002). Screening antimicrobial agents in relatively large batches of cheese can be costly, though combinations of organic acids and nisin have shown to exhibit inhibitory effects (Gadotti et al., 2014). Coelho et al (2014) used a much smaller batch size (0.5 L) to assess the antilisterial effects of incorporating bacteriocin-producing cultures into fresh cheeses, which raises the question of what scale is actually necessary for modeling fresh cheese production.

High-throughput, miniaturized cheese manufacturing models have been explored for their use in screening starter cultures and antimicrobials, resulting in cheeses with comparable structure to traditional equivalents despite preparation in 96-well microplates (Bachmann et al., 2009). We recently developed a similar model that now shows promise for screening antimicrobial additives in the production of miniature fresh cheeses (Van Tassell et al., 2015). Such miniaturized cheese models can be adapted to address numerous manufacturing parameters while benefitting from greater replication with minimal capital or resource consumption and improved biosafety management. In this way, antimicrobial treatments can be assessed *in situ* for intact cheeses under different treatment or contamination scenarios, reflecting commercial applications more appropriately.

Use of a surrogate organism for modeling listerial contamination of cheeses should also be addressed with care. Due to its close phylogenetic relatedness and similar physiology, nonpathogenic *L. innocua* can be used in place of *L. monocytogenes* for experimental work. However, variations in stress response may result in reactions to different antimicrobials that are

not comparable between organisms under different environmental conditions. Therefore, caution should be taken in extrapolating results from the use of a surrogate and validation with *L. monocytogenes* strains is encouraged for any given application. For a thorough review of the subject, see Milillo et al (2011).

1.6 Future directions

Relatively little is known about the association of *L. monocytogenes* with specific varieties of HSC. Most studies approach Queso Fresco as representative of the category, however this may not be the case. It is unclear to what extent different manufacturing steps and variations in finished product affect epidemiologic factors, as knowledge of *L. monocytogenes* presence in commercial products is largely outdated and limited to Queso Fresco, especially in the US. More current knowledge of incidence, particularly in other varieties of HSC, would be informative for epidemiological and preventative purposes.

Advances in monitoring and sanitation in dairy processing plants may also contribute significantly to preventing further outbreaks of listeriosis due to HSCs, especially considering the proportion of contamination likely due to post-pasteurization contact with environmentally persistent strains. One such area with room for growth involves the use of next-generation sequencing tools for microbial community profiling, such as already demonstrated in some wineries and artisanal cheese producers (Bokulich and Mills, 2013; Bokulich et al., 2016). More thorough knowledge of typical microbial communities and “abnormal” or “unsafe” deviations therefrom could perhaps help track potential sanitation issues and avoid environmental persistence of *L. monocytogenes*.

Furthermore, the extensive exploration of model cheese systems could promote deeper understanding of the interactions between antilisterial treatments, their targets, and food matrices. Such models could similarly facilitate the screening of combinatorial treatments that may exhibit synergistic mechanisms of antimicrobial action and the optimization of application parameters for their use in processing environments. Novel antimicrobials that may function as enhanced food preservatives include bioengineered bacteriophages or phage endolysins (Van Tassell et al., 2016). It would also be necessary, however, to overcome the confines of laboratory testing and confirm efficacy amongst the complexities of a commercial manufacturing setting.

Hopefully as more attention is drawn to the field, collaborative and interdisciplinary efforts will ultimately produce a cost efficient, consumer friendly solution to effectively limit *L. monocytogenes* in HSCs.

1.7 Tables and Figure

Table 1.1. Basic characteristics of selected fresh Hispanic-style cheeses

Name	Class	Cheese characteristics	Manufacturing distinction	Reference
Adobera	Fresh-pasta filata	Meltable, sliceable; final format resembles brick-like shape.	Rennet-set, milled texturized, uncooked, non-hot kneaded, non-stretched, pressed. Industrial manufacture includes the addition of mesophilic culture. No thermo-plastification step during cheese-making, its melting behavior is due its low pH (near 5.1-5.3)	Villegas de Gante, 2004
Asadero	Fresh-pasta filata	Meltable, shreddable; often confused with Oaxaca	Rennet-set, uncooked, hot kneaded, stretched, non-pressed. Industrial manufacture includes the addition of thermophilic culture. Cheddarized curd is heated in a small volume of whey to promote plastification and stretching. Different mold formats (e.g. blocks, balls or discs).	Villegas de Gante, 2004; Villegas de Gante and Messner Guillen, 2015
Oaxaca	Fresh-pasta filata	Meltable, shreddable; referred as Hispanic-style Mozzarella.	Rennet-set, uncooked, hot kneaded, stretched, non-pressed. Industrial manufacture can be performed by direct acidification (e.g. acetic acid) or by adding thermophilic culture. Stretching and kneading of acidified curd (near pH 5.1-5.4) in hot water. Typically molded like a ball of twine.	Villegas de Gante, 2004; Hnosko *et al*., 2009
Panela	Fresh	Soft, sliceable; final format as truncated cone shape; often whey oozes during cold storage	Rennet-set, generally uncooked, self-pressed. Curd is molded in baskets and cheeses are auto pressed by pilling them up on one another. Molding of the curd using a basket gives its particular shape.	Villegas de Gante, 2004
Queso Blanco	Fresh	Soft, mildly acid, sliceable; also named as Queso para Freir (frying cheese).	Acid-set, uncooked, pressed. Curd is rapidly set by isoelectric coagulation by adding acetic, lactic or citric acid to hot milk (approximately 80°C).	Villegas de Gante, 2004; Hnosko *et al*., 2009
Queso Fresco	Fresh	Soft, crumbly; most widespread Hispanic-style cheese in the U.S.	Rennet-set, generally uncooked, pressed. Salted curds are milled before pressing to enhance crumbling properties.	Hnosko et al., 2009
Ranchero	Fresh	Soft, crumbly; might be confused with Queso Fresco due to crumbly attributes.	Rennet-set, uncooked, non-pressed. Salt is added directly to dry milled curds and afterwards molded in a galvanized metal, plastic or wooden ring.	Villegas de Gante, 2004

Table 1.2. Basic characteristics of selected aged Hispanic-style cheeses

Name	Class	Cheese characteristics	Manufacturing distinction	Reference
Añejo	Aged	Crumbly; guajillo chili paste can be used to cover the ripened cheese.	Rennet-set, uncooked, non-pressed. Different degrees of crumbliness can be achieved during curd manipulation (multiple slicing, milling and kneading steps). Unmolded genuine Añejo cheese is ripened during approximately one month to up to 12 months at room temperature.	Villegas de Gante, 2004; Hernández-Morales et al., 2010; Hernández Morales et al., 2011
Chihuahua	Aged	Semi hard, sliceable, meltable; often named as Menonita or Quesadilla cheese	Rennet-set, uncooked or lightly cooked, pressed. Industrial manufacture includes the addition of mesophilic culture. Proper cheddarization (pH near 5.2-5.5) impacts on texture and ripening. Ripening period goes from 15 days to one month in cold room.	Villegas de Gante, 2004
Cotija	Aged	Semi hard, crumbly, acidic, salty; referred as the Hispanic-style Parmesan.	Rennet-set, uncooked, pressed. Genuine Cotija cheese made with raw milk is subjected to several months of ripening. Commercial Cotija cheeses might have ripening periods no longer than two weeks or even lack of aging.	Villegas de Gante, 2004
Manchego type	Aged	Semi hard, sliceable, meltable; Hispanic-style version of Protected Designation of Origin Manchego	Rennet-set, light cooked, pressed. Industrial manufacture includes the addition of mesophilic culture. Ripening period goes from 10 days to less than one month at 10-12°C.	Villegas de Gante, 2004; Hnosko et al., 2009

Table 1.3. Composition and pH values of selected Hispanic-style cheeses

Cheese variety	Moisture (%)	Protein (%)	Fat (%)	NaCl (%)	pH	Reference
Adobera	47	23.4	22.5	NA[1]	4.98-7.3	Villegas de Gante, 2004 Torres-Vitela et al., 2012
Añejo	31.8-41.9	21.44-30.3	25-33.8	1.8-2.8	5.1-5.4	Hernández-Morales et al., 2010
Asadero	42.16-53.01	20.74-29.93	18.36-32.23	0.76-1.93	NA	Alba et al., 1990
Chihuahua	35.14-42.4	22.1-27.3	27.3-37	0.78-2.18	5.19-5.42	Saltijeral et al., 1999 Van Hekken et al., 2007 Olson et al., 2011
Cotija	37.3-38.2	28.2-28.5	24-25	4.11-4.63	5.20-5.65	Villegas de Gante, 2004
Manchego type	38.7-45.5	24.41-27.59	24.89-31.91	0.88-2.5	5.09-5.51	Caro et al., 2014
Oaxaca	44.6-53	20-22.8	19.88-24.92	0.7-2.3	4.81-5.41	Caro et al., 2014 Fuentes et al., 2015
Panela	49-59.4	16.24-20.56	15.44-22.16	1.03-2.13	4.92-6.53	Saltijeral et al., 1999 Torres-Vitela et al., 2012 Caro et al., 2014
Queso Blanco	47.02-50	19.6-25	18.2-24.31	2.32-3.3	5.2-6.8	Villegas de Gante, 2004 Uhlich et al., 2006 Leong et al., 2014
Queso Fresco	49.4-58.9	14-19.7	20.5-31	1.02-2.7	5.26-6.77	Tunick and Van Hekken, 2010 Legget et al., 2012 Tunick et al., 2012 Van Hekken et al., 2012 Van Hekken et al., 2013
Ranchero	50.1-56.9	22.6-25.6	17.4-26.4	0.8-1.8	4.9-5.4	Solís-Méndez et al., 2013

[1]NA = data not available

Table 1.4. Prevalence of *L. monocytogenes* in some types of Hispanic-style cheeses.

Cheese variety	Prevalence (positive samples/total samples)	Country	Reference
Adobera	12 % (12/100)	MX	Torres-Vitela et al., 2012
	18.75 % (3/16)	MX	Rosas-Barbosa et al., 2014
Chihuahua	0 % (0/40)	MX	Saltijeral et al., 1999
	0 % (0/60)	MX	Alcázar Montañez et al., 2006
Manchego type	0 % (0/40)	MX	Saltijeral et al., 1999
Panela	15 % (6/40)	MX	Saltijeral et al., 1999
	0 % (0/60)	MX	Alcázar Montañez et al., 2006
	6 % (6/100)	MX	Torres-Vitela et al., 2012
	37.5 % (6/16)	MX	Rosas-Barbosa et al., 2014
Queso Fresco	2.75% (48/1746)	USA	CFSAN and FSIS, 2003
	3.4 % (5/149)	MX	Moreno-Enriquez et al., 2007
	9.3 % (7/75)	MX	Soto Beltran et al., 2014
	6.25 % (1/16)	MX	Rosas-Barbosa et al., 2014
Hispanic-style cheese, variety unespecified	2% (2/100)	USA	Genigeorgis et al., 1991a
	0.17% (5/2,931)	USA	Gombas et al., 2003
	6.3 % (7/111)	USA	Kabuki et al., 2004
	1% (2/204)	MX	Kinde et al., 2007

Table 1.5. Occurrence of *L. monocytogenes* isolates from Hispanic-style cheese processing plants

Type of sampling site	Prevalence (positive samples/total samples)	Reference
Milk	0 % (0/47)	Moreno-Enriquez et al., 2007
	0 % (0/16)	Rosas-Barbosa et al., 2014
Curds	10 % (3/30)	Rosas-Barbosa et al., 2014
Equipment	19.3 % (33/171)	Rosas-Barbosa et al., 2014
Food contact surfaces	1.7 % (2/119)	Kabuki et al., 2004
	33.3 % (10/30)	Moreno-Enriquez et al., 2007
	29.2 % (14/48)	Rosas-Barbosa et al., 2014
Floors	25.7 % (19/74)	Kabuki et al., 2004
	6.3 % (1/16)	Rosas-Barbosa et al., 2004
Cheeses	6.3 % (7/111)	Kabuki et al., 2004
	3.4 % (5/149)	Moreno-Enriquez et al., 2007
	17.5 % (11/63)	Rosas-Barbosa et al., 2014
Others	11.3 % (6/53)	Kabuki et al., 2004
	0 % (0/29)	Moreno-Enriquez et al., 2007
	31.3 % (10/32)	Rosas-Barbosa et al., 2014

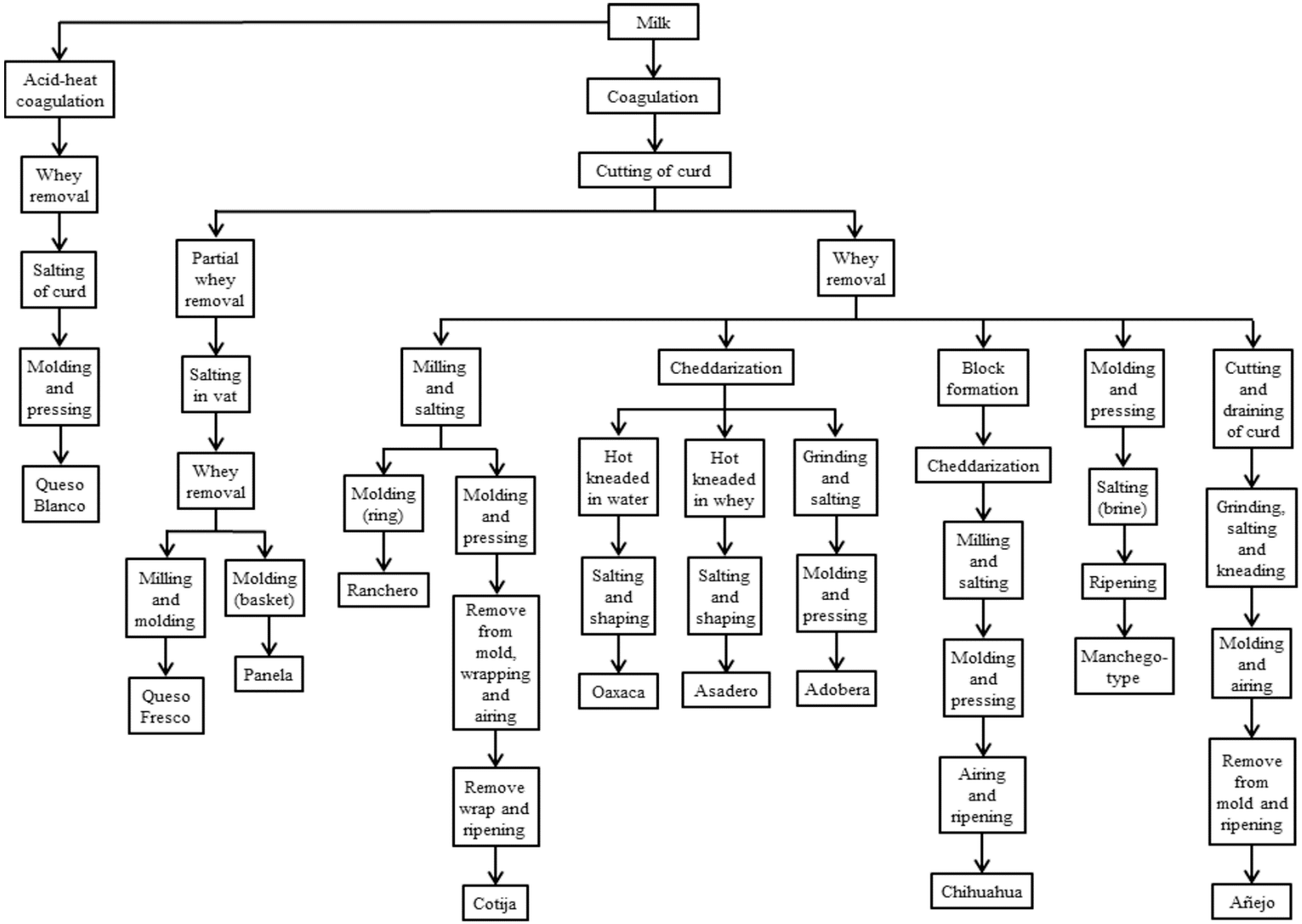

Figure 1.1. Main cheese making steps for manufacturing Hispanic-style cheeses.

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CHAPTER 2

ANTIMICROBIAL BEHAVIOUR OF PHAGE ENDOLYSIN PLYP100 AND ITS SYNERGY WITH NISIN TO CONTROL *LISTERIA MONOCYTOGENES* IN QUESO FRESCO[1]

2.1 Abstract

Hispanic-style fresh cheeses, such as Queso Fresco (QF), have been linked to numerous listeriosis outbreaks in the United States. In this work, we have studied the antilisterial behavior and effectiveness of the *Listeria* phage endolysin PlyP100 in QF, as well as the potential synergy between PlyP100 and nisin. PlyP100 showed similar bacterial reduction regardless of varying *L. monocytogenes* inoculum size in QF, and when the inoculation size was 1 Log CFU/g, no pathogen recovery after cheese enrichment was observed. PlyP100 was stable in QF for up to 28 days of cold storage exhibiting similar antilisterial activity regardless of when contamination with *L. monocytogenes* occurred. PlyP100 alone exhibited a strong listeriostatic effect in QF, on the contrary, nisin alone was not effective to control the pathogen in QF during cold storage. The combination of nisin and PlyP100 showed a strong synergy in QF with non-enumerable levels of *L. monocytogenes* after 4 weeks of refrigerated storage. Moreover, *L. monocytogenes* isolates from cheeses treated with nisin, PlyP100, and their combination did not develop resistance to nisin or PlyP100. Our results support the use of PlyP100 combined with nisin as an efficient *L. monocytogenes* control measure in QF.

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2.2 Introduction

While most cheese styles have not been associated with foodborne illness in the US, Hispanic-style fresh cheeses (HSFCs) represent a continued risk for listeriosis (Ibarra-Sánchez et al., 2017). They have been linked to both historic (Linnan et al., 1988) and recent outbreaks (CDC 2013, 2015). In fact, 4 of 26 listeriosis outbreaks listed in the CDC FOOD tool from 2011-2015 were linked to HSFCs, including 3 from pasteurized milk cheeses. In particular, Queso Fresco (QF) is the most widely produced and implicated HSFC in the US. Importantly, QF is known to support the growth of *L. monocytogenes* (Genigeorgis *et al*., 1991; Leggett *et al*., 2012; Leong et *al*., 2014; Van Hekken *et al*., 2012; Van Tassell et al., 2015*)*. In one study, *L. monocytogenes* was detected in environmental samples from 3 HSFC plants that exclusively purchased pasteurized milk for cheese production, and specifically in one plant the finished product samples were contaminated at a rate of 29% (7/24 samples) (Kabuki et al., 2004). Clearly, *L. monocytogenes* contamination of QF represents a significant health risk considering the high fatality rate of listeriosis infection among susceptible populations.

Nisin, a bacteriocin that is widely used in the food industry as a biopreservative, exhibits antimicrobial activity against Gram-positive bacteria such as *L. monocytogenes* (Field et al., 2015). Unfortunately, nisin stability and solubility is limited at neutral pH (Delves-Broughton et al., 1996), such as what is found in QF. Furthermore, studies have shown that *L. monocytogenes* can develop resistance to nisin (Gandhi and Chikindas, 2007). Nisin alone has not been successful at limiting *L. monocytogenes* growth in QF (Gadotti et al., 2014; Lourenço et al., 2017; Van Tassell et al., 2015)

Endolysins are bacteriophage-encoded enzymes that hydrolyze bacterial cell walls resulting in cell lysis and death (Van Tassell et al., 2016). While endolysins work from within

infected cells during a bacteriophage infection, endolysins can hydrolyze the cell walls of Gram-positive bacteria such as *L. monocytogenes* when applied exogenously. Endolysins can have a varied lytic spectrum, with many having broad activity within a genus, and are being exploited in numerous areas including food safety, diagnostics, and treatment of experimental infections in animals (Rodríguez-Rubio et al., 2016, Van Tassell et al., 2016) Endolysins have been able to eliminate or prevent the growth of relevant foodborne pathogens such as *Staphylococcus aureus* and *L. monocytogenes* when added to cow's milk (Chang et al., 2017a,b; García et al., 2010; Obeso et al., 2008), soya milk (Zhang et al., 2012), cheese (Guo et al., 2016), lean beef (Chang et al., 2017a) and ham (Chang et al., 2017b). However, to our knowledge, the behavior of endolysins incorporated into QF has not been rigorously investigated.

PlyP100 is an endolysin from the GRAS *Listeria* phage P100. Previously, the lytic activity of heterologously produced PlyP100 was characterized in a variety of environmental conditions and shown to lyse a wide range of *L. monocytogenes* strains. Additionally, PlyP100 is strongly bacteriostatic against *L. monocytogenes* in QF (Van Tassell et al., 2017). The objective of this study was to further evaluate the effectiveness, behavior, and potential synergism with nisin of PlyP100 against *L. monocytogenes* when incorporated into QF.

2.3 Material and methods

2.3.1 Microorganisms and culture conditions

Listeria strains (Table 2.1) were recovered from frozen glycerol stock (-80°C) and grown in brain heart infusion (BHI; Difco, Becton Dickinson and Co., Sparks, MD) broth, at 37°C for 24 h with 250-rpm agitation to obtain cell concentrations of ~ 9 Log CFU/mL. *L. monocytogenes* cocktails were prepared by combining equal volumes of stationary phase cultures of the five

different foodborne outbreak-associated strains. The serial dilutions of the *L. monocytogenes* cocktail were further prepared in PBS (KCl 200 mg/L; KH_2PO_4, 200 mg/L; NaCl, 8 g/L; Na_2HPO_4, 1.15 g/L, pH 7.2) to obtain the desired cell concentrations. *Listeria* enumeration was carried out on PALCAM Listeria-Selective agar (EMD-Millipore) supplemented with 20 µg/mL ceftazidime (Tokyo Chemical Industry Co. Ltd.) and incubated for 48 h at 37°C. *Escherichia coli* BL21 (DE3) containing pRSETB-plyP100 (chemically synthetized *ply*P100 gene using the NCBI reported sequence DQ004855.1) was used for overexpression of endolysin PlyP100 (Van Tassell et al., 2017) and cultured in LB broth (Fisher Scientific) supplemented with 50 mg/L ampicillin.

2.3.2 PlyP100 purification

PlyP100 was produced and purified as previously described (Van Tassell et al., 2017). Briefly, *E. coli* BL21 (DE3)-plyP100, were grown aerobically in shaking flasks (250 rpm) at 37°C to an optical density (OD_{600}) of 0.5-0.6 and were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cultures were then incubated for 8 hours at 33°C, pelleted via centrifugation at 4000 × *g* for 30 min at 4°C, washed in phosphate buffered saline (PBS; pH 7.2), and frozen overnight at -20°C. Recombinant PlyP100 was purified from the cell pellets using the QIAexpress Ni-NTA Fast Start Kit (Qiagen) according to the manufacturer's instructions. Purified PlyP100 was dialyzed against 100 mM NaCl via an Amicon Ultra-15 10K Centrifugal Filter Unit (10 kDa molecular weight cutoff; Merck Millipore) and diluted with an equal volume of glycerol, followed by sterile-filtration and storage at -20°C. Purity of the eluted protein was determined in 15% (w/v) SDS-PAGE in the BioRad Mini-Protean gel apparatus. PlyP100 concentration was quantified via the Quick Start Bradford Protein Assay (Bio-Rad) using BSA as the protein standard.

2.3.3 Quantification of PlyP100 activity

The PlyP100 activity was quantified via a turbidity reduction assay, using *L. innocua* cells for endolysin substrate due to their similar sensitivity as *L. monocytogenes* (Van Tassell et al., 2017). *L. innocua* were grown to OD_{600} = 1.0, centrifuged, and resuspended in 50 mM phosphate buffer (pH 7.0) to a final OD_{600} = 1.5. Bacterial suspensions (0.1 mL) were added to a PlyP100 preparation (20 µl purified endolysin, 80 µL phosphate buffer) in sterile microtiter plates, and the decrease in OD_{600} was monitored every 30 s for 30 min, at 25 °C, in a spectrophotometer. The activity of endolysin was calculated using the steepest slope of the obtained curve and its dilution factor; 1 U was defined as the amount of enzyme necessary to decrease the OD_{600} of *Listeria* cells in suspension by 0.01/min (Schmelcher et al., 2012).

2.3.4 PlyP100 retention coefficient in miniature laboratory Queso Fresco (MLQF)

The amount of PlyP100 retained in QF was calculated by measuring the amount of PlyP100 in whey compared to the total content of endolysin added to the milk or cheese curd. Batches of miniature laboratory Queso Fresco (MLQF) were prepared as previously described with minor modifications (Van Tassell et al., 2015). Three independent batches were prepared for both the addition of 100 µg PlyP100 directly into whole milk or mixed into drained curds during cheese-making. All whey was collected and then centrifuged at 20,000 × *g* for 2 min to separate the cheese fines from whey. The concentration of PlyP100 in the separated whey samples was measured using a His-Tag Protein ELISA kit (Cell Biolabs) according to the manufacturer's instructions. The retention coefficient of PlyP100 was calculated as follows:

$$\text{Retention coefficient} = \frac{(\text{PlyP100 content in milk basis}) - (\text{PlyP100 content in whey})}{(\text{PlyP100 content in milk basis})}$$

2.3.5 Effect of different inoculum sizes on the antilisterial activity of PlyP100 in MLQF

Briefly, pasteurized whole milk was used to prepare multiple small batches of QF as described above. Sample cheeses were inoculated with a five-strain *L. monocytogenes* cocktail directly into the curd prior to pressing, for final concentration of approximately 5, 4, 3, 2 or 1 Log CFU/g. In all batches, PlyP100 at concentrations of 0 or 10 U/g was incorporated into drained curd before pressing as follows: after inoculation and a 15-min incubation to allow *Listeria* to attach to the curd, tubes were centrifuged at 6,000 × *g* for 5 min for incomplete pressing. All accessible whey was removed, and 214.3 µL purified PlyP100 (7 U/mL and 13.86 U/mg of protein) were mixed with the curd for a final concentration of approximately 10 U of PlyP100/g of cheese. The curd was centrifuged for another 8 min at 6,000 × g, the remaining whey was removed and all cheese samples were stored at 4°C for up to 7 days until sampled. *L. monocytogenes* cells were enumerated by spread plating on PALCAM Listeria-Selective agar supplemented with 20 µg/mL ceftazidime. To expand the detection limits of plate-count enumeration, samples from each time point were also subjected to enrichment using the FDA-Bacteriological Analytical Manual standard enrichment/recovery method with some modifications (Hitchins et al., 2017) as shown below. Individual QF samples, approximately 0.15 g, were mixed with 1.35 mL of Listeria enrichment broth modified (Difco) supplemented with 1.1 g/L sodium pyruvate. Homogenized samples were incubated at 30°C for 48 h. After incubation, the mixture was streaked onto PALCAM plates and incubated at 37°C for 48 h. After incubation, the plates were examined for black colonies with a halo (esculin positive), and results were expressed as positive or negative for recovery.

2.3.6 Stability of PlyP100 in MLQF during cold storage

Three independent batches of QF were prepared as described previously with the following modification to the *Listeria* contamination procedure. Cheeses, manufactured with 0 or 10 U/g PlyP100 added to the drained and uninoculated curds, were surface-contaminated after 0, 7, 14, or 28 days of storage at 4ºC, by adding 50 µL of serially diluted *L. monocytogenes* cocktail for a final inoculum of approximately 5 Log CFU/g. Cells were allowed to attach for 30 min, liquid was completely removed and cheeses were sampled for enumeration after an additional 7 days of storage at 4ºC.

2.3.7 Dose-Response of PlyP100 in MLQF

QF batches were manufactured as previously described, but inoculated with a five-strain *L. monocytogenes* cocktail inoculum (described above) at approximately 5 Log CFU/g. Sample cheeses were treated with PlyP100 at concentrations of 0, 2.5, 5, 7.5 and 10 U/g into drained, contaminated curds followed by pressing. Cheeses were stored at 4°C and sampled after 0 and 7 days for enumeration of *Listeria*.

2.3.8 Effect of PlyP100 and nisin on *L. monocytogenes* in MLQF

Antimicrobial activity of purified PlyP100 combined with nisin was evaluated by their addition to QF, inoculated with the five-strain *L. monocytogenes* cocktail described above. PlyP100 (2.5 or 10 U/g) was added with or without 250 µg/g nisin (Nisaplin, Danisco, New Century, KS). Nisin was added into the milk before renneting while PlyP100 was incorporated into the drained, contaminated curds as described above. All QF samples were stored at 4°C, for up to 28 days, until sampled. Complementary to plate-count enumeration, samples from each

time point were also subjected to enrichment as described above. By the end of QF storage, three random *L. monocytogenes* isolates per cheese treatment (no antimicrobial, 2.5 U/g PlyP100 with or without 250 µg/g nisin), per independent experiment, were recovered from PALCAM plates and cultured individually for antimicrobial susceptibility testing.

2.3.9 Assessment of susceptibility of MLQF *L. monocytogenes* isolates to nisin and PlyP100

A total of 36 *L. monocytogenes* isolates from QF samples were individually prepared from single colonies inoculated into 10 ml BHI broth and incubated at 37°C for 24 h. The antimicrobial susceptibility tests were conducted as follows: susceptibility to nisin was measured by testing the minimum inhibitory concentration (MIC) via broth dilution method as described by Van Tassell et al. (2015); susceptibility to PlyP100 was determined via turbidity reduction assay as described above, and endolysin specific activity was calculated as the change in OD_{600} per min per mg protein.

2.3.10 Statistical Analysis

All experiments were performed independently three times with duplicate samples for each time point and dilutions were plated in triplicate. Experiments were analyzed as completely randomized designs using JMP 13.1 (SAS Institute Inc., Cary, NC). The Analysis of Variance (ANOVA) was performed to establish the significance of the factors, and the Analysis of Means (ANOM) was applied to determine which of the *L. monocytogenes* community isolates from QF exhibits antimicrobial susceptibility that are significantly different from the overall antimicrobial susceptibility in the population. Dose-response results were evaluated using Tukey's test to determine the statistical significance of mean differences between treatments.

2.4 Results

2.4.1 Retention coefficient of PlyP100 in MLQF

When added to cheese curds prior to pressing, PlyP100 exhibited a retention coefficient of 0.984 ± 0.004, indicating that approximately 2% of the endolysin was lost during whey removal. The retention coefficient of PlyP100 incorporated directly into milk, prior to cheese making, was 0.988 ± 0.003. There was no significant difference in retention observed (P = 0.4202), regardless of when the endolysin was introduced during manufacturing.

2.4.2 Effect of different inoculum sizes on the antilisterial activity of PlyP100 in MLQF

The antimicrobial effect of PlyP100, when it was enumerable, showed similar bacterial reduction regardless of varying size of *L. monocytogenes* cocktail inoculum in QF (P = 0.7264), suggesting that PlyP100 listeriacidal (bactericidal to *Listeria*) activity is inoculum independent for the range of concentrations evaluated (Table 2.2). In cases where the listerial population was enumerable, an approximate 0.54 to 0.75 Log CFU/g reduction occurred in the QF after 7 days of refrigerated storage. Additionally, no recovery of *L. monocytogenes* after an enrichment procedure was achieved when cheese samples treated with 10 U/g PlyP100 were inoculated with approximately 1 Log CFU/g.

2.4.3 Stability of PlyP100 in MLQF during cold storage

The stability of PlyP100 was assessed by inoculating the *L. monocytogenes* cocktail on QF surfaces at 0-28 days after manufacture. An approximate 0.5 Log CFU/g reduction of *L. monocytogenes* occurred regardless of whether the culture was added immediately upon manufacture or up to 28 days later (Figure 2.1). No significant difference in colony count

reduction of the pathogen across contamination after cheese manufacture was observed (P = 0.6927), indicating that PlyP100 remains active in QF during refrigerated storage over the course of its shelf life. As a control, the *Listeria* population increased by over 1 Log CFU/g when inoculated onto QF without PlyP100, regardless of the timing of inoculation.

2.4.4 Dose-Response of PlyP100 in MLQF

A range of concentrations of PlyP100 between 2.5 U/g and 10 U/g (the maximum concentration achievable due to endolysin purification yield) were added to QF inoculated with ~5 Log CFU/g of *L. monocytogenes*. PlyP100 exhibited a dose-dependent bacterial reduction (Table 2.3). At the lowest concentration tested, *L. monocytogenes* exhibited no significant growth after 7 days relative to the initial inoculum. Increasing the PlyP100 concentration resulted in an approximately 0.5 Log CFU/g reduction, relative to initial inoculum, similar to what was observed during the PlyP100's stability testing (Figure 2.1).

2.4.5 Effect of PlyP100 and nisin on *L. monocytogenes* in MLQF

After 28 days of storage at 4ºC, *L. monocytogenes* viable counts increased from an initial inoculum of 4.2 Log CFU/g to 8.1 Log CFU/g (Figure 2.2) in QF. The addition of nisin (250 µg/g) showed minimal impact on viable pathogen counts. Nisin initially slowed the growth of *L. monocytogenes* by approximately 1 Log CFU/g over the first week, but subsequent growth led to a final population comparable to the untreated QF at the end of four weeks of storage. The cheeses treated with PlyP100 alone were consistent with the observations made during dose-response testing; 2.5 U/g PlyP100 remained listeriostatic (bacteriostatic to *Listeria*) with no increase in viable cell count relative to the initial inoculum after 28 days and 10 U/g PlyP100

reduced cell viability by 0.5 Log CFU/g over the first 7 days and an additional 0.5 Log CFU/g by day 28. After 28 days of cold storage, all treatments combining PlyP100 with nisin reduced viable counts of *L. monocytogenes* below the enumeration detection limit. Additionally, about half of cheese samples did not yield *L. monocytogenes* growth after 48 hr of enrichment: 3 positive/3 negative and 4 positive/2 negative samples when cheeses were treated with 250 µg/g nisin + 2.5 U/g PlyP100 and 250 µg/g nisin + 10 U/g PlyP100 respectively.

2.4.6 Assessment of susceptibility of MLQF *L. monocytogenes* isolates to nisin and PlyP100

The susceptibility of *L. monocytogenes* community isolates from QF at the end of the cold storage, was evaluated by determining the MIC of nisin and specific activity of PlyP100. Due to the low yield of purified PlyP100 (7 U/mL; 13.86 U/mg of protein), it was not possible to assess the MIC for the endolysin. *L. monocytogenes* community isolates from QF samples treated with nisin (250 µg/g), PlyP100 (2.5 U/g) or their mixture (250 µg/g nisin + 2.5 U/g PlyP100) exhibited similar susceptibility to either nisin or PlyP100 ($P > 0.05$, ANOM) after antimicrobial exposure in QF, during cold storage over 28 days, compared to the susceptibility ranges (nisin MIC: 3.125-6.25 µg/mL; PlyP100 specific activity: 0.32 ± 0.02 – 0.50 ± 0.09) of pathogen isolates from untreated cheeses as reference control (Table 2.4). ,

2.5 Discussion

Queso Fresco is characterized by its high moisture content, low salt content and near neutral pH, which provide favorable conditions for *L. monocytogenes* growth. A limited number of studies have explored *Listeria* control measures in QF and the few new antimicrobials tested did not completely eliminate the pathogen or did not inhibit regrowth during cold storage. Novel

antimicrobials are needed to improve cheese safety in regards to *Listeria* contamination, and endolysins possess desirable antimicrobial traits for the food industry that need to be evaluated within a food matrix.

2.5.1 Retention coefficient of PlyP100 in MLQF

A high retention coefficient is a desirable property in cheese-making. Water soluble ingredients are prone to suffer loss in cheese whey (Giroux et al., 2013; Han et al., 2011), and are typically added to the curd, rather than to the milk, to improve their retention in the cheese matrix (Bermúdez-Aguirre and Barbosa-Cánovas, 2012; Heller et al., 2003). PlyP100 exhibited a high retention coefficient regardless of whether the endolysin was added to the milk or directly to curds. Particularly in QF manufacturing, dried curds are milled to enhance crumbly properties before pressing. Therefore by adding PlyP100 at this stage not only guarantees a high retention in cheese curd, but also does not alter the logistics already established in the manufacturing process for QF.

2.5.2 Effect of different inoculum sizes on the antilisterial activity of PlyP100 in MLQF

Some reports of *L. monocytogenes* antimicrobial susceptibility, in vitro and in situ, have shown direct dependence on inoculum size: the lower the pathogen inoculum the less antimicrobial concentration was required and vice versa (Bal'a and Marshall, 1996; Schillinger et al., 2001). However, the antimicrobial activity of PlyP100 (10 U/g) was not affected by the contamination level of the target pathogen (1-5 Log CFU/g), and also displayed the advantage of being listeriostatic at very high contamination levels. Although *Listeria* contamination levels are not typically reported in Hispanic-style cheeses survey studies, PlyP100 may be able to eliminate

L. monocytogenes in situations of low contamination levels (less than 100 CFU/g), as observed in the USDA-FSIS risk assessment (2003).

2.5.3 Dose-response and stability of PlyP100 in MLQF during cold storage

Similar to other reports of endolysins evaluated in food matrices, a dose-dependent antilisterial effect was observed for PlyP100 in QF (Table 2.3). Our results suggest that higher concentrations of PlyP100 than the maximum amount tested (10 U/g) may cause even greater reduction of *L. monocytogenes* populations in QF. Food antimicrobials are expected to be active in the food product during storage conditions in order to extend its shelf-life, and while several endolysins have been shown to retain their activity in solution after several months of storage at 4°C (Oliveira et al., 2012), no previous studies have explored endolysin stability in the food product during storage. PlyP100 maintained its activity in QF up to 28 days after manufacturing suggesting that PlyP100 may protect against *L. monocytogene*s contamination at the processing plant level as well as in consumer homes. The later representing a mitigation of the risk of listeriosis from the presence of *L. monocytogenes* in domestic refrigerators (Kennedy et al., 2005; Kilonzo-Nthenge et al., 2008; Macias-Rodriguez et al., 2013).

2.5.4 Effect of PlyP100 and nisin on *L. monocytogenes* in MLQF

In comparison to other studies that have focused on testing endolysins in liquid food matrices (Changet al., 2017a,b; Garcia et al., 2010; Obeso et al., 2008; Zhang et al., 2012), the antimicrobial activity of PlyP100 was reduced in the semi-solid QF matrix. Regardless, there was significant enough activity for a listeriostatic effect in QF, suggesting that a higher endolysin concentration would be necessary to enhance listeriacidal activity in QF. Factors that might have

an impact on the effectiveness of endolysins in QF during refrigerated storage, compared to reports in milk, may include: 1) enzyme destabilization from ionic interactions due to a higher cation concentration (sodium and calcium), 2) cold storage temperature of QF slowing down the enzymatic activity of PlyP100 (Van Tassell et al., 2017), and 3) the cheese semisolid matrix may limit endolysin diffusion disallowing its encounter with pathogenic cells.

The lack of pathogen regrowth observed in PlyP100 treated cheeses suggests that *L. monocytogenes* does not develop resistance to endolysins. Another study has shown regrowth of *L. monocytogenes* in QF treated with phage P100, most likely due to phage resistance development (Soni et al., 2012). This represents an advantage of using PlyP100 over its natural producer, the *Listeria* phage P100, when controlling for *L. monocytogenes* in QF.

Nisin has been widely used in the food industry as a preservative due to its ability to inhibit Gram-positive organisms, such as *L. monocytogenes*. Similar to our previous report (Van Tassell et al., 2015), nisin at the maximum permitted concentration of 250 ppm did not provide an effective barrier against *L. monocytogenes* in QF, as evident by the growth of the pathogen reaching similar colony counts to the untreated QF. The lack of nisin effectiveness in QF has to do with nisin's inherent lack of stability at the near neutral pH of QF (Van Tassell et al., 2015).

PlyP100 or nisin alone are not sufficient to ensure the safety of QF. Exploring the potential synergy helps to overcome individual limitations of antimicrobials. The combination of nisin with various antimicrobials has shown increased antimicrobial activity (Gharsallaoui et al., 2016). Also, the lytic activity of endolysins has shown improvement when combined with carvacrol (Chang et al., 2017b) or nisin (García et al., 2010) to control *Staphylococcus aureus* in foods.

Our results suggested a synergistic effect when combining PlyP100 with nisin in QF after 28 days of cold storage. The antimicrobial mixture not only reduced the initial pathogen contamination inoculum to non-detectable levels of enumeration, but also was effective enough that the recovery of *L. monocytogenes* was not possible in about half of the QF samples – an approximate 4 Log CFU/g reduction. Synergy between PlyP100 and nisin might be the result of the permeabilization of the cytoplasmic membrane caused by nisin which weakens the *L. monocytogenes'* cell wall allowing an increased hydrolytic action by PlyP100 (García et al., 2010); or nisin's gradual loss of stability in QF, might be complemented by PlyP100's listeriostatic effect, thus allowing residual nisin to be listeriacidal, causing reduction of *L. monocytogenes* populations without regrowth. It has been shown that nisin is more effective against exponential-phase cells (Dykes and Moorhead, 2002), and endolysins seem to display a similar trait (Van Tassell et al., 2017), suggesting that PlyP100 in combination with nisin may contribute to both preventing regrowth as well as eliminating actively growing *L. monocytogenes* cells.

2.5.5 Assessment of susceptibility of MLQF *L. monocytogenes* isolates to nisin and PlyP100

Foodborne pathogen resistance or adaptation development to food antimicrobials are of particular concern to the food industry, however, combining antimicrobial agents aids to minimize bacterial resistance and adaptation events (Nair et al., 2016). Our results showed that about half of the cheese samples treated with combinations of PlyP100 with nisin had viable *L. monocytogenes* recoverable after enrichment. However, no difference in susceptibility to nisin or PlyP100 was observed in 36 random *L. monocytogenes* isolates from a total of 12 QF samples, regardless of whether antimicrobials were added. It has been suggested that there is a low

probability for bacteria to develop resistance to endolysins (Oliveira et al., 2012). On the other hand, the development of nisin resistance has been reported in some Gram-positive pathogens such as *L. monocytogenes* (Zhou et al., 2014), representing a health risk due to multiplication of these resistant bacteria in nisin-containing food. However, the incomplete elimination of viable *L. monocytogenes* from these QF samples might not be attributed to resistance acquisition to either nisin or PlyP100. In our previous report, as well as this current study, we did not recover any nisin resistant *Listeria* isolates from samples treated with nisin (Van Tassell et al., 2015). This suggests that the stability loss of nisin, at the near-neutral pH of QF, might limit the occurrence of *Listeria* developing nisin resistance during refrigerated cheese storage. Surviving *L. monocytogenes* isolates exposed to nisin and PlyP100 combination in cheese might be the result of persister formation (bacterial subpopulations not killed by the antimicrobial, but are dormant cells and not intrinsically resistant to the antimicrobial). Recently, *L. monocytogenes* persister cell formation, following treatment with nisin in broth medium, has been reported (Wu et al., 2017). However, additional experiments are needed to determine if surviving *L. monocytogenes* are in fact persisters or if the concentrations of PlyP100 and nisin combination need to be optimized.

2.6 Conclusions

In summary, the *Listeria* phage endolysin PlyP100 showed promising antilisterial traits, in QF, such as a high retention coefficient in cheese curds, no observable pathogen inoculum size effect, high stability during cold storage and dose-dependent listeriacidal activity. Additionally, PlyP100 and nisin completely eliminated *L. monocytogenes* in about half of the cheeses samples

despite the ~ 4 Log CFU/g inoculation and without the pathogen developing resistance to either antimicrobial.

Future work could focus on optimizing the concentrations of PlyP100 and nisin when combined, and exploring combinations with other promising antilisterials for the reduction of *L. monocytogenes* in QF and other Hispanic-style fresh cheeses. Moreover, food grade production of recombinant endolysins and their safety assessment for human consumption need to be addressed before application in the food industry. However, the results of this work support the use of phage endolysins along with nisin as a biocontrol hurdle to inhibit *L. monocytogenes* in Queso Fresco to ensure cheese safety during cold storage.

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2.8 Tables and Figures

Table 2.1. Strains of *Listeria*

Listeria strains	Serogroup	Isolation source
Listeria innocua ATCC 33090	6a	Animal, cow brain
Listeria monocytogenes NRRL B-33104	4b	Food, epidemic, Jalisco cheese
Listeria monocytogenes NRRL B-33513	4b	Food, epidemic, pate
Listeria monocytogenes NRRL B-33420	4b	Food, epidemic, RTE[1] meat products
Listeria monocytogenes NRRL B-33424	1/2b	Human, epidemic, chocolate milk
Listeria monocytogenes NRRL B-33419	1/2a	Human, epidemic, sliced turkey

[1]Ready-To-Eat

Table 2.2. Effect of PlyP100[1] against different inoculum sizes of *L. monocytogenes* cocktail in Queso Fresco after 7 days of storage at 4°C.

Inoculum size	Survival (Log CFU/g)[2]	Recovery[3]
5 Log CFU/g	4.38 ± 0.24	+
4 Log CFU/g	3.46 ± 0.17	+
3 Log CFU/g	2.25 ± 0.10	+
2 Log CFU/g	ND[4]	+
1 Log CFU/g	ND	-

[1]10 U/g of cheese; 1 U = amount of enzyme necessary to decrease the OD_{600} of *Listeria* cells in suspension by 0.01/min

[2]Values are means ± SEM

[3]Isolation (+) or no recovery (-) of *L. monocytogenes* colonies after 48 h enrichment of cheese samples

[4]ND = below detection limit of enumeration

Table 2.3. Effect of different concentrations of PlyP100 on survival of *L. monocytogenes* cocktail in Queso Fresco after 7 days of storage at 4°C.

Concentration of PlyP100[1]	Survival (ΔLog CFU/g)[2]
0 U/g	$+1.83 \pm 0.05^{a}$
2.5 U/g	$+0.11 \pm 0.10^{b}$
5.0 U/g	-0.25 ± 0.13^{bc}
7.5 U/g	-0.45 ± 0.07^{cd}
10 U/g	-0.55 ± 0.14^{d}

[a,b,c,d]Means with dissimilar letters are significantly different ($P < 0.05$) when compared using Tukey's test
[1]1 U = amount of enzyme necessary to decrease the OD_{600} of *Listeria* cells in suspension by 0.01/min
[2]Survival of *L. monocytogenes*: ΔLog CFU/g = day 7 minus day 0. +, growth; -, no growth. *L. monocytogenes* initial inoculum of approximately 5 Log CFU/g. Values are means ± SEM

Table 2.4. Antimicrobial susceptibility of *L. monocytogenes* isolates[1,2] recovered after 28 days of exposure to nisin and PlyP100 in Queso Fresco at 4°C.

MLQF Treatments	Nisin MIC (µg/mL)[3]	PlyP100 specific activity[3,4]
No antimicrobial	3.125 – 6.25	0.32 – 0.50
250 µg/g nisin	3.125 – 6.25	0.41 – 0.71
2.5 U/g PlyP100	3.125 – 6.25	0.29 – 0.50
250 µg/g nisin + 2.5 U/g PlyP100	3.125 – 6.25	0.29 – 0.49

[1]Cheeses were initially inoculated with approximately 4 Log CFU/g of *L. monocytogenes* cocktail
[2]Three random *L. monocytogenes* isolates per cheese treatment per independent experiment (n = 36)
[3]No significant differences with the overall antimicrobial susceptibility in the population by ANOM ($P > 0.05$)
[4]Calculated as the change in OD_{600} per min per mg protein

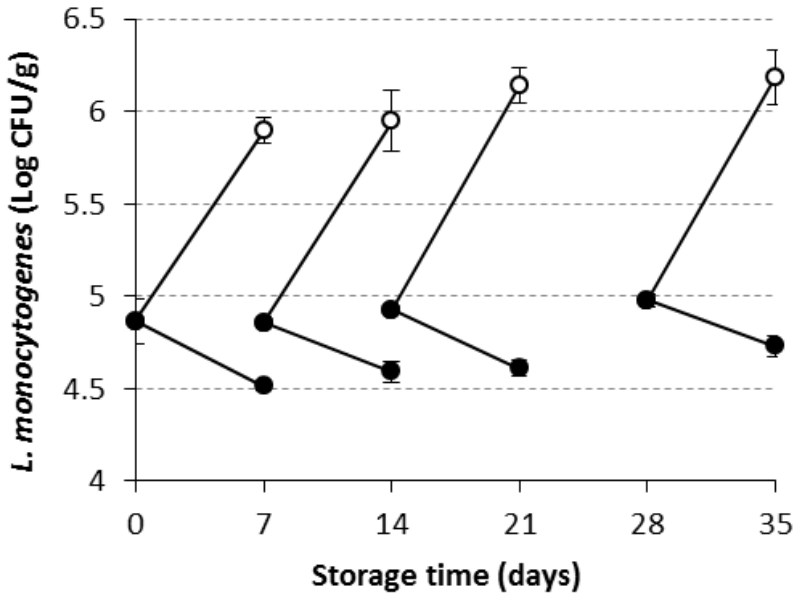

Figure 2.1. Antilisterial stability of PlyP100 in Queso Fresco during storage at 4°C. Survival of *L. monocytogenes* was assessed one week after initial inoculation at days 0, 7, 14, and 28 in cheeses treated with; 0 (○) or 10 (●) U/g PlyP100. 1 U = amount of enzyme necessary to decrease the OD_{600} of *Listeria* cells in suspension by 0.01/min. Values are means ± SEM

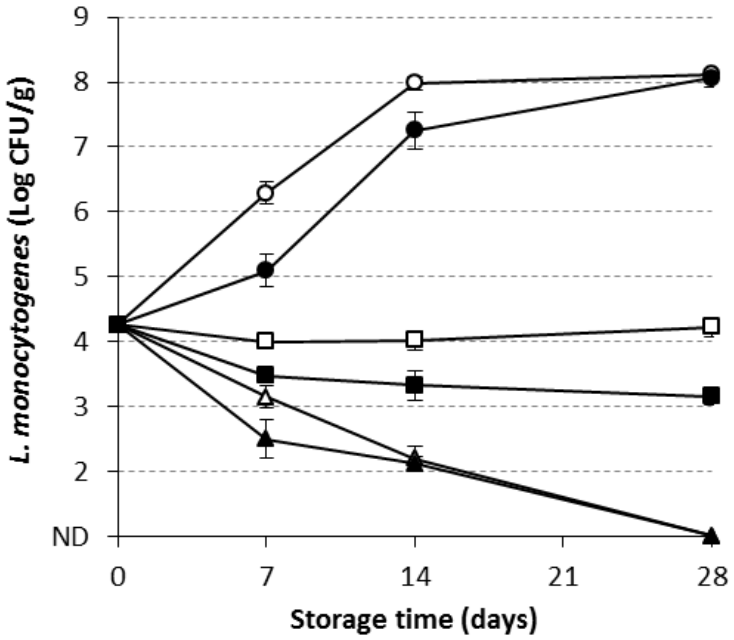

Figure 2.2. Effect of nisin and different concentrations of PlyP100 on survival of *L. monocytogenes* cocktail in Queso Fresco after storage at 4°C. Nisin and PlyP100 were added to cheeses by weight, with final concentrations of 0 (○) or 250 (●) µg/g nisin; 2.5 (□) or 10 (■) U/g PlyP100; 250 µg/g nisin + 2.5 U/g PlyP100 (Δ); and 250 µg/g nisin + 10 U/g PlyP100 (▲). 1 U = amount of enzyme necessary to decrease the OD_{600} of *Listeria* cells in suspension by 0.01/min. Values are means ± SEM. ND, not detected.

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CHAPTER 3

CO-ASSEMBLY OF NISIN AND ZEIN IN MICROFLUIDICS FOR ENHANCED ANTILISTERIAL ACTIVITY IN QUESO FRESCO[1]

3.1 Abstract

Nisin-loaded zein microcapsules were prepared in a microfluidic chip using an internal phase separation method. The release profiles of nisin from the microcapsules were modified by varying nisin loading and flow rate during the microfluidic process. Rapid release of nisin was achieved with low flow rate in dispersed phase (0.2 ml/hr) and high loading (9.67 – 12.64 μg/mg) of nisin in the microcapsule, while slow release of nisin was achieved with high flow rate in dispersed phase (0.3 ml/hr) and low loading of nisin (5.94 μg/mg) in the microcapsule. When tested in fresh cheese, all treatments with the nisin loaded zein microcapsules achieved overall lower viable *Listeria* cell counts relative to the treatment with free nisin, notably at early cheese storage. After 3 days of cold storage, the microcapsules with high flow rate and low nisin load reduced the initial viable population of *L. monocytogenes* by approximately 1 Log CFU/g, and the high nisin load capsules exhibited a reduction of approximately 0.5 Log CFU/g of the pathogen. Although subsequent regrowth of *L. monocytogenes* was observed after 7 days of cold storage for all the encapsulated nisin treatments, the high flow rate and low load microcapsules maintained the greatest difference in *L. monocytogenes* counts, approximately 2 Log CFU/g,

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[1]This chapter was the result of a collaboration and was published in its entirely in LWT. My primary contributions involved quantification of nisin of microcapsules samples by HPLC, modifying procedures for nisin release kinetics, evaluation of antilisterial effect of microcapsules in Queso Fresco, and composition of the manuscript. Feng Y, **Ibarra-Sánchez LA**, Luu L, Miller MJ, Lee Y (2019). Co-assembly of nisin and zein in microfluidics for enhanced antilisterial activity in Queso Fresco. LWT 111:355-362. This article is reprinted with permission from the Publisher.

compared to the untreated cheese. The cheese with the high flow rate and high load microcapsules was least effective on controlling *L. monocytogenes*. Nano-FTIR spectrum suggested that the distribution of nisin in the zein microcapsule was homogeneous, which indicates that a co-assembly of zein and nisin occurred during the internal phase separation process.

3.2 Introduction

Each year, the contamination by foodborne pathogens affects 48 million people in the United States and imposes over $15.5 billion of economic burden to the public (Hoffmann, Maculloch, & Batz, 2015). These foodborne illnesses along with the increase of antibiotic resistance in foodborne pathogens, present critical challenges for controlling disease and infection (Mulholland, Turpin, Bonev, & Hirst, 2016). Among those foodborne illness related outbreaks, many of them are caused by *L. monocytogenes* (Sarkar, Bhunia, & Yao, 2016). As an ubiquitous microorganism in the environment, *L. monocytogenes* is considered to be a serious concern for many ready-to-eat foods (Janes, Kooshesh, & Johnson, 2002), especially in fresh cheeses such as Queso Fresco (Luis A. Ibarra-Sánchez, Van Tassell, & Miller, 2018a). According to the literature, listeriosis, which is an illness caused by *L. monocytogenes*, is responsible for an estimated annual economic loss of $2.6 billion in the United States (Hoffmann and others 2012).

Recently, natural antimicrobial agents have gained increasing interests (Li et al., 2015; Matalanis, Decker, & McClements, 2012; S. Zhang, Zhang, Fang, & Liu, 2017), due to the potential health concerns and side effects with synthetic food preservatives such as nitrates, benzoates and sulfites (Pisoschi et al., 2018). One of the representative natural antimicrobial agents would be nisin (Liu & Hansen, 1990; Xiao, Davidson, & Zhong, 2011b) which is an antimicrobial peptide (composed of 34 aminoacids) produced by certain *Lactococcus lactis*

strains (Lins, Ducarme, Breukink, & Brasseur, 1999). To date, nisin has shown potent effect on inhibiting the growth of *Listeria monocytogenes* (Abee, Rombouts, Hugenholtz, Guihard, & Letellier, 1994; Imran et al., 2013) and even some gram negative pathogens(Vukomanović et al., 2017).

Application of nisin in food is limited due to its instability and insolubility in food matrices, where nisin can readily be degraded by other food ingredients such as sodium metabisulfite and titanium dioxide, and also Ca^{2+} and Mg^{2+} can inhibit the antimicrobial activity of nisin (Abee et al., 1994). Nisin can also be degraded in the neutral pH conditions (Rollema, Kuipers, Both, De Vos, & Siezen, 1995). Encapsulation of nisin using Generally Recognized As Safe (GRAS) materials were investigated in a previous study (Bernela, Kaur, Chopra, & Thakur, 2014) to improve and extend their efficacy in food matrices. Zein, a GRAS protein from corn, has been widely studied in food and pharmaceutical sciences as an emulsifier and packaging material, due to its excellent physiochemical property, safety, and relatively low cost (Feng & Lee, 2016; Shukla & Cheryan, 2001; B. Zhang & Wang, 2012). Previously nisin was encapsulated in zein using spray drying method. It lead to a porous microcapsule structure and resulted in poorer antilisterial properties than free nisin, then glycerol was used as a plasticizer to modify the morphology (Xiao et al., 2011b). Thermal treatment during conventional processing, such as spray drying, could also make a negative impact on the stability and antimicrobial activity of nisin (Holcapkova et al., 2017). On the other hands, those studies have demonstrated that zein, a water-insoluble protein, has a great potential as a carrier for antimicrobial agents (Kashiri et al., 2016; Mei et al., 2017; Y. Zhang et al., 2014). However, there is still lack of studies to understand the molecular structure of zein when nisin interacts with the wall materials.

Microfluidics has recently shown a great potential as a platform for encapsulation related applications (Feng & Lee, 2019a, 2019b; Olenskyj, Feng, & Lee, 2017). As an emerging technology developed in 1990s that capable of manipulating fluids in small scale (10^{-9} to 10^{-18} liters), microfluidics has been extensively investigated in the chemical engineering field (Whitesides, 2006). In the past a few years, a lot of food-grade materials have studied for microfluidic processing(Akay, Heils, Trieu, Smirnova, & Yesil-Celiktas, 2017; Feng & Lee, 2017, 2019b, 2019a; Kim, Park, Kim, Jeong, & Lee, 2017; Olenskyj et al., 2017). Microfluidic technique features many advantages including simplicity, precise control over product quality, and the capability to design various structures (Muijlwijk, Berton-carabin, & Schroën, 2016). Unlike the conventional encapsulation methods such as high shear and pressure homogenization, there is no heat generation during a microfluidic processing (Ali, York, & Blagden, 2009). As such, nisin will be able to retain its stability and antimicrobial activity during processing. In our previous study, zein could self-assemble to form hollow microcapsules in microfluidic chips, driven by diffusion induced internal phase separation (Feng & Lee, 2017, 2019b). The objective of this study was to encapsulate nisin in zein using a microfluidic device through an internal phase separation approach and control the release rate of nisin for the purposes of extending its antimicrobial efficacy when incorporated into Queso Fresco.

3.3 Materials and Methods

3.3.1 Materials

Zein (Z3625, Sigma-Aldrich, St. Louis, MO) dissolved in 70% (v/v) ethanol (200 Proof, Decon Laboratories, King of Prussia, PA) was used as the dispersing phase. Tributyrin (W222305, Sigma-Aldrich, St. Louis, MO) containing 2% (w/v) soy lecithin (MP Biomedicals

Inc, Solon, OH) was used as the continuous phase. Nisin (Nisaplin, Danisco, New Century, KS) was extracted with 70% ethanol at pH = 3.0 overnight to prepare the stock solution. The extracted nisin fraction was centrifuged at 4200 rpm (1000 × g) for 20 min into the dispersing phase at 2 mg/ml, to assess the rate of release. Hexane (Macron Fine Chemicals, Center Valley, PA) was used to wash the microcapsules. No. 42 (pore size 2.5 μm) Whatman filter paper (Whatman Inc., Florham Park, NJ) was used to collect final products.

3.3.2 Microfluidic fabrication

The dispersing phase was prepared by mixing 6% (w/v) of zein into an ethanol-water binary system containing 70% (v/v) ethanol with two nisin concentrations (approximately 2.2 mg/ml and 1.1 mg/ml), in order to obtain capsules with high load and low load of nisin. The concentrations were selected based on the preliminary tests, for the considerations of deliver enough amount nisin without interfering assembly process. The mixture was centrifuged at 2000 g for 20 min to remove insoluble components. The continuous phase was prepared by mixing 2% (w/v) of soy lecithin with tributyrin by stirring at 250 rpm for overnight, followed by centrifugation at 2000 g for 10 min to remove impurities. Nisin loaded microcapsules were then produced in a 100 μm T-junction microfluidic chip (Dolomite, UK) by internal phase separation that described in our previous study (Feng & Lee, 2017). The samples prepared in this study are listed in Table 3.1. Two flow rate combinations were chosen for this study and flow rates were controlled using a Harvard Model 11 Elite syringe pump (Harvard Apparatus Inc., Holliston, MA). Once collected, the capsules were immediately washed with hexane for three times on a filter paper with vacuum. The final microcapsules were obtained by transferring the filtered samples into a convective oven to remove excessive water at 30 °C for 6 hours. To quantify nisin

load, approximately 5 mg of microcapsules were dispersed in 500 µL of 70% (v/v) ethanol to disrupt microcapsules and release nisin before injecting to HPLC system.

3.3.3 Characterization of microcapsules

3.3.3.1 Scanning electron microscopy (SEM)

The surface morphology of microcapsules was imaged using a scanning electron microscope ((S-4700, Hitachi company, Japan) with an accelerating voltage of 5 kV located in Beckman Institute at the University of Illinois, Urbana-Champaign. Samples were adhered to a conductive carbon tape and sputter coated (model Dest-1 TSC, Denton Vacuum LLC., Moorestown, NJ, USA) with a gold layer before imaging.

3.3.3.2 Confocal laser scanning microscopy (CLSM).

A CLSM was used to visualize the internal structure of the microcapsules. Freshly collected microcapsule suspensions (1 ml) were stained with 40 µL of Nile blue (0.1%) for 2 h. A Zeiss LSM 700 confocal microscope (Zeiss, Germany) was operated using the excitation wavelength of 488 nm for the Nile blue dye to visualize the emulsions. Images were taken at a magnification of 20x and further processed using the instrumental software.

3.3.3.3 Circular dichroism spectrum (CD)

The CD spectra were recorded with a JASCO J-715 spectropolarimeter (Jasco Inc., Easton, MD). Samples were placed in a quarts cell with a 1 cm path length. The measurement was conducted over the range of 200-250 nm at 25 °C, with the scanning speed of 50nm/min, and the resolution of 1 nm. The content of α-helix, β-sheet, and random coil was calculated

using the BeStSel software (Micsonai et al., 2015), which could be accessed online at http://bestsel.elte.hu. The Savitsky-Golay smoothing algorithm was used, with a polynomial order of 3 and a smoothing window of 20 points, according to a previous study (Greenfield, 2006).

3.3.3.4 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra were acquired using an ATR-FTIR Nicolet Nexus 670 IR spectrometer (Thermo Nicolet Corp., Madison, MI) which was equipped with a Germanium attenuated total reflection (ATR) accessory. The IR transmittance was acquired from 4000 to 800 cm-1 with a resolution of 2 cm-1. 40 repeated scans were undertaken for each sample. All signals were collected against a background spectrum recorded from the mica substrate only.

3.3.3.5 Atomic Force Microscopy – Infrared Spectroscopy (AFM-IR)

The distribution of nisin was investigated with a AFM-IR equipment (NeaSNOM, Neaspec GmbH, Germany) following a method described in a previous work (Huth and others 2012). The nano-FTIR is based on an oscillating gold-coated AFM tip and a near-field interferometric signal from of scattered light. A wavenumber range from 1150 to 1800 was chosen to reflect the secondary structure, according to a previous study (Amenabar and others 2013). Three locations on each AFM scanning image were randomly selected to obtain the spectrum. The control blank zein microcapsule and high load 0.3-10 microcapsule were studied for comparison.

3.3.3.6 Nisin loading and release kinetics

To study the release kinetics, duplicated samples for each time point were prepared by dispersing approximately 7 mg of microcapsules in 500 µL of deionized (DI) water (pH = 7.0) and maintained with constant shaking rate at 250 rpm at room temperature. At 0, 1, 3, 10, 30, and 60 min, samples were immediately filtered (PTFE Syringe Filter 0.22 µm, Fisher Scientific, MA) to stop the release process by separating microcapsules (unreleased nisin) from the solution. The filtered samples were used to evaluate the rate of release by measuring nisin concentration by HPLC.

3.3.3.7 Nisin Quantification with HPLC

Nisin was quantified by the method proposed by Liu and Hansen (1990) with modifications. Waters 2695 Alliance HPLC system (Waters, Milford, MA) equipped with a Hewlett-Packard series 1050 photodiode array detector (Hewlett-Packard, Palo Alto, CA) was used. The HPLC system was interfaced with a computer using ChemStation software (Agilent Technologies Inc.). The analytical column was a reversed-phase Hypersil GOLD C18 (175 A, 250 x 4.6 mm, 5 µm) (Thermo Scientific, San Diego, CA). Solvent A was 0.1 % (v/v) trifluoroacetic acid (TFA) in ultra-pure water, and solvent B was 90% (v/v) HPLC-grade acetonitrile (Thermo Fisher Scientific) containing 0.1% TFA (v/v) in ultra-pure water. The column temperature was maintained at 40 °C, sample injection volume was 20 µL, and samples were measured at UV absorption of 215 nm. A linear gradient from 31% B to 43% B over 16 min was run at a flow rate of 1.0 mL/min. The standard curve was plotted using 10, 50, 100, 200 and 300 ppm nisin (Nisaplin®,2.5% nisin w/w), and the amount of nisin was calculated from the area of the peak at 215 nm. HPLC analysis was performed in duplicate for each sample.

3.3.4 Microbial test

3.3.4.1 Microorganisms and culture conditions

Listeria monocytogenes strains (Agricultural Research Service Culture Collection Northern Regional Research Laboratory strains B-33104, B33419, B-33420, B-33424, and B-33513) were recovered from frozen glycerol stock (-80 °C) and grown in brain heart infusion (BHI; Difco, Becton Dickinson and Co., Sparks, MD) broth, at 37 °C for 24 h with 250-rpm agitation to obtain cell concentrations of ~9 Log CFU/mL. *L. monocytogenes* cocktails were prepared by combining equal volumes of stationary phase cultures of the five different foodborne outbreak-associated strains. The serial dilutions of the *L. monocytogenes* cocktail were further prepared in PBS (KCl 200 mg/L; KH2PO4, 200 mg/L; NaCl, 8 g/L; Na2HPO4, 1.15 g/L, pH 7.2) to obtain the desired cell concentrations. *Listeria* enumeration was carried out on PALCAM Listeria-Selective agar (EMDMillipore) supplemented with 20 mg/mL ceftazidime (Tokyo Chemical Industry Co. Ltd.) and incubated for 48 h at 37 °C.

3.3.4.2 Antilisterial properties in Queso Fresco

Antimicrobial activity of free and encapsulated nisin were evaluated by their addition to Queso Fresco (QF) as previously described with minor modifications (Van Tassell and others 2015). Briefly, batches of QF were prepared with Nisaplin and microcapsules to an equivalent amount of 37.5 µg/mL nisin added into the milk before renneting. This corresponded to approximately 250 µg of nisin/g of cheese. Sample cheeses were inoculated with a five-strain *L. monocytogenes* cocktail directly into the curd prior to pressing, for a final concentration of approximately 4 Log CFU/g. All cheeses were stored at 4 °C for up to 28 days, until sampled for *Listeria* enumeration. Cheeses were individually homogenized and serially diluted in PBS, and

spread plated on PALCAM Listeria-Selective agar supplemented with 20 μg/mL ceftazidime to enumerate *L. monocytogenes*. Plates were incubated at 37 °C for 48 h.

3.4 Results and Discussion

3.4.1 Nisin loading and release kinetics of nisin

The loading of nisin was quantified by HPLC. The loading of nisin was 5.94 μg/mg in the low load sample and 9.67-12.64 μg/mg in the high load microcapsules (Table 3.1). The loading were different across samples primarily due to the initial differences in nisin concentration. The nisin loading in high load 0.2-10 (low flow rate) sample (9.67 μg/mg) is slightly lower than the high load 0.3-10 (high flow rate) sample (12.64 μg/mg), probably due to the cracked structure of microcapsules that lead to lower retention rate. Nisin is a hydrophobic peptide that has poor solubility in water but soluble in alcohols, and the low pH of alcohol environment could further increased the solubility of nisin in alcohols (Liu & Hansen, 1990). The release kinetics of nisin (μg) from the microcapsules per mg were plotted in figure 3.1. Comparing the two samples with both high loading but different flow rates, the high load 0.2-10 sample showed slightly greater release rate than the other two samples, due to its cracked morphology and exposed interior structure (Figure 3.2). On the other hand, the low load 0.3-10 microcapsule released significantly less amount of nisin per unit microcapsules, because of low nisin loading and its interaction with zein, which could potentially hinder its release from the matrix. The release of nisin from microcapsule is driven by diffusion, while the opened capsules structure helps to create greater interface between the nisin and liquid, which allows rapid release process (Calado, dos Santos, & Semiao, 2016). Since the initial concentration gradient between the capsule surface and liquid media is large, an abrupt release occurs. A very slightly difference in sample handling, an

initially different degree of microcapsule agglomeration as well as the random fraction of capsules sinking/floating at the beginning of release could all bring in significant variation during the first a few minutes of the release process. Therefore, typically a large variation is found in the first a few data points and the variation decreases gradually.

3.4.2 Morphology of microcapsules

The SEM image of microcapsules are shown in the figure 3.2. It showed that the capsules prepared with low flow rate has an open and cracked structure. The cracked particles during internal phase separation has been reported when the diffusion was either too fast or too slow (Dowding, Atkin, Vincent, & Bouillot, 2004). In this case, a fast diffusion rate was expected due to the formation of small droplet at low flow rate. The drastic shrinking of capsule during rapid diffusion leads to the formation of cracks before zein could self-assemble to from stable structure. When the flow rate of dispersing phase increased, the microcapsules has less tendency to crack. The cracked structure in the high load 0.2-10 sample allows a greater release rate of nisin, when compared other two capsules. The morphology was further confirmed with the confocal laser scanning microscopy (CLSM) in the figure 3.3. It was also found that there is a cavity reside in the microcapsule high load 0.3-10, which could function as a reservoir that enables sustained release.

3.4.3 Molecular characterization of zein-nisin interaction

The SEM image of microcapsules are shown in the figure 3.2. It showed that the capsules prepared with low flow rate has an open and cracked structure. The cracked particles during internal phase separation has been reported when the diffusion was either too fast or too slow

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3.4.4 Molecular characterization of zein-nisin interaction

The molecular-level interaction between zein and nisin was studied using FTIR, CD, and nano-FTIR spectrum. The FTIR spectrum is shown in the figure 3.4. The peaks were identified according to previous studies (Bernela et al., 2014; Khan et al., 2014), and most peaks remain the similar intensity, including the O-H stretch peak at 3299.63 cm-1, the C-H stretch at 2963.21 cm-1, the C=O stretch at 1744.25 cm-1, the O-H stretch at 1252.58 cm-1, and the C-O stretch at 1172.89 cm-1 and 1102.96 cm-1. On the other hand, an increased intensity of amide peaks (amide I at 1658.27 cm-1 that indicating C=O vibration, amide II at 1544.28 cm-1 that indicating N-H vibration, and amide III at 1452.80 cm-1 that indicating C-N bond) were observed in nisin-loaded samples. A previous study also reported the increased intensity of amide absorbance as the evidence of nisin loading (Khan et al., 2014). In addition, the nisin-loaded sample did not show new peaks, indicating no covalent bond formation between nisin and zein.

Figure 3.5 shows the secondary structure of microcapsules that were characterized by CD spectrum. According to the CD spectrum, the loading of nisin altered the secondary structures of protein. The distribution of α-helix, β-sheet and random coils were calculated with BestSel algorithms (Micsonai et al., 2015). In blank zein capsules, very small amount of α-helix (1.9%) presents, with relatively high fraction of β-sheet (43.3%) and random coil (54.8%), as we reported in our previous work (Feng & Lee, 2017). Self-assembly of zein converted the majority of α-helix into β-sheet (Wang & Padua, 2012). Similar distribution of secondary structures were also found in low nisin loaded sample (0% α-helix, 39.7% β-sheet, and 60.3% random coil). However, the microcapsules with high-loading of nisin contains much higher fraction of α-helix, 4.9% in the low flow rate sample and 12.2% in the high flow rate sample. The results suggest the self-assembly of zein is hindered by the increased concentration of nisin, which is probably due to the hydrogen bonding between nisin and the amino acids that reside in the α-helical structure of zein. On the other hand, it is also possible that the α-helix structure originally exists in nisin. However, previous study only suggested the formation of β-sheet in nisin when the pH is greater than 5.5 (Dykes, Hancock, & Hastings, 1998).

Unlike the microcapsules with core-shell structure, the interaction between peptide and protein makes it difficult to predict the spatial distribution of nisin in the zein microcapsule. It is possible that nisin is involved in the arrangement of self-assembled zein molecules and forms a co-assembled structure, instead of a core-shell structure. As such, we utilized a nano-FTIR technology to study the surface composition of the nisin-loaded microcapsules. A schematic diagram is shown in the figure 3.6 to illustrate the mechanism of nano-FTIR. Briefly, a gold-coated AFM tip is paired with an IR device to probe the near-field spectrum during scanning. As such, it can provide the fingerprint to reflect the composition at the material surface, which could

be used in this work to study the distribution of nisin. Two samples, a blank zein capsule and the high nisin loaded and high flow rate capsule (high load 0.3-10) were compared. For each capsule, three spots were randomly selected after the AFM scanning on the capsule surface (2μm * 1μm). All three spots showed similar spectrums, indicating a homogeneous surface composition. Comparing the spectrum from the nisin-loaded zein capsules and blank zein capsules, the nisin-loaded zein capsule has two characteristic peaks of amide I and amide II (Amenabar et al., 2013), which is shown in the yellow color. As mentioned in the previous sections, these amide peaks are an indication of the presence of nisin. Hence, it is reasonable to speculate that nisin and zein formed a co-assembled homogeneous structure.

3.4.5 Effect of encapsulated nisin on *L. monocytogenes* in Queso Fresco

Nisin is used in the food industry due to its antimicrobial effect against Gram-positive bacteria, notably *L. monocytogenes*, a pathogen of relevant concern in fresh cheeses (Luis A. Ibarra-Sánchez, Van Tassell, & Miller, 2018b). To assess the antilisterial properties of microcapsules in a food matrix, free and encapsulated nisin were added to the maximum permissible level of 250 ppm of nisin in Queso Fresco. Over 28 days of storage at 4 °C, *Listeria* grew from approximately 4 to more than 8 Log CFU/g in the untreated control cheeses (Figure 3.7). As shown in the previous reports (L.A. Ibarra-Sánchez, Van Tassell, & Miller, 2017; Luis A. Ibarra-Sánchez et al., 2018a), the addition of free nisin is ineffective to control *Listeria* in Queso Fresco, where only a discrete reduction of *Listeria* cells can be observed over the first 3 days of storage, but at the end of the four weeks of storage the final population of the pathogen is comparable to the untreated cheeses. The reduced effectiveness of nisin in dairy products, in particular Queso Fresco, is in part a combination of nisin's low stability at near neutral pH (Van

Tassell et al., 2015) and the absorption of nisin to fat globules due to nisin's hydrophobicity (Bhatti, Veeramachaneni, & Shelef, 2004) considering Queso Fresco's high fat content (> 20 % w/w). All treatments with nisin loaded zein microcapsules achieved overall lower viable *Listeria* cell counts relative to cheeses with free nisin, notably at early storage. After 3 days of cold storage, low load 0.3-10 capsules reduced the initial viable population of *L. monocytogenes* by approximately 1 Log CFU/g, and both high load capsules exhibited a reduction of approximately 0.5 Log CFU/g of the pathogen. Althought subsequent regrowth of *L. monocytogenes* was observed after 7 days of cheese storage for all nisin encapsulated treatments, low load 0.3-10 maintained the greatest difference in pathogen cell counts compared to untreated cheeses (aproximately 2.5 Log CFU/g), followed by high load 0.2-10 capsules (approximately 1.5 Log CFU/g), and finally in cheeses added with high load 0.3-10 capsules. This is different from other studies that have microencapsulated nisin using zein or other GRAS materials, in which discreet or no antimicrobial improvement than free nisin was observed in controlling *L. monocytogenes* when evaluated in milk (da Silva Malheiros, Daroit, da Silveira, & Brandelli, 2010; Martinez, Alvarenga, Thomazini, Fávaro-Trindade, & Sant'Ana, 2016; Xiao, Davidson, & Zhong, 2011a) or cheese (Malheiros, Sant'Anna, Barbosa, Brandelli, & Franco, 2012). Differences between the effectiveness of microcapsules preparations in Queso Fresco might be the result of their morphology and structure characteristics and the number of particles used depending on the load. In the case of high load 0.2-10 and high load 0.3-10, faster nisin realease profiles were found (Figure 1), probably driven by their high loading per microcapsule and concentration gradient. Although the release of nisin per unit microcapsules is high, while in the circumstance of cheese matrix where the mobility of nisin is greatly limited, the distribution of nisin could be inhomogenies in cheese. Those released nisin could form clusters surrounding zein

microcapsules without been transported throughout the cheese matrix. The low load 0.3-10 displayed the slowest nisin release profile (Figure 3.1), and the lowest concentration per unit microcapsule. Since more microcaspules were used to compensate the loading limit and reach the equivalent initial nisin being innoculated, a more even distribution of nisin in the cheese matrix may be achieved compared to the high loading samples. which hence helped to delay the growth of *L. monocytogenes* in the cheese matrix. High load 0.3-10 exhibits a similar nisin release profile as high load 0.2-10, and they showed similar results in the antimicrobial test. Our observation might be the result of adding larger number of low load microcapsules (5.94 μg nisin/mg microcapsules) into the cheese, compared to the number of high load microcapsules added, to achieve the same nisin concentration in all treatments (250 μg nisin/g cheese), this might allowed a better distribution of the low load microcapsules within the cheese matrix increasing the encounter between *Listeria* cells and released nisin.

3.5 Conclusions

In this study, an antimicrobial peptide nisin was encapsulated in zein using internal phase separation method. Different release patterns were achieved by adjusting the flow rates and nisin loading. The distribution of nisin in zein microcapsules was characterized with AFM-IR, and a homogeneous co-assemble pattern between nisin and zein was suggested. Microbial test infers that microbial activity of encapsulated nisin is highly dependent on the food matrices and environmental conditions. Sustained release of antimicrobial agent is helpful for inhibiting *Listeria* in cheese rather than instant release. Future work could focus on optimizing the microencapsulation preparation conditions and reveal more insights in terms of choosing the appropriate antimicrobial release media in different food matrices and environment. Moreover,

sensory evaluation of the zein microcapsules added into the food product need to be addressed before application in the food industry. However, our results support the use of encapsulation technology to improve nisin effectiveness to control *Listeria* in food matrices.

3.6 Table and Figures

Table 3.1. A list of samples prepared in this study

Sample name	Loading (μg/mg)	Dispersing flow rate (ml/hr)	Continuous flow rate (ml/hr)
Nisin	N.A.	N.A.	N.A.
Low load 0.3-10	5.94 ± 0.52	0.3	10
High load 0.2-10	9.67 ± 1.27	0.2	10
High load 0.3-10	12.64 ± 2.04	0.3	10

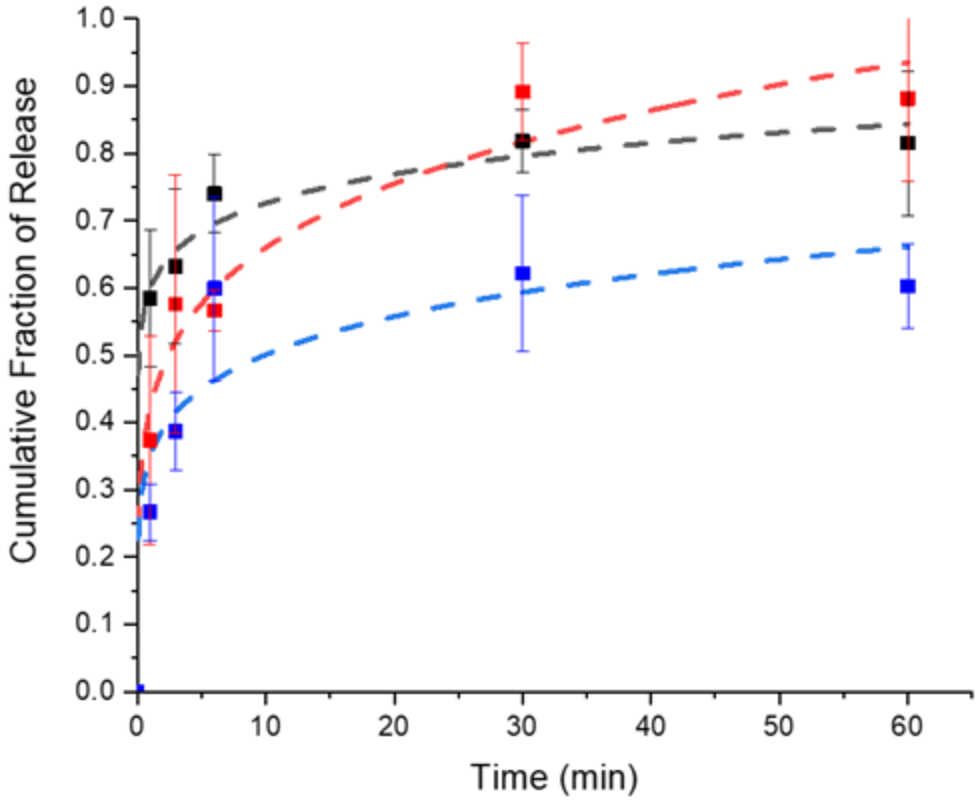

Figure 3.1. The release profiles of nisin from different capsules. Sample low load 0.3-10 is the zein capsule with 5.94 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate; sample high load 0.3-10 is the zein capsule containing 12.64 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of flow rate; sample high load 0.2-10 is the zein capsule containing 9.67 µg/mg of nisin, prepared with 0.2 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate. Values are means ± SEM.
Legend: Low load 0.3-10 (black), High load 0.2-10 (red), High load 0.3-10 (blue)

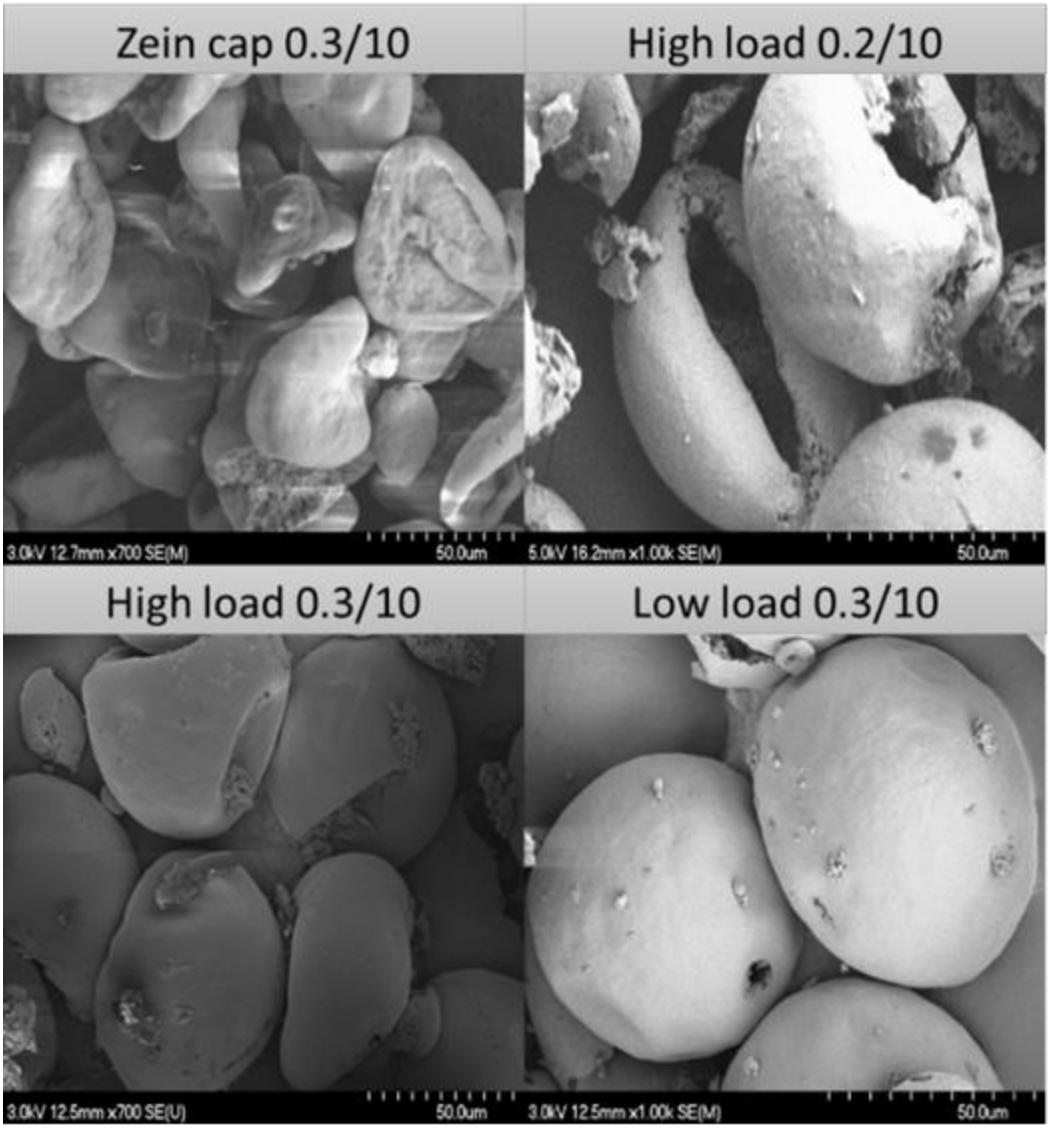

Figure 3.2. The scanning electron microscopy images of microcapsules formed at different conditions. Zein capsule is the blank that does not contain nisin; Sample low load 0.3-10 is the zein capsule with 5.94 μg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate; sample high load 0.3-10 is the zein capsule containing 12.64 μg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of flow rate; sample high load 0.2-10 is the zein capsule containing 9.67 μg/mg of nisin, prepared with 0.2 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate.

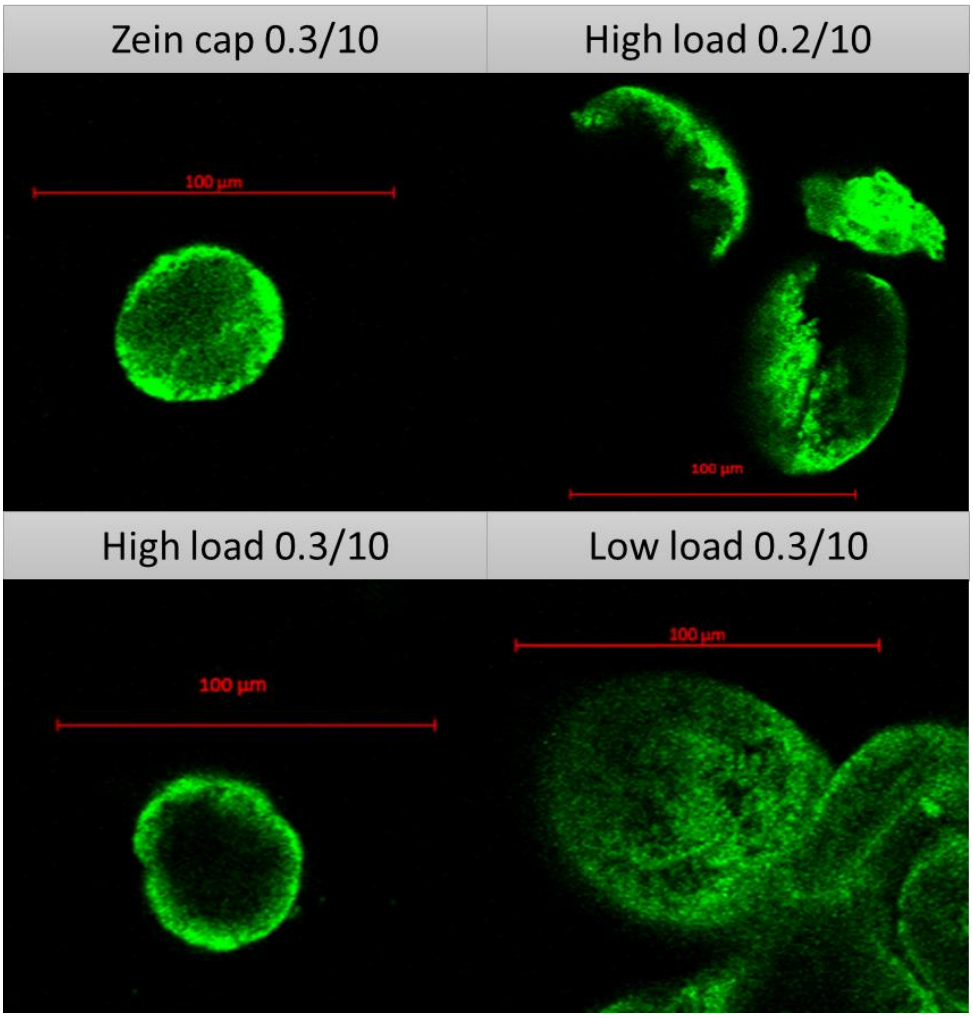

Figure 3.3. The confocal laser scanning microscopy (CLSM) images of microcapsules formed at different conditions. Zein capsule is the blank that does not contain nisin; Sample low load 0.3-10 is the zein capsule with 5.94 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate; sample high load 0.3-10 is the zein capsule containing 12.64 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of flow rate; sample high load 0.2-10 is the zein capsule containing 9.67 µg/mg of nisin, prepared with 0.2 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate.

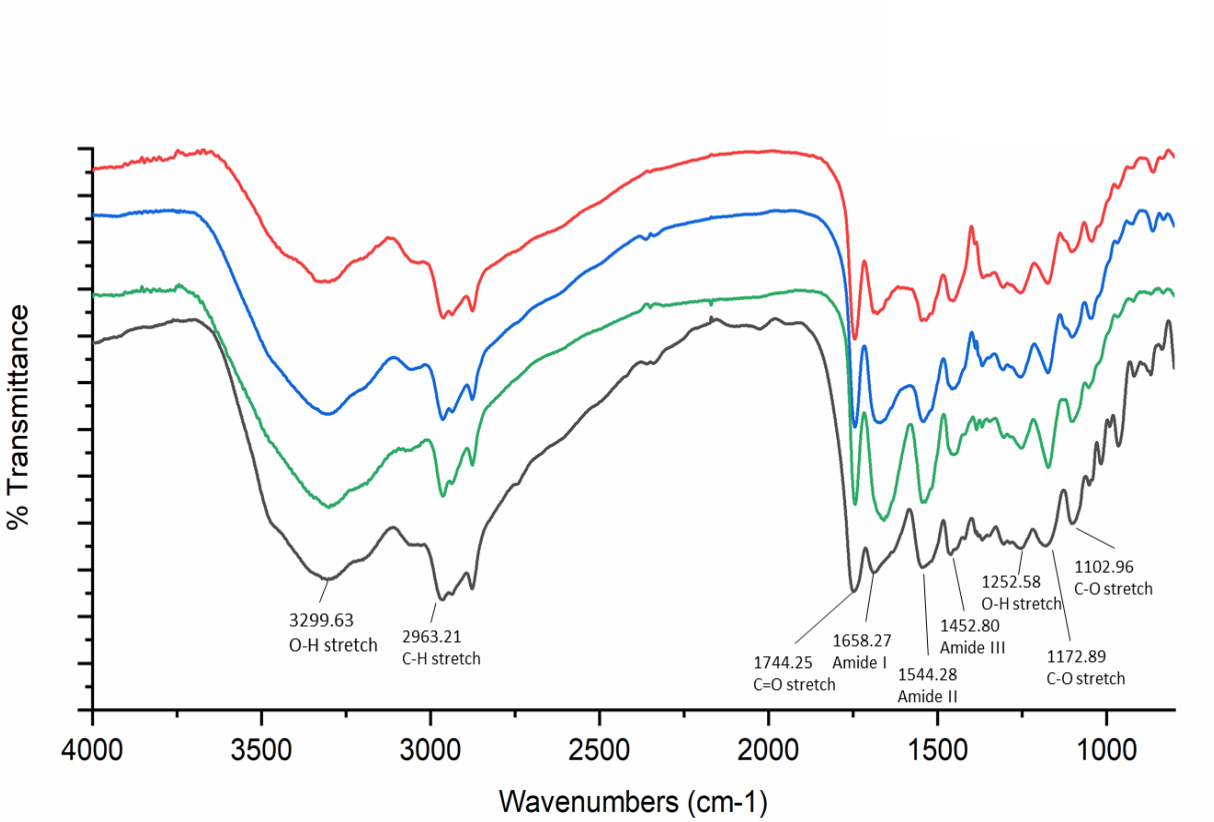

Figure 3.4. FTIR spectrum of microcapsules, zein capsule is the blank that does not contain nisin; Sample low load 0.3-10 is the zein capsule with 5.94 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate; sample high load 0.3-10 is the zein capsule containing 12.64 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of flow rate; sample high load 0.2-10 is the zein capsule containing 9.67 µg/mg of nisin, prepared with 0.2 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate.
Legend: zein microcapsule (black), low load 0.3-10 (red), high load 0.3-10 (blue), high load 0.2-10 (green)

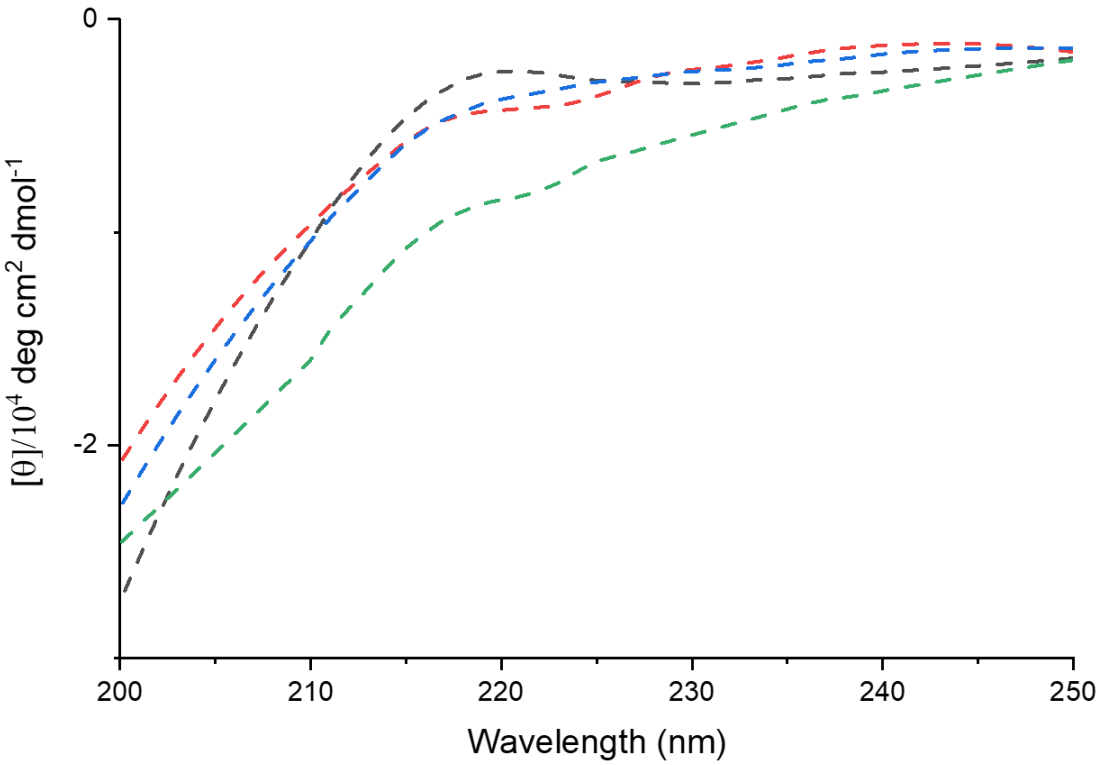

Figure 3.5. Circular dichroism spectrum of zein capsule and nisin-loaded zein capsules. zein capsule is the blank that does not contain nisin; Sample low load 0.3-10 is the zein capsule with 5.94 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate; sample high load 0.3-10 is the zein capsule containing 12.64 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of flow rate; sample high load 0.2-10 is the zein capsule containing 9.67 µg/mg of nisin, prepared with 0.2 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate.
Legend: zein capsule (green), low load 0.3-10 (black), high load 0.2-10 (red), high load 0.3-10 (blue)

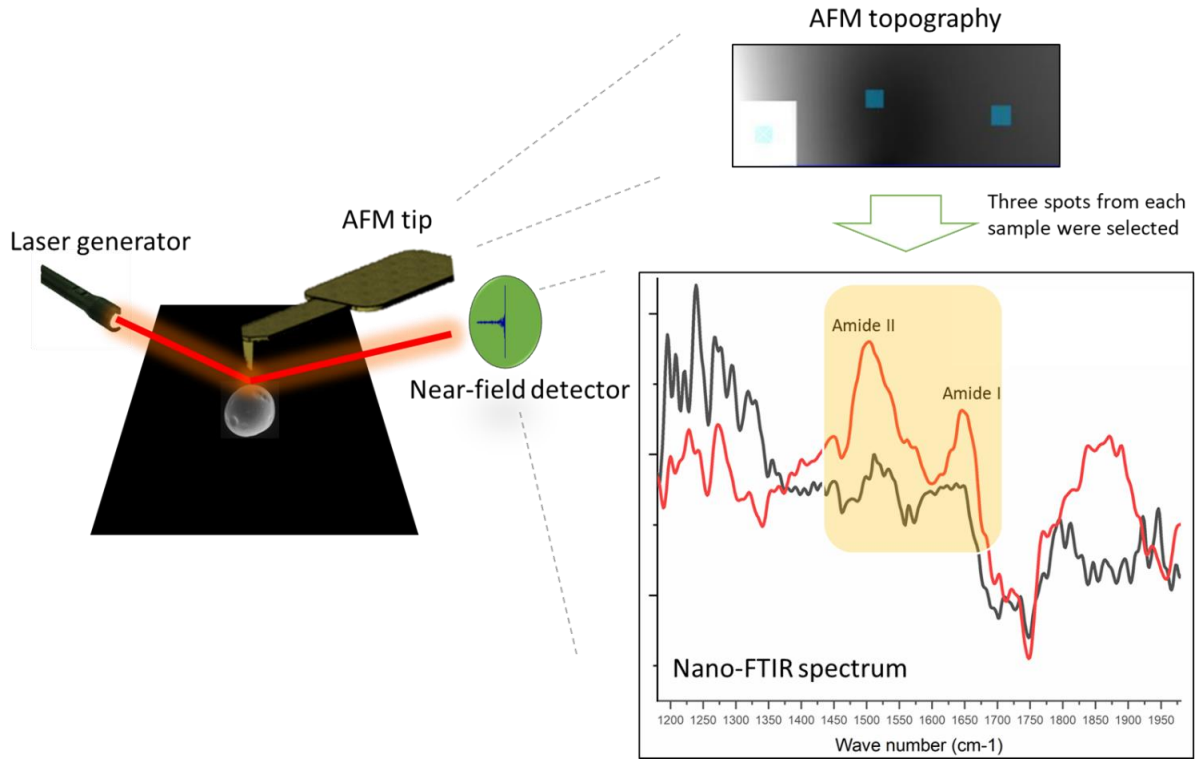

Figure 3.6. A schematic diagram of nano-FTIR measurement, and a spectrum to compare the surface composition between zein microcapsule and nisin-loaded zein microcapsule. The wavenumber range from 1150 to 1800 was chosen to indicate the difference in secondary structure. High load 0.3-10 microcapsule was used here as a representative nisin-loaded sample. Legend: zein microcapsule (black), nisin-loaded zein (red)

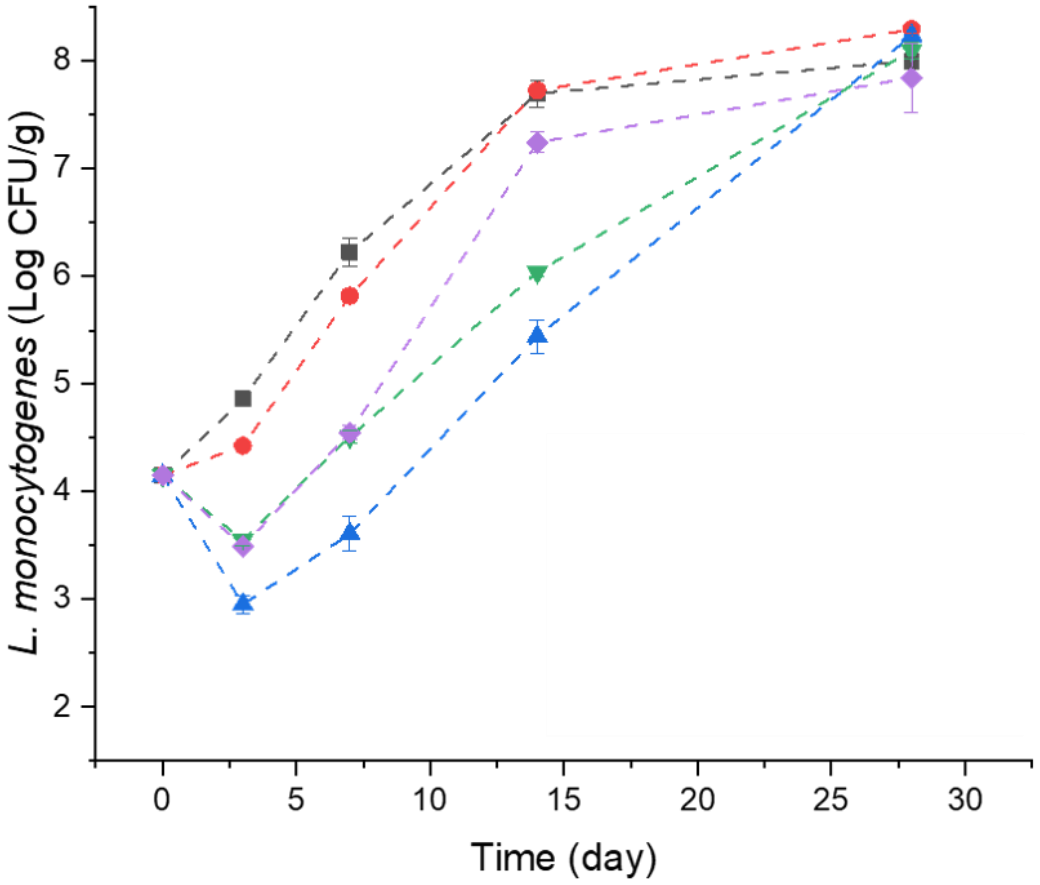

Figure 3.7. Effect of free nisin and nisin loaded zein microcapsules on survival of L. monocytogenes cocktail in Queso Fresco after storage at 4 °C. Microencapsulated and free nisin were added at a final concentration of 250 µg nisin/g cheese. Sample low load 0.3-10 is the zein capsule with 5.94 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate; sample high load 0.3-10 is the zein capsule containing 12.64 µg/mg of nisin, prepared with 0.3 ml/hr of dispersing flow rate and 10 ml/hr of flow rate; sample high load 0.2-10 is the zein capsule containing 9.67 µg/mg of nisin, prepared with 0.2 ml/hr of dispersing flow rate and 10 ml/hr of continuous flow rate. Sample nisin is the commercial nisin with the concentration of 2.5% (w/w). Values are means ± SEM.
Legend: No antimicrobial (black), Nisin control (red), Low load 0.3-10 (blue), High load 0.2-10 (green), High load 0.3-10 (purple)

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CHAPTER 4

BIOENGINEERED NISIN DERIVATIVES TO CONTROL *LISTERIA MONOCYTOGENES* IN QUESO FRESCO [1]

4.1 Abstract

Nisin is an antimicrobial peptide used as a food preservative and it is able to inhibit *Listeria monocytogenes*; however nisin has not been effective to control *L. monocytogenes* in Queso Fresco (QF). In this work, we have used bioengineering strategies to create a series of nisin A derivatives by substituting hydrophobic residues I30 and V32 of the C-terminal region of nisin with positively charged amino acids (H, K and R). Nisin derivatives produced exhibited reduced antilisterial activity as compared to nisin A. Nisin V32K displayed significantly higher stability at neutral pH and negligible absorption to milk fat. All nisin derivatives were more stable under QF-like experimental conditions (pH 7 + 22% milk fat), on the contrary, >95% of initial amount of nisin A was loss at the same conditions. Nisin A was not effective to prevent growth of *L. monocytogenes* in QF, however, single substituted nisin derivatives (I30H, I30K, I30R, V32H, V32K and V32R) exhibited a listeriastatic effect in QF for up to 3 days of cold storage despite of their reduce antilisterial activity. Our results highlight the potential benefits of bioengineering nisin to generate nisin derivatives with enhanced stability and extended antilisterial activity in QF.

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4.2 Introduction

Listeria monocytogenes is an opportunistic and zero-tolerance foodborne pathogen that causes listeriosis, a rare illness but with a high fatality rate (Jackson et al., 2018). Listeriosis outbreaks have often been linked to the consumption of Hispanic-style cheeses (HSC), accounting for 17% of total listeriosis outbreaks in the U.S. on the past ten years (CDC-NORS, 2018). In particular, Queso Fresco (QF), the most widespread HSC in the U.S., is characterized by having a near neutral pH, high fat (>20%) and low salt (< 3%) content and a short shelf-life requiring refrigeration, and importantly QF is known to support *L. monocytogenes*' growth (Ibarra-Sanchez et al., 2017).

Nisin A, the natural nisin variant approved for use as food preservative in the U.S. (21CFR184.1538), is a 34-amino acid antimicrobial peptide known for being highly effective at inhibiting *L. monocytogenes* and other Gram-positive bacteria (Liu & Hansen, 1990; Field et al., 2015a). However, nisin alone has shown limited effectiveness to control *L. monocytogenes* in QF (Van Tassell et al., 2015; Ibarra-Sanchez et al., 2018; Feng et al., 2019). Nisin's inestability at neutral pH (Liu & Hansen, 1990) and absorption to milk fat globules (Jung et al., 1992) are likely contribuiting factors that need to be addressed to make nisin effective in QF.

Nisin is gene-encoded which allows for its manipulation to generate nisin derivatives that could potentially perform better than the unmodified peptide. Multiple studies have generated nisin derivatives by random mutagenesis of the nisin gene (*nisA*) or by intensive modifications targeting certain residues (e.g. K12 and S29) or the hinge region (residues 20-22) (Field et al., 2015a). It is not yet clear what nisin modifications are the most beneficial for food applications, however, the C-terminal part of nisin (residues 29-34) is the least conserved among all seven natural variants of nisin that have been reported: nisin A, Z, F, Q, H, U and U2 (Field et al.,

2015a; O'Connor et al., 2015). Additionally, the C-terminal region contains two hydrophobic residues at position 30 (isoleucine) and 32 (valine), which could be substituted with charged amino acids to potentially decrease the hydrophobicity and increase the solubility of the peptide (Pace et al., 2009), making both residues (I30 and V32) good candidates for manipulation to create nisin derivatives.

Bioenginnering of nisin has been mainly focused on identifying derivatives with enhanced antimicrobial activity, including those able to inhibit Gram-negative bacteria (Field et al., 2012), and few reports have aimed to generate derivatives with improved biochemical properties such as better solubility at neutral pH (Rollema et al., 1995). Moreover, only a small number of nisin derivatives with enhanced antimicrobial activity have been tested in food matrices such as chocolate milk (Field et al., 2015b; Rousse et al., 2012), chicken noodle soup (Field et al., 2015b), apple juice and powderer infant milk formula (Campion et al., 2017), where in most cases nisin derivatives were more effective than unmodified nisin in controlling foodborne pathogens.

Our hypothesis was that increasing the hydrophilicity of nisin will result in increased stability in QF conditions while maintaining antilisterial activity. The objective of this study was to generate a series of nisin A derivatives targeting the hydrophobic residues I30 and V32, to evaluate their biochemical properties and efficacy against *L. monocytogenes* in QF.

4.3 Methods

4.3.1 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 4.1. *L. lactis* strains were grown in M17 broth supplemented with 0.5% glucose (GM17) at 30°C. *L. monocytogenes* strains were

grown in brain heart infusion (BHI, Difco, Becton Dickinson and Co., Sparks, MD) broth, at 37°C for with 250-rpm agitation to obtain cell concentrations of ~9 Log CFU/mL. *L. monocytogenes* cocktails were prepared by combining equal volumes of stationary phase cultures of the five different foodborne outbreak-associated strains. The serial dilutions of the *L. monocytogenes* cocktail were further prepared in PBS (KCl 200 mg/L; KH2PO4, 200 mg/L; NaCl, 8 g/L; Na2HPO4, 1.15 g/L, pH 7.2) to obtain the desired cell concentrations. *Listeria* enumeration was carried out on PALCAM Listeria-Selective agar (EMD Millipore) supplemented with 20 mg/mL ceftazidime (Tokyo Chemical Industry Co. Ltd.) and incubated for 48 h at 37 °C.

4.3.2 Creation of nisin derivatives

A two-plasmid complementation platform was developed to generate nisin derivatives. The platform contains two plasmids: a larger size plasmid pWK6-delA was constructed to express nisin modification and immunity genes, and a small size plasmid pleiss-Pcon-nisA was constructed to generate point mutations in nisin A precursor by PCR due to its smaller size. The plasmid pWK6-delA was constructed by deletion of *nisA* gene from the constitutive nisin expression plasmid pWK6-PlctA/F through selection and counter selection (Kong et al., 2016). The plasmid pleiss-Pcon-nisA was constructed by replacing of *gfp* gene with nisA from the plasmid pleiss-Pcon-gfp (Kong et al., 2018). To create mutations in nisA gene, two pairs of primers were used: a pair of reverse complementary forward and reverse primers nisA5F/nisA5R targeting the start codon region of nisin gene, and a pair of reverse complementary primers mutF/mutR targeting the region for mutation and containing required mutations within the oligo. Two fragments generated by PCR with primer pairs of nisA5F/mutR (nisA fragment) and

nisA5R/mutF (vector) were assembled by Gibson assembly and then transformed into *L. lactis* MG1363 to create plasmid pleiss-Pcon-nisAmut. The plasmid pleiss-Pcon-nisAmut was first verified by sequencing and then transformed into MG1363 competent cells which already contain the plasmid pWK6-delA. The strain MG1363/ pWK6-delA/ pleiss-Pcon-nisAmut producing nisin variant was used for subsequent experiments.

4.3.3 Generation of nisin derivative containing fermentates

L. lactis strains were first cultured overnight in GM17 at 30°C, and then flasks with GM17 were inoculated with the overnight culture (1:50 inoculation) and incubated for 10 h at 30°C. The cells were removed by centrifugation at 20,000 x g for 10 min at 4°C. The pH of supernatants were adjusted to 3-3.5 using HCl 0.1 M, followed by sterile filtration (SFCA bottle-top filter 0.2 μm, Fisher Scientific, MA) and heating for 10 min at 80°C to inactivate enzymes. The supernatants were frozen and lyophilized in a freeze dryer (Labconco FreeZone, MO), and the powder was stored at -20°C.

4.3.4 Semi purification of nisin A derivatives

Semi purification of nisin derivatives was performed by the method proposed by Gough et al. (2017) with modifications. Briefly, 3 g of freeze-dried supernatant and 5.6 g of NaCl were suspended in 18 ml of sterile deionized water at 40°C, and allowed to sit at room temperature for 4 h. This was centrifuged at 20,000 x g for 45 min at 4°C. The supernatant was discarded and the pellet was resuspended in 2 mL of sterile deionized water before use.

4.3.5 Nisin Quantification with HPLC

Nisin was quantified by the method proposed by Liu and Hansen (1990) with modifications. Waters 2695 Alliance HPLC system (Waters, Milford, MA) equipped with a Hewlett-Packard series 1050 photodiode array detector (Hewlett-Packard, Palo Alto, CA) was used. The HPLC system was interfaced with a computer using ChemStation software (Agilent Technologies Inc.). The analytical column was a reversed-phase Hypersil GOLD C18 (175 A, 250 x 4.6 mm, 5 µm) (Thermo Scientific, San Diego, CA). Solvent A was 0.1 % (v/v) trifluoroacetic acid (TFA) in ultra-pure water, and solvent B was 90% (v/v) HPLC-grade acetonitrile (Thermo Fisher Scientific) containing 0.1% TFA (v/v) in ultra-pure water. The column temperature was maintained at 40°C, sample injection volume was 20 µL, and samples were measured at UV absorption of 215 nm. A linear gradient from 31% B to 41.5% B over 14 min was run at a flow rate of 1.0 mL/min. The standard curve was plotted using 10, 50, 100, 200 and 300 ppm nisin (Nisaplin®,2.5% nisin w/w), and the amount of nisin was calculated from the area of the peak at 215 nm. HPLC analysis was performed in duplicate for each sample. Identification of nisin derivatives was performed prior quantification to determine their retention times.

4.3.6 Identification of nisin A derivatives

Semi purified nisin derivative suspensions were injected to HPLC system as described above, and peaks eluting from the column were collected manually in 1.5 mL polypropylene centrifuge tubes. For each nisin derivative, fractions from three injections were pooled and acetonitrile was removed by air-drying overnight at room temperature before testing. To determine the peak corresponding to each nisin derivative, all fractions were first screened for

their antimicrobial activity and then analyzed with MALDI-TOF, as follows: overnight culture of *L. lactis* subsp. *cremoris* ATCC 19257 (indicator strain) grown in GM17 was diluted to approximately 5 Log CFU/mL in fresh GM17. Indicator strain culture (150 µL) was mixed with HPLC fraction (50 µL) into 96-well microtiter plate, and incubated at 30°C for 24 h. Only the fractions causing inhibition of visible growth of the indicator strain were analyzed with MALDI-TOF by the UIUC Mass Spectrometry Laboratory to identify the nisin derivatives by molecular weight for further quantitation using HPLC.

4.3.7 Minimum inhibitory concentrations

Minimum inhibitory concentration (MIC) of nisin derivatives was tested via the broth dilution method as described by Van Tassell et al. (2015), with minor modification. Briefly, overnight culture of single *L. monocytogenes* strain (NRRL B-33104, B-33513 and B-33420) was inoculated at approximately 5 Log CFU/mL into 96-well microtiter plates containing wells with serial 2-fold dilutions of nisin A (Nisaplin) and nisin derivative, in BHI broth supplemented with 50 mM phosphate buffer (pH 7.0). MIC was recorded as the lowest concentration that visibly prevented growth after overnight incubation at 37°C.

4.3.8 Milk fat preparation

Milk fat was separated from commercial unsalted butter. Briefly, 30 g of unsalted butter were heated up to 90°C for 10 min in a water bath. The upper layer (separated proteins) was removed, and melted butter was centrifuged at 1,700 x g for 5 min at 20°C. Milk fat (top layer) was separated carefully from water-protein bottom layer, and milk fat was stored at 4°C.

4.3.9 Chemical stability and absorption to milk fat

Nisin A (Nisaplin®, confirmed by MALDI-TOF) and nisin derivative solutions (150 µg/mL nisin) were prepared in 50 mM phosphate buffer at pH 3 and 7, and mixed with melted milk fat to a final concentration of 0% and 22% fat. Level of fat was chosen to represent that found in the model QF used for further experiments (Van Tassell et al., 2015). Samples were mixed vigorously using a vortex and incubated with shaking at 250 rpm for 24 h at 37°C. Samples were taken at 0 and 24 h and the nisin content was quantified from the aliquots of the aqueous phase using RP-HPLC. Residual nisin (%) was calculated by difference between initial added nisin (0 h) and the amount of nisin in the aqueous phase after 24 h incubation.

4.3.10 Effect of nisin derivatives on *L. monocytogenes* in Queso Fresco

Antimicrobial activity of nisin A and nisin derivatives was evaluated by their addition to Queso Fresco (QF) as previously described with minor modifications (Van Tassell et al., 2015). Briefly, batches of miniature laboratory QF were prepared with nisin A (Nisaplin) and nisin derivatives to an equivalent amount of 37.5 µg nisin added into the dry curd (approximately 250 µg of nisin/g of cheese). Sample cheeses were inoculated with a five-strain *L. monocytogenes* cocktail directly into the curd prior to pressing, for a final concentration of approximately 4 Log CFU/g. After inoculation and a 15-min incubation to allow *Listeria* to attach to the curd, tubes were centrifuged at 6,000 x g for 5 min for incomplete pressing. Accessible whey was removed, the antimicrobial treatments were added, and the curd was centrifuged for another 8 min. The remaining whey was removed and all cheeses were stored at 4 °C for up to 14 days, until sampled for *Listeria* enumeration. Cheeses were individually homogenized and serially diluted in

PBS, and spread plated on PALCAM Listeria-Selective agar supplemented with 20 µg/mL ceftazidime to enumerate *L. monocytogenes*. Plates were incubated at 37°C for 48 h.

4.4 Results

4.4.1 Generation and identification of nisin A derivatives

A bank of *L. lactis* (MG1363) strains producing nisin A derivatives was generated. Two hydrophobic residues in the C-terminal region of nisin (I30 and V32) were substituted with all three positively charged amino acids (H, K and R). The resultant nisin derivatives included 6 single substituted derivatives (I30H, I30K, I30R, V32H, V32K and V32R) and a set of 4 double substituted derivatives (I30H/V32H, I30H/V32K, I30H/V32R and I30K/V32K). Nisin derivatives were semi purified and concentrated by salting out. To allow quantification of each nisin derivative for further experiments, eluted peaks from HPLC of semi purified preparations were screened for bioactivity against *L. lactis* ATCC 19257 (indicator strain). For all nisin derivative preparations, only one eluted peak exhibited inhibition of the indicator strain, suggesting that there may not be other compound different from nisin with antimicrobial activity in the semi purified preparation. Mass spectrometric analysis confirmed that the eluted peak with antimicrobial activity, of each semi purified preparation, corresponded to a nisin derivative (data not shown).

4.4.2 Minimum inhibitory concentrations

The MIC of nisin derivatives against *L. monocytogenes* NRRL B-33104 (strain isolated from Jalisco cheese) in BHI was determined to be 3.12 µg/mL for nisin A, 25 µg/mL for all three I30 substitutions (I30H, I30K and I30R), V32H and V32R. The MIC was 50 µg/mL for V32K

(Table 4.2). All four double substituted nisin derivatives displayed higher MIC (100-200 µg/mL) against the same *Listeria* strain, indicating a greater decrease of their antilisterial activity relative to single substituted nisin derivatives. Based on MIC determination, single substituted nisin derivatives tested displayed at least 8-fold decrease in their antilisterial activity compared to unmodified nisin (nisin A).

4.4.3 Chemical stability and absorption to milk fat

The residual percentage of nisin A and nisin derivatives as a function of pH and milk fat was evaluated (Table 4.3). There was an interaction between pH and milk fat on the residual nisin of nisin A, I30H, I30K and V32H ($P < 0.05$). For nisin V32R, there was an effect of pH and an effect of milk fat on residual nisin, but no interaction ($P > 0.05$). Only the main effect of pH was significant ($P < 0.05$) for all other nisin derivatives (I30R, V32K, I30H/V32H, I30H/V32K, I30H/V32R and I30K/V32K). At pH 3, nisin A exhibited its highest stability (100% residual nisin) after 24 h incubation at 37 ºC. There was no statistical difference between residual percentage of nisin A and nisin derivatives ($P > 0.05$) after incubation at pH 3, however a loss of up to approximately 10% of initial nisin was observed for certain nisin derivatives (I30K, I30H/V32H and I30H/V32R). After incubation at pH 7, 83.78 ± 4.33% of nisin V32K was quantified in the solution, while 65.02 ± 2.90% nisin A was quantified. Nisin derivatives, except V32K, showed similar or lower stability at pH 7 compared to nisin A (at least 35% loss of nisin). Nisin A and nisin derivatives were incubated at pH 3 with the addition of 22% milk fat, with the purpose of observe the absorption of nisin to milk fat in a condition of high stability (pH 3). Negligible absorption to milk fat was observed for nisin V32K (99.83 ± 0.40% residual nisin), while nisin A had the highest absorption to milk fat (86.18 ± 0.83% residual nisin). When

comparing the residual nisin after incubation at pH 3 with and without 22% milk fat, all nisin derivatives showed up to 5% loss of nisin, while nisin A had a loss of approximately 14%. Residual nisin A dramatically decreased (> 95% loss of initial nisin) after incubation at pH 7 with milk fat, experimental condition that resembles some of the features in Queso Fresco. On the contrary, all nisin derivatives exhibited at least 35% residual nisin under the same experimental condition (pH 7 + 22% milk fat), notably V32K and I30R showed the highest residual nisin (approximately 23 and 38% loss of initial nisin, respectively).

4.4.4 Effect of nisin derivatives on *L. monocytogenes* in QF

L. monocytogenes cocktail grew in untreated QF until it reached a population of up to approximately 8 Log CFU/g over 14 days of storage at 4 ºC (Figure 4.1). In cheeses added with nisin A, an initial difference of approximately 0.4 Log CFU/g compared to untreated cheeses was observed, but regrowth of *Listeria* resulted in final pathogen counts similar to control cheeses by day 14 of storage. Overall, nisin derivatives displayed modest antilisterial enhancement in QF. At day 3, all single substituted nisin derivatives (I30X and V32X) caused no increase in viable cell counts relative to the initial inoculum, this represents approximately 1 Log CFU/g difference relative to untreated cheeses, however, subsequent regrowth was observed from day 7 of storage.

4.5 Discussions

Nisin is known to be an effective antilisterial compound, however, reduced antimicrobial activity has been observed when incorporated in foods, notably in QF, where the near neutral pH of the cheese and their high fat content (> 20%) are important factors on limiting the effectiveness of nisin. In this study, a non-nisin producing *L. lactis* strain was bioengineered with

a modified nisin biosynthetic pathway yet lacking *nisA* to generate a series of nisin A derivatives, targeting the hydrophobic residues I30 and V32 in the C-terminal region and substituted with positively charged amino acids (H, K and R).

Bioengineering of nisin may result in nisin derivatives with altered antimicrobial activity that may be similar, lower or increased relative to unmodified nisin (Field et al., 2015c). Our results showed that all nisin derivatives generated, independently of the residue (I30 or V32) or the amino acid introduced (H, K or R), had reduced antilisterial activity *in vitro* compared to nisin A (at least 8-fold difference in MIC). Nisin is a cationic peptide, and the ionic interaction between nisin and the negative charges of the cell envelope is important on nisin's antimicrobial action (O'Brian et al., 2018), and it was surprising that the introduction of additional positively charged residues led to decreased antilisterial activity. In other study, the introduction of lysine and histidine into the hinge region resulted in nisin derivatives with similar antimicrobial activity as nisin A (Field et al., 2008). In the present study we targeted two residues in the C-terminal region of nisin A, and it has been suggested that the C-terminal region needs to translocate across the bacterial membrane as part of nisin's pore formation mechanism (van Kraaij et al., 1998). The reduced activity observed in the nisin derivatives generated by introducing positively charged amino acids into the C-terminal region may be the result of structural changes in the resulting peptide caused by the introduction of bulkier amino acids (H, K and R) leading to less effective translocation of the C-terminal region; or the introduction of additional positive charges may led to stronger ionic interaction of the C-terminal region with phospholipids in the cell wall resulting in incomplete translocation of nisin to form pores effectively and kill the bacteria (van Kraaij et al., 1998). Although an increased antimicrobial activity is a desirable trait when

screening and selecting for nisin derivatives, the resulting peptides with enhanced activity might not overcome the stability limitations of unmodified nisin for its use in food matrices.

Nisin is a hydrophobic peptide that is highly stable and soluble at low pH (Liu and Hansen, 1990), but nisin's traits do not make it suitable for its use in QF (near neutral pH and high fat food), however those biochemical traits may be altered in nisin derivatives and might potentially work better in cheese. A general strategy to increase the solubility of a protein or peptide, which could be associated with higher residual amount of the protein or peptide, consists on replacing hydrophobic residues with charged amino acids (Pace et al., 2009). In the present study we targeted two hydrophobic residues (I30 and 32V) that were replaced with positively charged amino acids (H, K and R). Overall, almost all nisin derivatives generated and tested (except nisin V32K) in this study displayed similar or lower stability at neutral pH, relative to nisin A. However all nisin derivatives exhibited lower absorption to milk fat and higher stability under QF-like conditions (neutral pH combined with milk fat), suggesting that the hydrophobic nature of nisin A was altered towards more hydrophilic properties in the nisin derivatives, and that extended antimicrobial activity due to residual nisin might be expected. On the contrary, nisin A was almost completely loss under QF-like conditions (pH 7 + 22% milk fat). The low stability and solubility of nisin A at neutral and higher pH values is well known (Liu and Hansen, 1990; Davies et al., 1998), additionally reduced activity of nisin due to absorption to milk fat globules has been observed in milk (Jung et al., 1992; Bhatti et al., 2004). Rollema et al. (1995) reported that nisin Z derivatives with additional lysine residue (N27K or H31K) displayed significantly increased solubility at pH 7, however we observed stability improvement at pH 7 only for nisin V32K (higher residual nisin). Our results suggest that the generated nisin derivatives might be better candidates than nisin A for their use in QF based on their biochemical

properties. Additionally, to the best of our knowledge, this is the first study to examine the individual effect of milk fat and its interaction with neutral pH on the residual amount of nisin A, and to characterize biochemical properties of nisin derivatives beyond their solubility and antimicrobial activity.

Different to other studies that have tested nisin derivatives with enhanced antilisterial activity in chocolate milk (Field et al., 2015b; Rouse et al., 2012), modest antilisterial enhancement relative to nisin A was observed for all single substituted nisin derivatives (I30H, I30K, I30R, V32H, V32K and V32R) when tested in QF matrix. It is important to recognize that all nisin derivatives tested in this study exhibited reduced antilisterial activity *in vitro*. Regardless, a listeriastatic (bacteriostatic for *Listeria*) was observed for all nisin derivatives at early cheese storage (between 3 to 7 days), while regrowth of *Listeria* was evident in cheeses with nisin A at the same time point of storage. Our results suggest that the reduced antilisterial activity of the nisin derivatives was compensated by their enhanced biochemical properties (low absorption to milk fat and more residual nisin under QF-like conditions) allowing them to display a listeriastatic effect in QF between 3 to 7 days of cold storage. It is possible that nisin derivatives with similar or enhanced antilisterial activity compared to nisin A but with biochemical properties more suitable for QF might provide extended protection against *Listeria*. However, more nisin derivatives need to be characterized for their biochemical properties and tested in food matrices to determine the most beneficial modifications in nisin's structure for specific applications in the food industry.

4.6 Conclusions

In summary, the nisin derivatives generated in this study showed promising biochemical traits for their application into QF such as comparable stability at neutral pH relative to unmodified nisin, lower to negligible absorption to milk fat and significantly higher stability under QF-like conditions. Additionally, different to unmodified nisin, single substituted nisin derivatives displayed a listeriastatic effect in QF at early cold storage despite of their reduced antilisterial activity.

Future work could focus on generating nisin derivatives targeting different residues in the C-terminal region and substituting them with charged or less hydrophobic amino acids, also combining them with other antimicrobials to increase their efficacy to control *Listeria* in cheeses. However, our results support that altering residue 30 or 32 of nisin result in nisin derivatives with biochemical and antilisterial properties more suitable for their use in QF.

4.7 Tables and Figure

Table 4.1. Strains used in this study

Bacterial strains	Relevant characteristics or source of strains
Lactococcus lactis subsp. *cremoris* MG1363	Non-nisin producer strain
Lactococcus lactis subsp. *cremoris* ATCC 19257	Nisin sensitive indicator
Listeria monocytogenes NRRL B-33104	Serotype 4b. Food, epidemic, Jalisco cheese
Listeria monocytogenes NRRL B-33513	Serotype 4b. Food, epidemic, pate
Listeria monocytogenes NRRL B-33420	Serotype 4b. Food, epidemic, RTE[1] meat products
Listeria monocytogenes NRRL B-33424	Serotype 1/2b. Human, epidemic, chocolate milk
Listeria monocytogenes NRRL B-33419	Serotype 1/2a. Human, epidemic, sliced turkey

[1]Ready-To-Eat

Table 4.2. Minimum inhibitory concentration of nisin A and nisin derivatives against *L. monocytogenes*

Nisin derivative	*L. monocytogenes* strain		
	NRRL B-33104 (µg/mL)	NRRL B-33513 (µg/mL)	NRRL B-33420 (µg/mL)
Nisin A[1]	3.12	3.12	6.25
I30H	25	-	-
I30K	25	-	-
I30R	25	-	-
V32H	25	-	-
V32K	50	-	-
V32R	25	-	-
I30H/V32H	200	50	100
I30H/V32K	>100	-	-
I30H/V32R	100	50	100
I30K/V32K	>100	-	-

[1] Nisaplin® (2.5 % nisin w/w), commercial preparation of nisin A. Confirmed by MALDI-TOF.

Table 4.3. Effect of different pH values and milk fat content on residual nisin A and nisin derivatives after 24 h of incubation at 37ºC.

	Residual nisin (%)[1]			
	pH 3		pH 7	
Nisin derivative	0% milk fat	22% milk fat	0% milk fat	22% milk fat
Nisin A[2]	100.48 ± 0.30^{A}	86.18 ± 0.83$^{B, a}$	65.02 ± 2.90$^{C, ac}$	<4.6[3, D, a]
I30H	92.66 ± 1.10^{A}	90.17 ± 1.37$^{A, ab}$	68.88 ± 1.19$^{B, ab}$	59.23 ± 1.83$^{C, bc}$
I30K	91.06 ± 3.23^{A}	91.19 ± 1.89$^{A, ab}$	57.15 ± 0.76$^{B, acd}$	47.60 ± 1.51$^{C, cde}$
I30R	96.63 ± 0.86^{A}	95.53 ± 1.50$^{A, ab}$	67.97 ± 1.68$^{B, ac}$	62.59 ± 1.89$^{B, b}$
V32H	95.36 ± 2.73^{A}	91.49 ± 1.31$^{A, ab}$	52.41 ± 1.99$^{B, cd}$	35.57 ± 2.39$^{C, e}$
V32K	97.17 ± 1.28^{A}	99.83 ± 0.40$^{A, b}$	83.78 ± 4.33$^{B, b}$	77.26 ± 2.95$^{B, f}$
V32R	93.70 ± 3.59^{A}	87.47 ± 4.00$^{A, ab}$	54.53 ± 1.92$^{B, acd}$	40.84 ± 2.60$^{C, de}$
I30H/V32H	90.48 ± 1.29^{A}	88.97 ± 1.93$^{A, ab}$	48.04 ± 3.79$^{B, d}$	38.28 ± 2.53$^{B, e}$
I30H/V32K	98.27 ± 1.45^{A}	93.71 ± 1.32$^{A, ab}$	63.93 ± 5.89$^{B, ac}$	53.84 ± 5.21$^{B, bcd}$
I30H/V32R	89.58 ± 3.09^{A}	87.12 ± 3.53$^{A, ab}$	45.69 ± 3.64$^{B, d}$	35.97 ± 3.84$^{B, e}$
I30K/V32K	92.46 ± 3.63^{A}	89.02 ± 5.63$^{A, ab}$	69.81 ± 1.68$^{B, ab}$	60.62 ± 2.20$^{B, bc}$

^{A,B,C,D}Means with dissimilar letters in a row are significantly different ($P < 0.05$)

^{a,b,c,d,e,f}Means with dissimilar letters in a column are significantly different ($P < 0.05$). Means with no letters in a column are not significantly different from each other ($P > 0.05$)

[1] Determined by RP-HPLC after incubation at 37ºC for 24 h. Values are means ± SEM.

[2] Nisaplin® (2.5 % nisin w/w), commercial preparation of nisin A. Confirmed by MALDI-TOF.

[3]Below limit of detection for nisin (6.9 µg/mL)

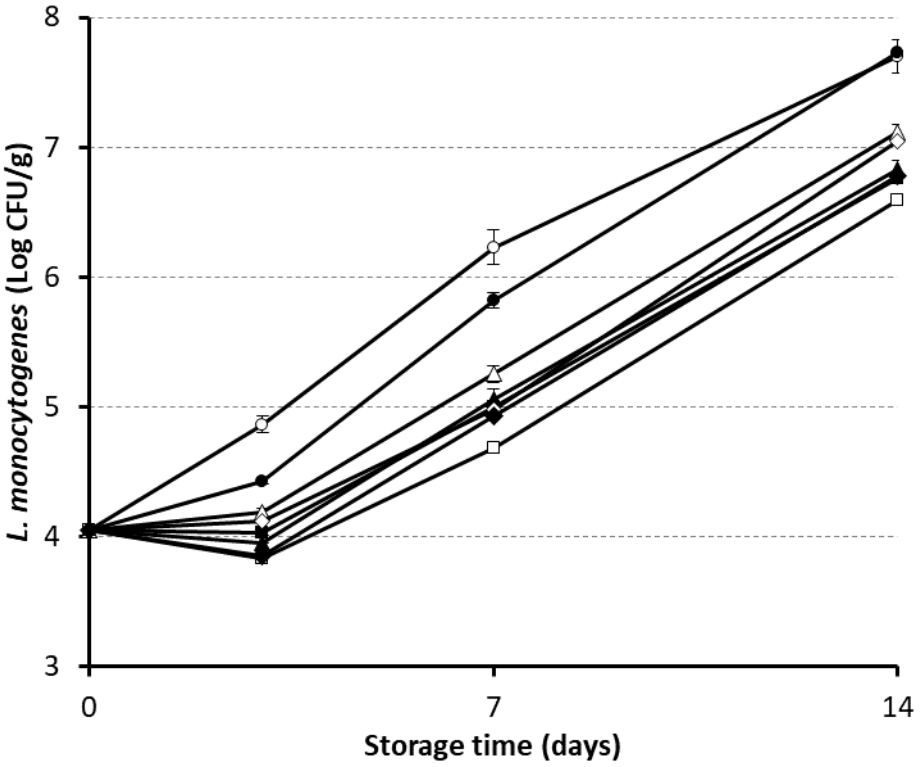

Figure 4.1. Effect of nisin A and nisin derivatives on survival of *L. monocytogenes* cocktail in Queso Fresco after storage at 4°C. Nisin A and nisin derivatives were added to cheeses by weight, with final concentrations of 250 µg/g nisin; (○) No antimicrobial; (●) Nisin A; (▲) I30H; (Δ) I30K; (■) I30R; (□) V32H; (◆) V32R; and (◇) V32K. Values are means ± SEM.

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Jung, D-S., Bodyfelt, F.W., Daeschel, M.A. 1992. Influence of fat and emulsifiers on the efficacy of nisin in inhibiting Listeria monocytogenes in fluid milk. Journal of Dairy Science 75, 387-393.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Hispanic-style cheeses have been implicated in historical and current listeriosis outbreaks in the U.S., additionally *L. monocytogenes* has been isolated from cheese manufacturing facilities and commercial samples, altogether representing a health risk for the consumers. In Chapter 2, I demonstrated that the *Listeria* phage endolysin PlyP100 could be a potentital candidate as a antilisterial preservative in Queso Fresco. The combination of PlyP100 with commercial nisin showed to be an effective antilisterial treatment in cheese resulting in complete elimination of the pathogen in most of the cheese samples. While the phage P100, the natural producer of PlyP100, is approved for its use in foods, endolysins in general still need to be assessed for their safety and be regulated for their use in food industry. However, PlyP100 is a product from a GRAS bacteriophage, and the safety and regulation of PlyP100 may be less complex than for those antimicrobials that have no history of consumption.

The quest of effective antilisterial treatments can be focused on improving natural GRAS antimicrobials, such as nisin. In Chapter 3, I reported a significant enhancement of the antilisterial activity of nisin when microencapsulated with zein. Although microencapsulated nisin was more effective than free nisin at early cheese storage showing subsequent regrowth, the results showed that a combination of sustained release and protective barrier provided by zein microcapsules could compensate at some degree the instability issues of nisin in Queso Fresco matrix. Further optimization of the microencapsulation preparation to obtain microcapsules with tunable properties such as different capsule loading, release rate, encapsulating material, or co-encapsulation with additional antimicrobials, could help to better understand which

characteristics in a microencapsulated system provide a sustained release of nisin to effectively provide extended inhibition of *Listeria* in cheese and other food matrices.

Similarly, in the research presented in Chapter 4, I demonstrated that the biochemical properties of nisin can be altered by substituting hydrophobic residues in the C-terminal region of nisin with positively charged amino acids, resulting in nisin derivatives that are more stable in the Queso Fresco matrix. Interestingly, different from unmodified nisin, the nisin derivatives were listeriastatic at early storage of the cheese samples despite of their reduced antilisterial activity, highlighting that increasing the stability of nisin in the cheese matrix is necessary to control *Listeria*. It is not known what other modification in the structure of nisin can improve the stability of nisin in non-acidic and high-fat foods. I would suggest characterizing and testing existing nisin banks for their biochemical properties and importantly their evaluation in food matrices. This knowledge can provide insights on targeted mutations to ultimately identify nisin derivatives for effective biocontrol in the food industry. Similar to PlyP100, nisin derivatives are considered as different substances to nisin A, requiring their safety assessment and regulation. Overall, these studies provide information on antimicrobial interventions to ensure the safety of fresh cheeses, a public concern in the U.S. and with high relevance in Latin American countries, where fresh cheeses are a staple food.

APPENDIX A

ADDITIONAL PUBLISHED RESEARCH CONTRIBUTIONS

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http://dx.doi.org/10.3168/jds.2015-9967

Use of a miniature laboratory fresh cheese model for investigating antimicrobial activities

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Figure A.1 This research article was the result of collaboration that developed a miniaturized Queso Fresco model for testing of antimicrobials. We showed that our miniature Queso Fresco model has comparable chemical composition (moisture, protein and fat) compared to traditional-sacale Queso Freso. Additionally we used our model to evaluate the antilisterial activity of nisin and ferulic acid. My primary contributions were the modification of the initial proposed miniature Queso Fresco model to its final version, determination of the chemical composition of the cheese model, conduct experiments in the Queso Fresco model with nisin and ferulic acid, determination of the texture profile analysis, performing the sensory evaluation, statistical analysis of data and revision of the manuscript.

Van Tassell, ML, **Ibarra-Sánchez LA**, Takhar SR, Amaya-Llano SL, Miller MJ. (2015). Use of a miniature laboratory fresh cheese model for investigating antimicrobial activities. Journal of Dairy Sceince 98(12): 8515-8524.

J. Dairy Sci. 100:2482–2487
https://doi.org/10.3168/jds.2016-11990

Hot topic: Antilisterial activity by endolysin PlyP100 in fresh cheese

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Figure A.2 This research article was the result of collaboration that explored the potential application of the *Listeria* phage endolysin PlyP100 in Queso Fresco. We showed that PlyP100 displays inhibitory activity against 18 *Listeria monocytogenes* strains, and importantly, exhibits a strong listeriastatic effect against a *L. monocytogenes* cocktail in Queso Fresco. My primary contributions were the selection and plasmid design for synthesizing the gene *ply*P100, transformation of *Escherichia coli* BL21(DE3) with *ply*P100 containing plasmid, conduct challenge experiments in Queso Fresco, and revision of the manuscript.

Van Tassell, ML, **Ibarra-Sánchez LA**, Hoepker GP, Miller MJ. (2017). Hot topic: Antilisterial activity by endolysin PlyP100 in fresh cheese. Journal of Dairy Sceince 100(4): 2482-2487.

J. Dairy Sci. 101:7736–7745
https://doi.org/10.3168/jds.2017-14037

Microbial analysis of commercially available US Queso Fresco

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Figure A.3 This research article was the result of collaboration that explored the microbial communities in commercially available Queso Fresco in Champaign County. All 64 cheese samples yielded negative to *Listeria* and all 9 different brands supported the growth of a *Listeria monocytogenes* cocktail regardless of the preservatives or culture-containing labeling. We showed that microbial communities in Queso Fresco sampled cluster together by brand despite of being from different produced lots. My primary contributions were the collection of all Queso Fresco samples and their testing for *L. monocytogenes*, establishing procedures for chemical analyses performed in cheese samples, conduct *L. monocytogenes* growth experiments in sampled commercial cheeses, extraction of DNA from selected cheeses for microbial analysis, composition and revision of the manuscript for submission.

Holle MJ, **Ibarra-Sánchez LA**, Liu X, Stasiewicz MJ, Miller MJ. (2018). Microbial analysis of commercially available US Queso Fresco. Journal of Dairy Science 101(4): 7736-7745.

APPENDIX B

ADDITIONAL SUBMITTED CONTRIBUTIONS

B.1 A manuscript with tentative title "Evaluation of mixtures of endolysin PlyP100, nisin, lauric arginate and ε-polylysine with antimicrobial effect on *Listeria monocytogenes* in Queso Fresco" has been prepared for its submission on August 2019. Co-authors Martínez-Ramos AR, **Ibarra-Sánchez LA**, Amaya-Llano SL and Miller MJ.

B.2 A review paper focused on advances in nisin use for preservation of dairy products has been prepared in collaboration with Dr. Layal Karam from Notre Dame University (Lebanon) for its submission on August 2019. Co-authors Karam L, **Ibarra-Sánchez LA**, and Miller MJ.