The inhibitory effect of nisin on *Mycobacterium avium* ssp. *paratuberculosis* and its effect on mycobacterial cell wall

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ABSTRACT

Infection with *Mycobacterium avium* ssp. *paratuberculosis* (*M. paratuberculosis*) is a widespread problem in the United States and worldwide, and it constitutes a significant health problem for dairy animals with a potential effect on human health. *Mycobacterium paratuberculosis* is easily transmitted through consumption of contaminated milk; therefore, finding safe methods to reduce the mycobacterial load in milk and other dairy products is important to the dairy industry. The main objective of the current study was to investigate the effect of natural products, such as bacteriocins designated as “generally regarded as safe” (GRAS), on the survival of *M. paratuberculosis* in milk. Commercially synthesized bacteriocin (nisin) was used to examine its effect on the survival of laboratory and field isolates of *M. paratuberculosis* and in contaminated milk. Surprisingly, nisin had a higher minimum inhibitory concentration (MIC) against the laboratory strain (*M. paratuberculosis* K10), at 500 U/mL, than against field isolates (e.g., *M. paratuberculosis* 4B and JTC 1281), at 15 U/mL. In milk, growth of *M. paratuberculosis* was inhibited after treatment with levels of nisin that are permissible in human food at 4°C and 37°C. Using both fluorescent and scanning electron microscopy, we were able to identify defects in the bacterial cell walls of treated cultures. Our analysis indicated that nisin reduced membrane integrity by forming pores in the mycobacterial cell wall, thereby decreasing survival of *M. paratuberculosis*. Thus, nisin treatment of milk could be implemented as a control measure to reduce *M. paratuberculosis* secreted in milk from infected herds. Nisin could also be used to reduce *M. paratuberculosis* in colostrum given to calves from infected animals, improving biosecurity control in dairy herds affected by Johne’s disease.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, milk, nisin, Johne’s disease

INTRODUCTION

Johne’s disease or paratuberculosis is a chronic granulomatous infection of the intestines of wild and domestic ruminants caused by *Mycobacterium avium* ssp. *paratuberculosis* (*M. paratuberculosis*; Collins, 1997). Johne’s disease can be transmitted from cows to calves through ingestion of *M. paratuberculosis*-infected colostrum or milk (Grant et al., 2002). Milk obtained under aseptic conditions from an asymptomatic *M. paratuberculosis*-infected cow could contain <10 cfu/50 mL of milk, whereas feces of the infected animal could contain $\geq 10^8$ cfu/g (Sweeney et al., 1992). Most likely, the major contamination risk of milk is through the indirect fecal route rather than via direct shedding of *M. paratuberculosis* into milk from infected animals (Nauta and van der Giessen, 1998). Although Johne’s disease is not currently recognized as a zoonotic disease, a possible link exists between *M. paratuberculosis* and Crohn’s disease in humans (Grant, 2005). Historically, *M. paratuberculosis* has been detected in both raw and pasteurized milk from cows, sheep, and goats, with isolation of the organism from several dairy products (Ikonomopoulos et al., 2005). Moreover, several laboratory pasteurization studies have reported that *M. paratuberculosis* can survive pasteurization of milk spiked with $\geq 10^4$ cfu/mL *M. paratuberculosis* (Chiodini and Hermon-Taylor, 1993; Grant et al., 1996, 1998; Meylan et al., 1996). Reducing the milk load of *M. paratuberculosis* will reduce the risks to the health of both animals and humans. Currently, there is no approved protocol to eliminate *M. paratuberculosis* from contaminated milk, emphasizing the risk for more cycles of transmission to newborn calves and humans who consume contaminated milk and posing a significant public health concern (Lu et al., 2013). Although antibiotic use to reduce *M. paratuberculosis* in milk and dairy products is a potential control strategy, the excessive use of

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antibiotics and the potential of developing antibiotic resistance (Phillips et al., 2004) make this alternative impractical (WHO, 2001). Therefore, we focused our efforts on investigating whether bacteriocins, a group of effective antimicrobial peptides, could be used to help control *M. paratuberculosis* in milk and dairy products.

Bacteriocins are antimicrobial peptides that are active against several bacterial species, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and other pathogens (Rilla et al., 2003, 2004). Nisin is a member of the class I bacteriocins also known as lantibiotics, which are ribosomally synthesized and post-translationally modified antimicrobial peptides (Willey and van der Donk, 2007). Lantibiotics are produced in an inactive form and then activated by cleavage of the specific N-terminal leader peptide. These active lantibiotics have the ability to kill mainly gram-positive bacteria and some gram-negative bacteria (Chatterjee et al., 2005). Nisin is one of the most studied bacteriocins produced by lactic acid bacteria (Cotter et al., 2005), especially by strains of *Lactococcus lactis* ssp. *lactis*, and it is most frequently used to control both spoilage and pathogenic bacteria (Lubelski et al., 2008). In addition, nisin is the only bacteriocin that has been granted “generally regarded as safe” (GRAS) status by the US Food and Drug Administration and the World Health Organization (Balcunas et al., 2013). Previously, attempts to inactivate *M. paratuberculosis* in milk included the use of UV irradiation (Altic et al., 2007), high hydrostatic pressure with mild temperature (López-Pedemonte et al., 2006), and pulsed electric field (Rowan et al., 2001), none of which provided an efficient means for control. However, nisin has been found to be active against specific mycobacterial species such as *Mycobacterium smegmatis* and *Mycobacterium bovis* strains (Chung et al., 2000). More recently, nisin was considered a less potent agent against *M. paratuberculosis*, *Mycobacterium kansasii*, and *Mycobacterium tuberculosis* compared with lacticin 3147, another lantibiotic (Carroll et al., 2010). Another study (Kralik et al., 2018) reported no effect of nisin against *M. paratuberculosis*. To better examine the effect of nisin in controlling *M. paratuberculosis* in milk, we studied the survival *M. paratuberculosis* in laboratory cultures and in milk treated with different concentrations of nisin. Our analysis indicated that variable concentrations of nisin could reduce both laboratory and clinical isolates of *M. paratuberculosis* in culture and in milk. Specifically, nisin was able to introduce holes in *M. paratuberculosis*, leading to a “bulging” phenotype in treated cells when examined by scanning electron microscopy and illustrating the mechanism by which nisin acts on *M. paratuberculosis*.

MATERIALS AND METHODS

Bacterial Cultures

Four strains of *M. paratuberculosis* (K10, 4B, JTC 1281, and JTC 1285) were tested in this study; K10 is a laboratory strain, 4B is a human isolate, JTC 1281 has been isolated from the oryx, and JTC 1285 has been isolated from goats (Hsu et al., 2011). All strains were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 0.05% (wt/vol) Tween 80 (Sigma Chemical Co., St. Louis, MO), 10% (vol/vol) oleic acid albumin-dextrose-catalase (OADC) supplement (Difco Laboratories), and 2 µg/mL mycobactin J (Symbiotics Europe SAS, Lyon, France), pH 6.6. All cultures were incubated at 37°C with gentle shaking (100 rpm) for 3 to 4 wk until the optical density at 600 nm (OD_{600}) was between 0.7 and 0.9, as detailed previously (Wu et al., 2007). *Lactococcus lactis* LL108 is an indicator bacterial strain used as a control for nisin; it was propagated in de Man, Rogosa, and Sharpe broth medium and incubated at 30°C for 18 h at 150 rpm (de Man et al., 1960).

Broth Susceptibility Testing

Commercially synthesized nisin was obtained from Sigma Chemical Co. A stock solution of 5,000 U/mL for nisin in 0.02 N HCl was freshly prepared directly before each experiment. Different concentrations of nisin were prepared in Middlebrook 7H9 broth to obtain final concentrations of 500, 250, 125, 62.5, 31.25, and 15.62 U/mL and then used to spike 100 µL of the prepared bacterial suspension (approximately 1×10^6 cfu/mL). The tubes were incubated at 37°C and OD_{600} was monitored at 5-d intervals for a period of 50 d. Growth curves based on OD_{600} readings were plotted to compare the results with those cultures with no nisin added (negative control). The growth curves were repeated at least twice for the 4 *M. paratuberculosis* strains before statistical analysis. The lowest concentration of the bacteriocin in the test tubes with no visible or detectable bacterial growth (turbidity) was considered to represent the MIC (Wong et al., 2008).

Microplate Alamar Blue Assay

A colorimetric, microplate Alamar Blue assay (MABA) was used to estimate the MIC of nisin against tested *M. paratuberculosis* isolates (including field and laboratory strains). Twofold serial dilutions of nisin were prepared in a 96-well plate, with final concentrations of nisin ranging from 7.8 to 1,000 U/mL, covering the range used for the broth susceptibility assay. One

hundred microliters of *M. paratuberculosis* inoculum was added to each nisin-containing well. Wells containing inoculum only served as positive controls, whereas wells containing no bacterial cells served as negative controls. The plates were sealed and incubated at 37°C for 7 to 14 d. Fifty microliters of a freshly prepared 1:1 mixture of 10× Alamar Blue (Accumed International, Westlake, OH) reagent and 10% Tween 80 was added to one positive control. The plates were re-incubated at 37°C for 24 h. When the positive well (inoculum only) turned pink, the reagent mixture was added to all wells in the microplate and the color of all wells was recorded. A blue color in the well was interpreted as no growth, and a pink color was scored as growth. The MIC was defined as the lowest bacteriocin concentration that prevented a color change from blue to pink (Franzblau et al., 1998).

Nisin Treatment of *M. paratuberculosis*-Spiked Milk

To test the antimycobacterial activity of nisin in milk and dairy products under different storage temperatures, samples of pasteurized skim cow milk (50 mL) were spiked with 2 *M. paratuberculosis* strains (K10 and JTC 1285, representing laboratory and field isolates, respectively) to approximately 1.2×10^6 cfu/mL. Different concentrations of nisin (1000, 500, and 100 U/mL) were added to the spiked milk samples and incubated at 2 temperatures (37°C and 4°C). The growth rate of each bacterial strain before and after treatment with nisin was obtained for each strain up to 20 d after nisin treatment at 5-d intervals (Gao et al., 2005). At each time point, culture aliquots were plated on Middlebrook 7H10 medium supplemented with OADC and mycobactin J, as detailed previously (Wu et al., 2007).

Live/Dead Assay for Bacterial Viability

The Live/Dead BacLight Bacterial Viability assay kit for fluorescence microscopy (Invitrogen/Thermo Fisher Scientific, Waltham, MA) was used to evaluate viability of *M. paratuberculosis* following nisin treatment using the manufacturer's directions. In this assay, a mixture of stains, SYTO 9 and propidium iodide (PI), was used to evaluate membrane integrity. The green-fluorescing SYTO 9 is able to enter all cells and is used to assess total cell counts, whereas red-fluorescing PI enters only cells with damaged cytoplasmic membranes. Three microliters of the stain mixture was added to bacterial cells previously treated with different concentrations of nisin (1,000, 500, and 100 U/mL) for 2 h, and untreated cells were used as controls. The samples were incubated for 15 min in the dark at room temperature; then, 5 µL of each stained sample was trapped between a cov-

erslip and glass slide to be viewed under a confocal fluorescence microscope (Olympus BX-51, Olympus, Tokyo, Japan), using a 60× objective sequentially with the fluorescence setting for fluorescein isothiocyanate (FITC; green/SYTO 9-positive cells) and PI (red/PI-positive cells) filters, followed by phase-contrast and bright-field settings. Images were acquired and analyzed using Olympus BX-51 software for quantification of live (green fluorescence) and dead (red fluorescence) cells.

Scanning Electron Microscopy

To investigate the effect of nisin on the membrane integrity of mycobacteria, we examined nisin-treated cultures using scanning electron microscopy (Biological and Biomaterials Preparation, Imaging and Characterization Facility, BBPIC, University of Wisconsin, Madison). Bacterial suspensions of 10^8 cfu/mL *M. paratuberculosis* K10 were mixed with nisin at the MIC (500 U/mL) and incubated at 37°C for 2 h, *Lactococcus lactis* LL108 were incubated with nisin at the MIC (10 U/mL) as a control. After incubation, cells were centrifuged at $3,000 \times g$ for 10 min, washed with PBS (pH 7.4) several times, and fixed with 2.5% glutaraldehyde in the same buffer overnight at 4°C. After fixation, cells were washed 2 or 3 times in 0.1 M PBS (pH 7.4), dehydrated in series of graded ethanol solutions (30 to 100%), and finally dried in a desiccator under vacuum. An automatic sputter coater (Polaron OM-SC7640, Quorum Technologies, Ashford, UK) was used for coating the specimens with 20-nm gold particles. The samples were then viewed using scanning electron microscopy (EVO 40, Carl Zeiss, Germany) (Singh and Mukhopadhyay, 2011).

Statistical Analysis

Kolmogorov-Smirnov and Shapiro-Wilk tests for normality with P -value ≥ 0.005 ; thus, all the data were in normal distribution. One-way ANOVA with repeated measures was followed by post hoc test (Tukey honestly significant difference test) for bacterial growth rate studies and 2-way ANOVA, Tukey's multiple comparison test when several growth kinetics were evaluated. The unpaired t -test with P -value ≤ 0.005 was used for testing differences in dimensions of mycobacterial bacilli.

RESULTS

Nisin Reduces the Viability of Mycobacteria

To evaluate the bactericidal activity of nisin on different strains of *M. paratuberculosis*, we examined the

growth of 4 strains of *M. paratuberculosis* over 50 d following treatment with varying concentrations of nisin. At different concentrations, nisin was found to inhibit the growth of all 4 strains of *M. paratuberculosis* tested in vitro during incubation at 37°C for up to 15 d after treatment, and the MIC for each strain was estimated (Figure 1). For some concentrations, the inhibitory effect continued up to 50 d after treatment. Interestingly, the tested strains had variable responses to nisin with

different MIC recorded, ranging from 15.6 U/mL for all strains except the K10 strain, which had a MIC of 500 U/mL at 15 d. At 30 d after treatment, the MIC for K10 remained at 500 U/mL, whereas that for JTC1285 was 62.5 U/mL and that for strains JTC1281 and 4B was 15.6 U/mL (Figure 1). Overall, strain K10 was the most nisin-resistant strain tested, suggesting that an adaptive response to nisin treatment could develop over time in *M. paratuberculosis*.

Figure 1. Growth kinetics of nisin-treated *Mycobacterium avium* ssp. *paratuberculosis* (MAP) cultures over time. Different strains of *M. paratuberculosis* were treated with decreasing concentrations of nisin and incubated at 37°C for 50 d and followed by optical density spectroscopy (OD at 600 nm). Each panel represents the growth curve for one strain over time. MAP K10 = laboratory strain; MAP 4B = human isolate; JTC 1281 = MAP strain isolated from oryx; JTC 1285 = MAP strain isolated from goats. The difference between the control and treated cultures at d 30 was statistically significant (**** $P < 0.0001$). Error bars represent the standard error of the mean.

Table 1. The antibacterial activity of nisin expressed as MIC (mean $\pm$ SD, $n = 3$) determined by the microplate Alamar blue assay

Bacterial strain ¹	Nisin MIC (U/mL)
<i>Lactococcus lactis</i> II 108	10.0 $\pm$ 0.0
MAP K10	500.0 $\pm$ 0.0
MAP 4B	15.0 $\pm$ 0.0
JTC 1281	15.0 $\pm$ 0.0
JTC 1285	62.5 $\pm$ 0.0

¹MAP K10 = *Mycobacterium avium* ssp. *paratuberculosis* (MAP) K10 (laboratory strain); MAP 4B = human isolate; JTC 1281 = MAP strain isolated from oryx; JTC 1285 = MAP strain isolated from goats.

Differential Effect of Nisin on Viability of Laboratory and Field Strains

To confirm the differences observed in the sensitivity of different strains of *M. paratuberculosis* to nisin, we used MABA, which has more dynamic readings than can be obtained using a standard growth curve. The MABA test showed that all strains were inhibited by nisin at the end of the observation time at 14 d after treatment. The MIC for each strain tested are shown in Table 1. The *M. paratuberculosis* strains 4B and JTC1281 were the most sensitive (15 U/mL), whereas *M. paratuberculosis* K10 was the most resistant (500 U/mL) and strain JTC1285 had an intermediate MIC at 62.5 U/mL. Overall, results from the MABA confirmed results obtained from standard growth curves (Figure 1).

Nisin Reduces *M. paratuberculosis* Survival in Milk

Following inactivation of *M. paratuberculosis* in laboratory culture medium, we sought to examine its survival in a more complex but natural environment for *M. paratuberculosis*—milk. A field strain, JTC1285, was selected for these studies, as well as a laboratory strain, *M. paratuberculosis* K10, using pasteurized cow milk stored at 4°C and 37°C. Because of the nature of milk and its normal turbidity, optical density measurement was not suitable so we used a standard bacterial plating method to examine cell viability and determine how many cells would die following nisin treatment. Three nisin concentrations (100, 500, and 1,000 U/mL) were used; all of these concentrations are permissible in milk for human consumption (Delves-Broughton et al., 1996). As expected, the highest nisin concentration had a bactericidal effect on both strains at 4°C and 37°C (Figure 2). The lowest nisin concentration had only a bacteriostatic effect on both strains at both temperatures; that is, preventing their further growth compared with the no-treatment groups. Finally, the intermediate concentration (500 U/mL) of nisin had a bacteriostatic effect at 4°C but a bactericidal effect at

37°C, which was more pronounced at 15 d after treatment ($P \leq 0.05$). Generally, the presence of nisin in milk significantly reduced ($P < 0.0001$) the survival of *M. paratuberculosis*, especially at concentrations of 500 and 1,000 U/mL.

Nisin Affects Mycobacterial Membrane Integrity

Because nisin treatment greatly affected the growth kinetics of *M. paratuberculosis*, we used the Live/Dead bacterial viability assay to examine the integrity of the mycobacterial cell wall. Compared with control samples, treatment with all concentrations of nisin triggered variable degrees of cell death (Figure 3), especially when *M. paratuberculosis* cultures were treated with 500 U/mL of nisin. As expected, the laboratory strain of *M. paratuberculosis* was least affected by nisin treatment, whereas the other strains were more sensitive.

An earlier report indicated that nisin works by creating pores in the target bacterial cell membranes, leading to cell death (Field et al., 2012). To test this hypothesis in the case of *M. paratuberculosis*, we used scanning electron microscopy to examine cultures treated with nisin. As expected, morphological changes were noted in *M. paratuberculosis* cells and in *Lactococcus lactis* LL 108, the nisin-sensitive strain used as a control. In addition to pore formation, analysis of the scanning electron micrographs of *M. paratuberculosis* cells revealed many structural changes in cells morphology (Figure 4). Treated *M. paratuberculosis* cells appeared to be bulged and shortened (i.e., wider and shorter) compared with untreated cells where they maintained normal appearance. Nisin-treated *M. paratuberculosis* had distorted contours and, in some cases, had noticeable membrane pores. Untreated *M. paratuberculosis* cells were long, smooth, and intact. Similar morphological changes were observed for the indicator bacteria, *Lactococcus lactis* LL108, exposed to nisin (Figure 4). Treated cells were shorter than controls with a significant difference observed for length ($P = 0.0027$; Figure 5A) and width ($P < 0.0001$; Figure 5B) of *M. paratuberculosis* bacilli before and after treatment with nisin. Overall, exposure of *M. paratuberculosis* to 500 U/mL nisin resulted in membrane damage, a distorted appearance, pore formation, and, in some cases, complete leakage of cell material.

DISCUSSION

Johne's disease is a chronic granulomatous enteritis of ruminants with great economic impact on the dairy industry (Berger et al., 2007). Infected animals can shed *M. paratuberculosis* in milk (Slana et al., 2008), and

recent studies have shown that *M. paratuberculosis* can survive pasteurization (Grant et al., 2005) with potential risk to consumers. The public health concern stems from the potential ingestion of *M. paratuberculosis* from contaminated milk, with possible involvement in the development of Crohn's disease in a sub-population of individuals (Grant, 2005). Pasteurized milk replacer contaminated with *M. paratuberculosis* (Grant et al., 2017) can also constitute a potential source of infection for young calves. Previously, nisin has been shown

to reduce the viability of many food-borne pathogens, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and oxacillin-resistant gram-positive bacteria (Cotter et al., 2005). Nisin has a long history as an antimicrobial agent to control both spoilage and food-borne pathogens (Sobrino-López and Martín-Belloso, 2008); however, the impact and mechanism of action of nisin on *M. paratuberculosis* is less understood. In this report, we focused our efforts on analyzing the antibacterial effect of nisin on *M.*

Figure 2. Growth kinetics of 2 *Mycobacterium avium* ssp. *paratuberculosis* strains (MAP K10 and JTC 1285) in milk following nisin treatment at 4 and 37°C. Each panel represents the growth curve for one strain at one temperature. On d 20, the difference in mycobacterial survival between treatments was statistically significant: **** $P < 0.0001$, *** $P = 0.0006$, ** $P = 0.0011$. Error bars represent the standard error of the mean.

Figure 3. Percentage of dead mycobacteria following treatment with nisin at 0, 100, 500, or 1,000 U/mL. Different strains of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and the nisin indicator species *Lactococcus lactis* LL108 were treated with permissible levels of nisin and stained with a mixture of fluorescent dyes (propidium iodide and Syto 9) and examined under the fluorescent microscope to calculate the percentage of dead bacteria. MAP K10 = laboratory strain; MAP 4B = human isolate; JTC 1281 = MAP strain isolated from oryx; JTC 1285 = MAP strain isolated from goats. Asterisks indicate significant differences compared with the control treatment (0 U/mL nisin): **** $P < 0.0001$, ** $P = 0.0037$. Error bars represent the standard error of the mean.

paratuberculosis and how nisin could be used to reduce the mycobacterial load in milk.

One of the promising findings of this study was that, except for the laboratory strain K10, *M. paratuberculosis* failed to grow at nisin concentrations <100 U/mL. An earlier study (Carroll et al., 2010) found that 60 U/mL was below the MIC₉₀ (the concentration inhibiting growth of 90% of isolates) for *M. paratuberculosis* and *M. tuberculosis*. The laboratory strain K10 could become tolerant to nisin using mechanisms similar to the adaptation of laboratory strains to stress conditions (Kralik et al., 2014). The different levels of sensitivity to nisin treatment by *M. paratuberculosis* observed in this study, even among field strains, further support the notion that genotyping variations among isolates could be responsible for resistance to nisin. Field strains of *M. paratuberculosis* were the most sensitive to nisin in both in vitro and in milk cultures. Similar to our results, the effect of nisin on *M. tuberculosis* and *M. kansasii* was shown to be dose and time dependent (Carroll et al., 2010). In an earlier study, nisin concentrations up to 1,000 U/mL did not affect *M. paratuberculosis* viability (Kralik et al., 2018), which is probably related to the genotype of the strain used. Gaggia et al. (2010) indicated that nisin at 2,500 U/mL had a bactericidal effect on *M. paratuberculosis* within 30 min. Previously, nisin was shown to be effective against many important spoilage and pathogenic microbes in several dairy products (Sobrino-Lopez and Martín-Belloso, 2006). Milk is the raw material for a variety of dairy prod-

ucts, including cheese, butter, cream, yogurt, ice cream, and infant formula. *Mycobacterium paratuberculosis* has been isolated from raw milk and can survive the process of pasteurization; therefore, *M. paratuberculosis* could be present in dairy products (Grant et al., 2001). Fortunately, nisin at 1,000 U/mL, a dose lower than the permissible dose for nisin treatment of milk for human consumption, was very effective in reducing milk contamination with *M. paratuberculosis* and was effective against both laboratory and clinical isolates.

In this report, we used 2 approaches to better define the mechanism of action of nisin on *M. paratuberculosis*. First, we used the Live/Dead bacterial viability assay that confirmed the dose-dependent effect of nisin on *M. paratuberculosis* viability by changing the mycobacterial membrane integrity. Previously, nisin was shown to decrease both components of proton motive force in a time- and concentration-dependent manner in *M. smegmatis* and *M. bovis* bacillus Calmette-Guérin (BCG; Chung et al., 2000). It is suggested that nisin, a positively charged peptide, is able to bind to the negatively charged plasma membrane through non-specific electrostatic interactions. Permeabilization of the cytoplasmic membrane and interference with the cell-wall biosynthesis of sensitive bacteria underlie the antimicrobial activity of nisin (Naghmouchi et al., 2012). To further elucidate the action of nisin on *M. paratuberculosis*, we used scanning electron microscopy, which demonstrated pore formation in the mycobacterial membrane accompanied by significant shape dis-

Figure 4. Morphological changes in *Mycobacterium avium* ssp. *paratuberculosis* (MAP) following treatment with nisin. Untreated cells of *Lactococcus lactis* LL108 (A; nisin-sensitive indicator strain) and MAP strain K10 (D; laboratory strain) were intact with regular shape and surface. After treatment with nisin at the MIC, cells appeared misshapen (B, E) with holes in the cell wall (denoted by arrows in B, C). Treated cells of MAP K10 appeared shorter (arrows in E) and bulged (arrows in F) with some ruptured cell walls. All images were captured at a scale of 1 cm = 5 μ m, except panel F, where 1 cm = 2.5 μ m.

tortion of bacilli. It is possible that nisin prevents the addition of the lipid II cell wall component (Breukink et al., 2003), which inhibits amino acid transport and affects cytoplasmic membrane integrity, as was shown for *Staphylococcus aureus* and *Bacillus subtilis* (Montville et al., 1999). In other words, nisin uses lipid II as a “docking molecule” to form pores on the cell

membrane surface in a targeted manner and effectively kill bacteria (Breukink and de Kruijff, 2006), similar to other lantibiotics (Willey and van der Donk, 2007). In *M. bovis* BCG, pores formed by nisin were large enough for export of protons but too small for export of ATP (Chung et al., 2000), whereas in *M. smegmatis*, the formed pores allowed export of both protons and

Figure 5. Dimensional differences in mycobacteria bacilli following nisin treatment. Scanning electron micrographs illustrated differences between *Mycobacterium avium* ssp. *paratuberculosis* (MAP) K10 bacilli before and after treatment with nisin and dimensional differences were clear. Treated cells were shorter ($1.87 \pm 0.37 \mu$ m) and wider ($0.81 \pm 0.14 \mu$ m) than untreated cells (length: $2.57 \pm 0.60 \mu$ m; width: $0.41 \pm 0.05 \mu$ m), resulting in the appearance of bulging. Unpaired *t*-test indicated a significant difference before and after treatment with nisin: *****P* < 0.0001, ***P* = 0.0027. Data represent average of 3 independent replicates plus standard deviation.

ATP (Montville et al., 1999). More analysis is needed to further characterize the pores formed by nisin treatment of *M. paratuberculosis*. Nonetheless, our analysis indicated the possibility of using nisin to control *M. paratuberculosis* in milk consumed by both animals and humans and improve the microbial quality of feed and food products, respectively.

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