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Potential of Nisin and Newly Discovered Bacteriocins as Preservatives for Pasteurised Milk

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ABSTRACT

Spoilage of pasteurized milk is mainly caused by the presence of organisms that either survive pasteurization (psychrotolerant spore-formers) or re-contaminate milk in the processing environment (post-pasteurisation contaminants). Pasteurization of bovine milk by heat treating at 72°C for 15-30 seconds ensures milk quality without impairing its organoleptic and nutritional status and extends shelf life to 12-14 days at refrigeration temperatures. Nisin A is a class I bacteriocin known to inhibit gram positive bacteria and approved by the European Food and Safety Authority as a food preservative. It is commercially available as Nisaplin®, which contains a concentration of 2.5% w/w of nisin. This study examined the effect of Nisaplin® at different concentrations on spoilage of refrigerated commercial whole pasteurized milk over a period of 59 days. At a high concentration of 4 mgml^{-1}, Nisaplin® reduced the total bacterial count below the limit of detection in the milk, and inhibition was visible for at least 14 days at 4°C. Previously isolated milk spoilage bacteria were identified using 16s rRNA gene sequencing and utilised as target indicators for bacteriocin production. *Lactobacillus delbrueckii* ssp. *bulgaricus* LMG6901, *Microbacterium lacticum,* and *Pseudomonas aeruginosa* were utilised as indicator strains in a screen of milk spoilage organisms for bacteriocin production. This resulted in identifying the putative bacteriocin producer *Carnobacterium divergens*, a lactic acid bacterium active against *L. bulgaricus*. The study concludes that Nisaplin® is effective in the reduction of microbial load and its effectiveness could be increased when combined with other preservative methods thus forming an extra hurdle in the milk.

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Introduction

Pasteurization of bovine milk extends shelf life to 12-14 days without impairing its organoleptic and nutritional qualities (El Dessouky Abdel-Aziz et al., 2020). The treatment entailing heating at 72°C for 15 seconds eliminates most susceptible bacterial species present in raw milk ensuring safety and standardizing shelf-life (Nasr & Elshaghabee, 2019). Further extension the shelf life of pasteurized milk would benefit consumers and production industries, enabling longer transport and storage times and consequently greater export potential. However, extended shelf-life milk must be minimally processed and retain a clean label (Radha et al., 2014). Shelf-life extension of commercial fluid milk beyond the simple 5 to 14 days at 4°C to 8°C of high-temperature short-time (HTST) pasteurization increases distribution distances and times, lengthens shelf life for the consumer prior to spoilage, and enhances food safety (Trmčić et al., 2015).

Milk spoilage is typically caused by either Gram-positive psychrotolerant endospore forming bacteria, usually *Bacillus* spp. and *Paenibacillus* spp. present in raw milk which can survive the pasteurization process, or post-pasteurization contaminants such as *Pseudomonas* spp. that gain access to milk due to poor post-processing conditions(Ziyaina et al., 2018) and capable of growth at refrigeration temperatures. Heat-resistant proteinases of psychrotrophic bacteria cause spoilage in processed milk because of enzyme-retaining activity after the heat treatment (Machado et al., 2017). Post pasteurization contamination (PPC) often results in spoilage within 7 to 14 days and represents psychrotolerant Gram-negative bacteria, including *Pseudomonas spp*, coliforms such as *Escherchia coli*, and other members of the *Enterobacteriaceae* family (Martin et al., 2018). Spoilage due to the outgrowth of aerobic, Gram-positive, spore

forming bacteria such as *Paenibacillus* spp. and *Bacillus* spp. typically occurs around 17 to 21 days of shelf-life if milk is stored around 6°C (Doll et al., 2017). The onset can be rapid, within (7) days, suggesting contamination with viable bacterial cells, which are not delayed by the germination process of spores (Ranieri et al., 2012). Non-endospore forming bacteria such as *Enterococcus* spp. and *Lactobacillus* spp. have also been found to tolerate temperatures of 60–80°C surviving in large numbers. Those surviving pasteurization, though their growth is severely reduced by storage at refrigeration temperatures take a few weeks to spoil milk (Rawat, 2015).

There are several methods currently used to extend the shelf-life of milk including ultra-pasteurization (UP), ultra-high temperature (UHT) pasteurization and microfiltration (MF). Extended shelf-life (ESL) milk has gained a substantial market share in many countries and is produced by thermal processing using conditions between those used for traditional HTST pasteurization and those used for UHT sterilization (Deeth, 2017) (Schmidt et al., 2012).The most fundamental rationale for producing ESL milk is to inactivate all vegetative bacteria and spores of psychrotrophic bacteria and to cause a minimal chemical change that can result in cooked flavour development (Castillejo et al., 2016). The primary focus of UHT is to eradicate *Bacillus cereus*, as many strains of this organism are pathogenic and can grow at temperatures below 7°C. Spores of certain *B. cereus* strains are extremely heat-resistant and cause spoilage of milk (Pujol et al., 2015). The ESL is produced using high temperatures which could affect the milk organoleptic qualities (Deeth, 2017). The ESL or ultra-pasteurized milk is produced by thermal processing using conditions between those used for traditional HTST pasteurization and those used for UHT sterilization. It should have a refrigerated shelf-life of more than 30 days.

Bacteriocins are small (<10 kDa), ribosomal produced antimicrobial peptides produced by bacteria that are typically heat stable. Nisin A is the prototypical bacteriocin which has been studied extensively since its discovery. It is a 34 amino acid heat stable bacteriocin peptide produced by strains of *Lactococcus lactis* subsp, *lactis*, and over 10 natural variants from multiple species of nisin: A, Z, F, Q, U, U2, O, P, J and H (Cotter et al., 2013). Nisin is one of two bacteriocins currently registered for use as a food additive to extending its shelf-life by inhibiting spoilage and pathogenic microorganisms (Ibarra-Sánchez et al., 2020). Nisin is classified as generally recognized as safe (GRAS) by the U.S Food and Drug Administration (FDA) and employed as a food bio-preservative approved by the European Food Safety Authority (EFSA) under the E number E234. It is non-toxic, flavourless, odourless, and is employed as a partially purified powder under the product name Nisaplin® (Danisco). Once ingested, nisin is inactivated by trypsin and pancreatin, therefore it has no effect on the gut microbiome. Moreover, the fact that humans have probably been exposed to it for centuries and having a daily acceptable intake (DSI) of 0.13 mg of nisin /kg of bodyweight deems it safe as a bio preservative (Soltani et al., 2021).

Nisaplin® has been shown to be effective in the microbial control of several dairy products and its use has been widely assessed in cheese manufacturing (Martinez et al., 2016). It is made through the fermentation of skimmed milk that has been digested by enzymes. The yeast is then added by *L. lactis* sbsp. *lactis* strains that also produce nisin. (Shimizu et al., 1999)This process is maintained at a pH of 6-7 and once done, the product is concentrated by foam extraction or membrane filtration. It is then precipitated and subjected to spray drying after which sodium chloride is added to the powder to standardize it to 1,000 international units (IU) nisin A/mg. (25% nisin A wt/wt) (Younes et al., 2017). In cheese making, nisin-producing and nisin-resistant starter cultures are both incorporated to maintain the process while at the same time controlling food-borne pathogens and spoilage bacteria (Radha et al., 2014) (Melini et al., 2017). It was reported that the concoction of nisin with other antimicrobial compounds, such as the monoester of lauric acid, monolaurin, and the milk lactoperoxidase system (LPS) or other bacteriocins can induce the sensitization of resistant spoilage and food-borne microorganisms (Zhang et al., 2014). Monolaurin and nisin combined have proved to successfully exert a bactericidal effect against different *Bacillus* species in skim milk, and inhibited their regrowth and sporulation (Rawat, 2015).

There are limitations that curb the use of Nisaplin® in dairy products, such as adsorption of the peptide to fat and the surface of protein globules (Silva et al., 2018). Studies have reported an interaction between milk fat and nisin activity, which may limit its application in fat containing dairy products. The peptide is positively charged and targets bacterial membranes where it may form pores, but it can be sequestered by milk fat globule membranes (Hantsis-Zacharov & Halpern, 2007). The activity of nisin against *Listeria monocytogenes* in milk was found to decrease as the milk fat concentration and increased with a maximum anti-listerial effect of nisin in skim milk and a reduced effect in milk with 17.5% fat (Chen & Zhong, 2017). It has also been noted that homogenization of milk reduces the anti-listerial activity of nisin (Wang et al., 2023).

Nisin has been approved for use in over 50 countries and was granted generally recognized as safe (GRAS) status by the FDA in 1988 (Saad et al., 2019). The World Health Organization Codex Committee on milk and milk products allows pure nisin as a food additive for processed cheese at a concentration of 12.5 mg/kg product, whereas up to 250 mg/kg is permitted by the US FDA (Zhang et al., 2014). Nisin, either added directly in purified form or produced, is used in several dairy food applications to ensure safety, extend shelf-life, and preserve quality (Chen & Zhong, 2017).

Consumers are increasingly becoming health conscious and averse to preservatives perceived as ‘chemical’ or ‘unnatural’ in foods. Nisin in contrast is a safe naturally expressed peptide partially purified in the form of Nisaplin®. Utilization of bacteriocins such as nisin as a bio-preservative alone or in combination with other treatments represents a method to extend milk shelf life and ensure its sensory properties are not altered (Radha et al., 2014). In this study Nisaplin® at a range of concentrations was assessed as a preservative for pasteurized commercial whole milk over a period of 59 days. Total aerobic plate counts were determined and 16s rRNA gene sequencing was utilised to determine the effect on the pasteurised milk spoilage bacteria microbiota. In addition, milk spoilage organisms were assayed for bacteriocin production against

Lactobacillus delbrueckii ssp. *bulgaricus* LMG6901, *Microbacterium lacticum* and *Pseudomonas aeruginosa* to identify novel bacteriocins and bacteriocin producers with activity against milk spoilage organisms.

Materials and Methods

Effect of Nisaplin® on commercial pasteurised milk

Unless otherwise stated, all the experiments were performed in biological and technical triplicate at laboratory scale in 250 ml Durham bottles, as illustrated in figure 1. Experiments were performed through different time frames spread over 59-day intervals. One Litre of commercial milk (Dawn, Ballinahina) was purchased on successive weeks for each biological replicate. The milk was then divided into four and aliquoted into sterile vessels. Nisaplin® (2.5% nisin from *L. lactis* subsp. *lactis* balanced with sodium chloride and denatured milk solids, Sigma-Aldrich, Wicklow, Ireland) was added to the pasteurised whole milk in different concentrations; 0 mg ml^{-1}, 0.04 mg ml^{-1}, 0.4 mg ml^{-1} and 4 mg ml^{-1}. This was first done by making a small solution of the Nisaplin® with a portion of the milk in a 50 ml sterling tube, vortexing it and then adding it to the milk sample and gently rolling it to ensure even distribution while avoiding bubbles. The solutions were then vortexed until homogenous, aliquoted into three volumes for technical replicates and stored at 4°C for 59 days. Serial dilutions and plating were subsequently done at day 0, 1, 3, 7, 14, 28, 49 and 59. 100 µl of the milk sample was aseptically removed and spread plated on plate count skim milk agar (MPCA) made in the lab (comprising of peptone from casein 5 g/L Yeast extract 2.5 g/L Skim milk powder 1 g/L Glucose 1 g/L and Agar 15 g/L), following serial dilution in maximum recovery diluent (MRD; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). The plates were then incubated at 30°C for 72 h and then plate counts were carried out after each time points.

Milk spoilage microorganisms (culture strains and conditions)

Bacterial isolates from a previous study (Hill et al., 2018) were retrieved from -80°C glycerol stocks and streaked on brain heart infusion (BHI) (Oxoid) and skimmed milk plate count agar (MPCA) and incubated for 72 h at 30°C. Pure streaks were subject to Gram stain and colony PCR for identification. For colony PCR a single colony was touched with a 200 µl pipette tip and swirled in 50 µl molecular grade H_2O. The solution was then subject to freeze thaw and utilised as template for the reaction. PCR amplification was performed using B27F and U1492R universal primers and the PCR product purified (GenElute, Sigma-Aldrich). The DNA was quantified using a Qubit 4.0 fluorometer and samples were sent to Genewiz, Germany, for Sanger sequencing of amplified 16s rRNA gene to identify the milk spoilage microorganisms.

Potential Bacteriocin Production

Initial screening of antimicrobial activity

Following quantification of TBC from Nisaplin containing milk, over 6000 colonies were screened against milk spoilage organisms *M. lacticum,* and *P. aeruginosa* in addition to an acid resistant indicator, *L. bulgaricus* LMG6901, for potential bacteriocins using the agar based deferred antagonism assay as described by Twomey et al., 2021 illustrated in Figure 2, with minor adjustments.

These bacteria were selected as potential indicator microorganisms due to their abundance in the composition of the spoilage microorganisms. The different indicator strains and were grown overnight in MPCA, BHI and MRS broths and incubated overnight at 30°C and 37°C respectively depending on their optimal conditions. They were then seeded (0.25% vol/vol) into the subsequent 7.5 g/L sloppy agar that had been tempered to 50°C and poured onto the milk spoilage microorganism plates, allowed to dry, and incubated overnight based on their optimal conditions. They were then examined for the presence of zones of clearing in the overlay indicative of antimicrobial activity.

Isolates which resulted in distinct zones of inhibition were further cultured in broth for well diffusion assays. 20 ml each of tempered sloppy agar seeded with the indicator strains above were poured and allowed to set into which 6mm-wide wells were punctured into it using a glass Pasteur pipette. Grown cultures were centrifuged at maximum speed (16,000 x *g*) for 4 min to pellet cells. The resulting supernatant was filter sterilised and 50 ul was added to bored wells. Plates incubated overnight according to the respective optimal conditions of the indicator strains. They were then examined for zones of inhibition the next day (Sugrue et al., 2020). Strains found to produce active supernatant were repeated with cell free supernatant brought to neutral pH through addition of 1M NaOH.

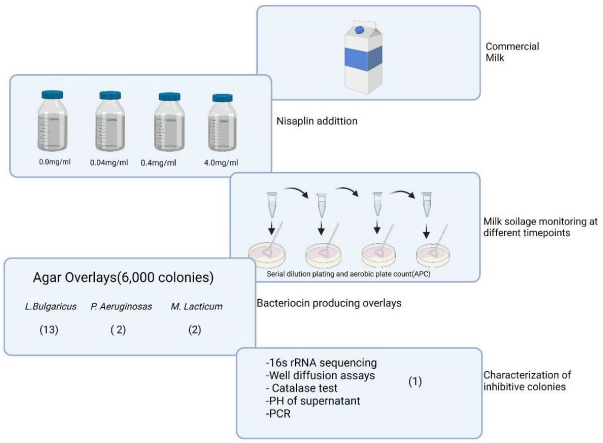


Figure 1. Experimental Overview

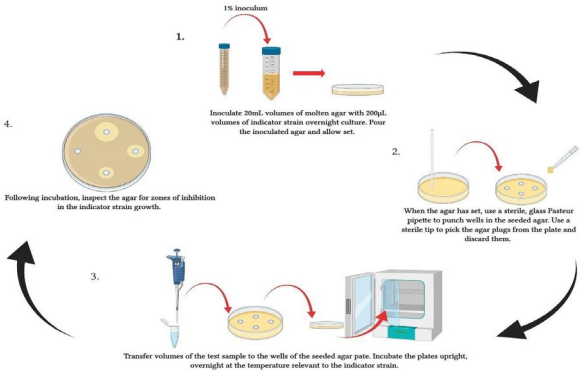


Figure 2. Agar- based well diffusion assay to detect antimicrobial activity.

The Zones of inhibition in the growth of the indicator strain indicate antimicrobial activity. Source (Twomey et al., 2021).

Strain identification and genome sequencing

To identify the potential bacteriocin producing microorganism, DNA was extracted following the protocol from the GenElute bacterial genomic DNA kit (Sigma). PCR amplification was then done following the Meridian Bioscience™ protocol with B27F and U1492R as the universal amplification primers. Quantification was also done using the Qubit 4.0 broad range kit and then sent for 16s rRNA gene was subject to sanger sequencing (Genewiz, Germany).

Data Analysis

The data on microbial levels of milk treated with varying concentration of Nisaplin® was analyzed as Mean±SD of triplicate treatments. The mean value ± standard deviation (SD) was used in data analysis. The Analysis of variance, means, standard deviation and Bonferroni test was used to test for significance using GraphPad prism version 8 (GraphPad Software, Inc. CA, USA). Means of different treatments were significantly different at p-value ($P< 0.05$).

Results

Effect of Nisaplin® on Commercial Pasteurised Milk

The effect of Nisaplin® on commercial pasteurised milk is presented in figure 3. Three different biological replicates were tested against different Nisaplin® concentrations in triplicates. The limit of detection for the total bacterial count (TBC) (log_{10} $CFUml^{-1}$) of the milk samples was 2.48 log CFU ml^{-1} and pre-addition of Nisaplin® (0 $mgml^{-1}$, Day 0) the mean TBC at baseline was 2.96 log_{10} $CFUml^{-1}$. Nisaplin® impacted at all concentrations but by day 14 the spoilage organisms rebound in the 0.04 and 0.4 $mgml^{-1}$ groups. However, it is observed that Nisaplin® effectively controlled milk spoilage up to 14 days at a high concentration of 4 mg ml^{-1}, at refrigeration temperatures of 4°C at the concentration of 4.0 $mgml^{-1}$ in two replicates out of three replicates.

The limit of detection for the total bacterial count (TBC) (Log $CFUml^{-1}$) of the milk samples was 2.48 log CFU ml^{-1}. Pre-addition of Nisaplin® the milk samples had a baseline average TBC of 2.96 log CFU ml^{-1} with replicate 3 being below detection level. At day 0, replicate 1 had a TBC of 2.67 log CFU ml^{-1} pre-addition but soon after addition at 0.04 $mgml^{-1}$ the TBC was below detection levels. It was observed that the Nisaplin® impacts at all concentrations but is most significant at 4 mg ml^{-1} concentration, where up to day 14 the TBC is below detection as shown in figure 3.

It was also noted that in the sample without Nisaplin®, the TBC increased steadily throughout the timeframe from an average of 2.96 log CFU ml^{-1} at day 0 to 8.66 log CFU ml^{-1} at day 56 at refrigeration temperatures. The count at 4mg ml^{-1} remained below detection levels until day 14 and then had a sudden surge of growth with high bacterial counts of 9.69 log CFU ml^{-1} and 9.86 log CFU ml^{-1} between day 28 and 56, respectively.

The higher the Nisaplin® concentration the longer the bacterial inhibition. The blank sample did not exhibit much inhibition and acted as the control in the experiment. There was spoilage organism rebound in the 0.04 and 0.4 mg ml^{-1} groups. However, it is observed that Nisaplin® effectively controlled milk spoilage up to 14 days at a high concentration of 4mg ml^{-1}, at refrigeration temperatures of 4ºC at the concentration of 4.0 mg ml^{-1} in two replicates out of three replicates. The strongest effect was recorded between day 0 and day 14 where the TBC was below the limit of detection, 2.48 log CFU ml^{-1}.

Milk spoilage microorganism identification from stock

Thirty-three isolates from a previous shelf-life study of milk at refrigeration and room temperature were identified for use as indicators in the bacteriocin screen. Thirty-two of the isolates were gram positive, as presented in Table 1. The isolates were identified as *Microbacterium lacticum* (87.5%), *Kocuria varians* (3.12%), *Microbacterium flavum* (3.12%) and *Brachybacterium nesterenkovii* (6.25%). *M. lacticum* were found from milk spoiled at 20°C (23 isolates) and 4°C (4 isolates).

Isolating Bacteriocin producers from the milk spoilage microbiome

An estimated 6000 bacterial colonies from the pasteurized commercial milk samples were screened for bacteriocin production. Out of this number, seventeen exhibited a zone of inhibition in agar overlay against at least one indicator. Thirteen isolates produced zones against *L. bulgaricus* LMG6901, two against *M. lacticum* and two produced zones against *P. aeruginosa*. The zones of inhibition are illustrated in table 2. Through 16s rRNA gene sequencing, the antimicrobial producing spoilage microorganisms identified included *P. aeruginosa, Paenibacillus polymyxa, Bacillus licheniformis, C. divergens*, *Paenibacillus rhizoplanae and Pseudomonas gessardii* as summarized in Table 3. *P. aeruginosa, Paenibacillus polymyxa, Bacillus licheniformis, Paenibacillus rhizoplanae and Pseudomonas gessardii* all produced zones in the sloppy gar overlays but did not show any activity in the well assays with the cell free supernatant.

A single isolate had exhibited a 4mm zone in agar overlay against *L. bulgaricus* LMG6901 which was also produced activity into supernatant measured by well diffusion assay. Activity was confirmed with neutralised CFS in well diffusion assay eliminating the potential inhibitive effect of acidic pH. 16S rRNA gene sequencing was used to identify the bacteria as *C. divergens* with 98% percentage identity.

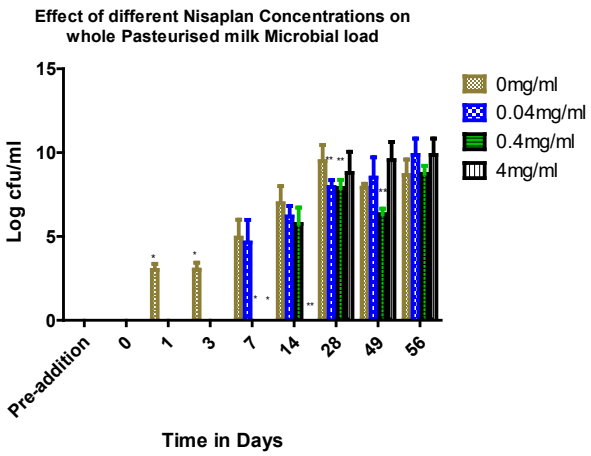


Figure 3. Graph illustrating the effects of Nisaplin® on commercial whole pasteurised milk across different time frames (days). PZF format from GraphPad Prism.

Table 1. Milk spoilage bacterial indicator species identification by 16S rRNA sequencing

16s Identification Species	Percent	Source	Gram stain Identity
Microbacterium lacticum	99.9	4°C	positive cocci
Microbacterium lacticum	97.67	4°C	positive rods
Microbacterium lacticum	98.3	20°C	positive rods
Microbacterium lacticum	98.3	20°C	positive rods
Microbacterium lacticum	99.06	20°C	negative cocci, with gram variables
Microbacterium lacticum	99.4	20°C	positive cocci
Microbacterium lacticum	99.61	20°C	positive cocci
Microbacterium lacticum	99.55	20°C	positive rods
Microbacterium lacticum	99.99	20°C	positive rods
Microbacterium lacticum	99.54	20°C	positive rods
Microbacterium lacticum	99.54	20°C	positive rods
Staphylococcus succinus	100	20°C	positive cocci
Microbacterium lacticum	100	20°C	positive rods
Microbacterium lacticum	100	20°C	Positive rods
Microbacterium lacticum	99.89	20°C	positive cocci
Kocuria varians	100	20°C	positive cocci
Microbacterium lacticum	97.95	4°C	positive rods
Microbacterium lacticum	97.95	4°C	positive rods
Microbacterium lacticum	100	20°C	positive rods
Microbacterium lacticum	99.5	20°C	positive rods
Microbacterium lacticum	99.72	20°C	positive rods
Microbacterium lacticum	99.58	20°C	positive rods
Microbacterium lacticum	100	20°C	positive rods
Microbacterium lacticum	100	20°C	Positive rods
Paenibacillus mucilaginous	99.81	4°C	Positive rods
Microbacterium flavum	100	20°C	Positive rods
Brachybacterium nesterenkovii	99.46	20°C	Positive cocci
Brachybacterium nesterenkovii	99.46	20°C	Positive cocci
Microbacterium lacticum	100	20°C	Positive rods
Microbacterium lacticum	99.73	20°C	Positive rods
Microbacterium lacticum	99.58	20°C	Positive rods
Microbacterium lacticum	99.72	20°C	Positive rods
Microbacterium lacticum	100	20°C	Positive rods

Table 2. Bacteriocin screening and well diffusion assays

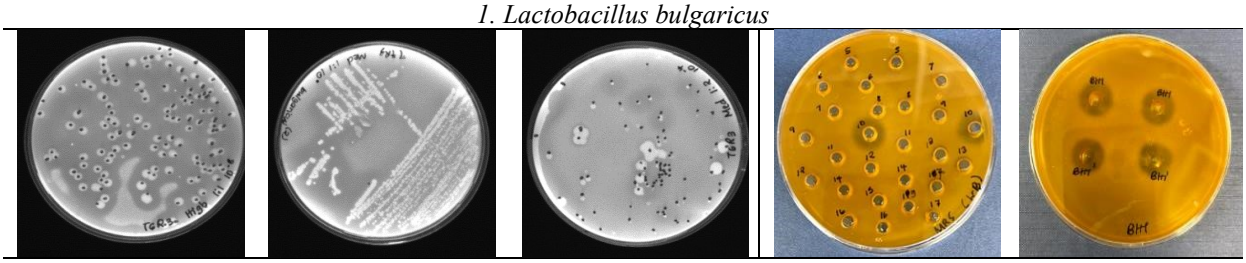
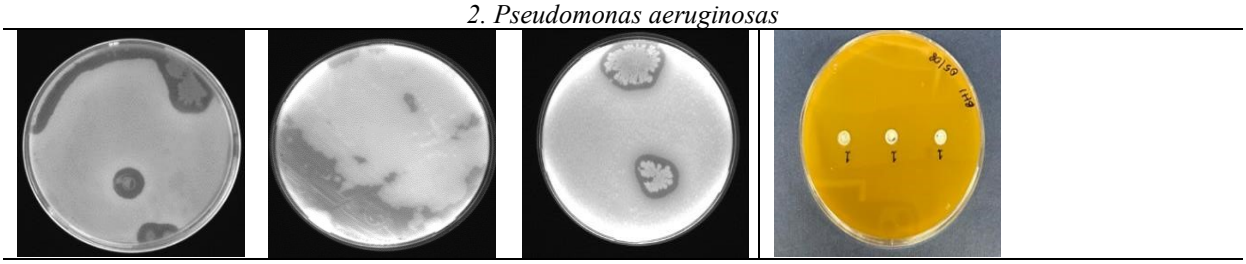
Overlays	Well diffusions with CFS	Well diffusions with neutral CFS
1. Lactobacillus bulgaricus		
2. Pseudomonas aeruginosas		
3. Microbacterium lacticum		

Table 3. Putative bacteriocin producers identified by 16s rRNA gene sequencing and indicator strains inhibited.

Milk spoilage microorganism (16S.rRNA)	Gram stain	Growth conditions	Percentage ID	Indicator strains
Pseudomonas aeruginosas	negative rods	aerobic	99.55	*Microbacterium lacticum*
Paenibacillus polymyxa	positive rods	aerobic	99.42	*Microbacterium lacticum*
Pseudomonas aeruginosas	negative rods	aerobic	99.82	*Pseudomonas aeruginosas*
Bacillus licheniformis	positive rods	aerobic	100	*Lactobacillus bulgaricus*
Bacillus licheniformis	positive rods	aerobic	99.9	*Lactobacillus bulgaricus*
Bacillus licheniformis	positive rods	aerobic	99.9	*Pseudomonas aeruginosas*
Pseudomonas aeruginosas	negative rods	aerobic	100	*Lactobacillus bulgaricus*
Pseudomonas aeruginosas	negative rods	aerobic	99.68	*Lactobacillus bulgaricus*
Pseudomonas aeruginosas	negative rods	aerobic	99.34	*Lactobacillus bulgaricus*
Carnobactrium divergens	positive rods	facultative anaerobe	98.77	*Lactobacillus bulgaricus*
Paenibacillus polymyxa	positive rods	aerobic	98.27	*Lactobacillus bulgaricus*
Pseudomonas aeruginosas	negative rods	aerobic	98.08	*Lactobacillus bulgaricus*
Pseudomonas aeruginosas	negative rods	aerobic	95.64	*Lactobacillus bulgaricus*
Paenibacillus rhizoplanae	positive rods	aerobic	97.82	*Lactobacillus bulgaricus*
Bacillus licheniformis	positive rods	aerobic	99.46	*Lactobacillus bulgaricus*
Pseudomonas gessardii	negative rods	aerobic	99.52	*Lactobacillus bulgaricus*
Pseudomonas aeruginosas	negative rods	aerobic	99.91	*Lactobacillus bulgaricus*

Discussion

Effect of Nisaplin® on Commercialized Whole Milk

According to (Meliani & Bensoltane, 2015), milk is generally regarded as spoilt when the bacterial count exceeds 10^6 cfu. Since the microorganisms produce enzymes that degrade milk, off odors and curdling were also considered as signs of spoilage. The fact that after day 14, no significant difference was recorded in the total bacteria count with a high concentration of 4 $mgml^{-1}$ can be explained by variability in the initial total bacteria count of the milk replicates and different batches. This was mainly dependent on the batch numbers and various days of purchase of the pasteurized milk, although the samples were sourced from the same location and the brand was consistent. It could also be possible that the third replicate may particularly be spoiled by Gram negative bacteria which are inherently resistant to nisin. The early spoilage in sample milk with no nisin concentration could been attributed to the highly developed acidity caused by the multiplication of lactic acid bacteria. It could also be that the milk spoiled as it normally does in its pasteurised shelf life. In the milks containing Nisaplin®, this process may have been inhibited and thereby delayed the development of acidity thus prolonging the shelf life of the milk sample.

Initial microbial load of the milk post pasteurisation is a key contributor towards milk spoilage. This is evident given by the third replicate and how its initial count was slightly higher than the other two. Nisin is not effective against Gram-negative bacteria, and this could be another explanation for the variations between the replicates. It is possible that the milk microbiome could have been altered from Gram-positive dominant bacteria to gram negatives like *Pseudomonas* spp thus accelerating the milk spoilage. A combination of treatments like high pressure treatment (HPT) to effectively eliminate the Gram-negatives could be implemented in this case (Arqués et al., 2008). It was observed an extension in the shelf life of nisin added pasteurized milk from 72 h to 132 h under tropical conditions. The difference in total bacterial count between the concentrations shows Nisaplin® effect in a short time - a matter of minutes. This means that its action is almost immediate. A study by (Wirjantoro et al., 2001) reported seven days extension in shelf life of pasteurized milk added with the very low level of Nisaplin® (10 $IUml^{-1}$). Nonetheless, it was observed that total bacteria count with high concentration of 4 $mgml^{-1}$ increased significantly, particularly from day 28 to 56. This signified possible resistant cells given that some bacteria can survive extreme conditions and become resistant to some harsh conditions. It is vital to know the composition of the food product and its processing conditions to ascertain proper preservation methods and dosage. The fact that the efficiency of Nisaplin® was observed to be reducing over time has been credited to the interaction of fat globules and the peptide.

It is also fundamental to note that the refrigeration temperature of 4^oC, where the milk was stored, was a hurdle to microbial growth and could also be a factor hindering growth of the microorganisms. Its combination with Nisaplin® could have been an additional factor in optimizing the antimicrobial effect on the milk. The composition of Nisaplin® is such that it contains denatured milk solids and sodium chloride at 50% wt/wt to 75% wt/wt. (Younes et al., 2017). Moreover, there is 1000 IU nisin A/mg of Nisaplin® in the milk sample, which translates to 0.025 µg of pure nisin (Younes et al., 2017). This means that 40 mgL^{-1} of Nisaplin® would add 20-35 mg of sodium chloride to the milk sample. This would therefore complement the already existing hurdles and the inhibitory effect in general.

Despite the modern-day consumer being conscious of food additives and that Nisaplin® is generally viewed as a clean label, high quantities may negatively impact the consumer and the organoleptic properties of the milk may also end up being altered. The Nisin concentration to pasteurized milk according to (Wirjantoro et al., 2001), maintains the quality of the milk product and consumers are satisfied with it as a clean preservative. Moreover, (Susanto, 2017)argued that Nisaplin® is preferred as a clean preservative and does not change the nature and taste of the food in most cases.

According to the Central Statistics Office (CSO) statistical released on 31st of August 2021, 1,018 million litres of milk was sold in Ireland in the month of July alone, as pasteurized whole milk direct for human consumption, representing an increase of 3.3% from July of 2020 (https://www.cso.ie/en/releasesandpublications/er/ms/milkstatisticsjuly2021/).Kenya reported approximately 5.2

billion litres of milk production in the year 2015. Of these, only 616 million litres of milk were processed, with the bulk of it, 70% sold as is un-chilled raw fresh milk through informal market outlets (https://www.3r-kenya.org/dairy/). The cost of production per litre was at approximately 26.8 cents in Ireland n 2018 according to (Teagasc, 2020) in comparison to Ksh.23 (€0.18) in Kenya currently (https://www.the-star.co.ke/news/2021-06-13-cost-ofmilk-production-still-high-at-sh23-a-litre-says-munya/). The Kenyan market for UHT and powdered milk is considered higher that fresh pasteurized whole milk due to less developed storage systems that usually see constant power outage situations. This translates to inconsistencies in storage temperatures especially in the rural parts of the countries where access to these amenities is a luxury. A study done by (Njarui et al., 2011) revealed that 99% and 84% of rural and urban households prefer raw respectively. It also reported that the more processing milk undergoes the less likely it is to be consumed by the average market because it was more affordable and widely available. Given that 1 kg of Nisaplin® would cost anywhere from $26 to $75 USD (€22.05 to €63.62) (https://www.alibaba.com/product-detail/Nisin-Nisinnisin-Best-Price-Food-Grade_1600288836248.html?spm=a2700.7724857.normal_offer.d_image.33904e4dExL5Jg &s=p) it would therefore mean that incorporating it at 4 $mgml^{-1}$ in the production process would minimally increase the cost of the milk. This marginal increase may be tolerated by the consumer if offset by shelf-life extension and no detectable differences in sensory properties were found.

Milk Spoilage Microorganism Identification from Stock

Cooling milk controls microorganism multiplication especially mesophilic bacteria that acidify milk causing the proteins to be unstable. Some of these though, are psychrotrophs that survive refrigeration temperatures by synthesizing phospholipids, permitting them to adapt and function at these conditions and excrete extracellular enzymes (McAuley et al., 2016). This then causes the carbohydrates present in milk to ferment, mainly the lactose to lactic acid and other products. The enzymatic reaction over time, is responsible for rancidity and makes fermented dairy product get the thick consistency as compared to fresh milk (Baglinière et al., 2017).

Pseudomonas spp. is a major known spoilage psychrotroph and its absence in this study could be explained by good hygienic conditions that minimize contamination levels in the processing plant where the samples were sourced. Due to the advances in the mechanization process of dairy, PPC spoilage has greatly reduced, and milk spoilage is now mainly because of aerobic, gram-positive spore formers, especially psychrotolerant *Bacilli sp.* (Ribeiro Júnior et al., 2018). If milk is refrigerated though at 4°C, spoilage due to these microorganisms would essentially take longer be visible; between 17-21 days. In cases where both spore forming and non-spore forming gram positive bacteria are responsible for spoilage, then the process takes a shorter time since the cells are not delayed by the spore germination process (Trmčić et al., 2015).

Most of the bacteria here were gram positive with *M. lacticum* being the most dominant species. It is common bacteria found on/in milk equipment, and this may be considered the reason why it appeared in large numbers in the milk samples (Speck, 1943). These cells required 72 h on skimmed milk place count agar (SMPCA) at 30°C to fully grow on a plate and develop colonies and were properly visible in 24 hours. This could be a reason as to why it is usually missed in routine checks as plates are typically incubated at 37°C for 24 h (Speck, 1943). The spoilage microorganisms seem to thrive in the pasteurized milk due to the bacteria that contaminate the milk after the pasteurization process. The spoilage microorganisms drive the deterioration of milk in colour, odour, or flavour to the point where it is unsuitable for human consumption.

This microorganism does not form spores, is non-motile and appears as smooth, small, round, off white colonies on SMPCA. They are characterized to be aerobes and are also weak acid producers. They have been demonstrated to produce sufficient lactic acid in 7 days at 30°C to cuddle milk (Trmčić et al., 2015). An exceptional characteristic, however, is that *M. lacticum* is heat resistant and has been reported to survive up to 76°C for up to 30 min, indicating that it may survive HTST pasteurization. A previous study by (Trmčić et al., 2015) also reports that some isolates withstood up to 85°C for 10 min and would only be eliminated at 30 min. It is interesting though that no growth was recorded at these conditions. Further, *M. lacticum* is not considered pathogenic and its presence in the milk sample should not raise alarm. The microorganism has its origins in in the intestines and faecal material of animals therefore mainly accessing the milk through the farm milking equipment (Speck, 1943). Their high tolerance to heat also helps them to survive the high temperatures of the water used in cleaning and once present in the milk they then carry on even through the pasteurization process as well.

The microbial content of milk can be used to estimate its quality and the conditions of production. In some cases, bacteria in milk can cause spoilage when permitted to multiply since milk is potentially susceptible to contamination with pathogenic microorganisms (Martin et al., 2018). Precautions must be taken to minimize this possibility and to destroy pathogens that may gain entrance. Certain microorganisms produce chemical changes that are desirable in the production of dairy products such as cheese, yogurt, and the addition of nisin is known to prevent the multiplication of lactic acid bacteria and thereby delay the development of acidity and prolonged the shelf life (Melini et al., 2017).

The proper pasteurization process can be conducted to eliminate spoilage microorganisms. Pasteurization of milk requires temperatures of 63°C maintained for 30 min or heating to a higher temperature, 72°C and holding for 15s before repackaging (Deeth, 2017). Pasteurization aims to making milk and milk products safe for human consumption by destroying all pathogenic bacteria. There are different ways to pasteurize milk and each process depends on the length of time and the temperature milk is heated. The pasteurized product group includes whole milk, skim milk, standardized milk, and various types of cream (Eisner, 2021). It is meant to wipe out pathogenic microbes in milk with minimal effect to its taste or nutritional value.

Isolating Bacteriocin Producers from the Milk Spoilage Microbiome

The indicator species used for bacteriocin screening *M. lacticum, P. aeruginosas* and *L. bulgaricus*. These microorganisms form a considerable part of the thermoduric bacterial flora of raw and pasteurized milk, powdered milk, cheese, and dairy equipment. Report by (Thorat, 2013) noted that *Lactobacilli* have been used in many fermentation processes and is characterized as gram positive, catalase negative, non-sporulating, non-pigmented bacteria. A study conducted by (Saad et al., 2019) also indicates that the most common milk spoilage microorganisms include *P. aeruginosas, C. divergens* and *P. polymyxa*. In addition, (Silva et al., 2018) notes that *Bacillus licheniformis, Pseudomonas gessardii, Pseudomonas aeruginosas* and *Bacillus licheniformis* are the main milk spoilage microorganisms active in the pasteurized milk.

The zones produced are indicators of antimicrobial activity, they cannot be used to determine potency, where the size of the peptide and the media components may have an effect (Daba & Elkhateeb, 2020). The zone produced by *C. divergens* against *L. bulgaricus LMG6901* had a 4mm radius. Well diffusion assays determine the bacteriocin activity; whether it remains bound to the cell or is available in the surrounding media (Twomey et al., 2021). Low pH facilitates the release of bacteriocins into the surrounding media and therefore this is a method of purifying various peptides. The fact that *C. divergens* demonstrated a zone even after neutralizing the pH indicated that the activity is not due to acidic pH which can also produce zones of inhibition. Future work may confirm the proteinaceous nature of the activity by heat and protease treatment of the supernatant.

C. divergens is a lactic acid bacterium that was in 2002 included in the authoritative list issued after a joint venture between the International Dairy Federation and the European Food and Feed Cultures Association (Bourdichon et al., 2012) as a food bio preservative. The *C. divergens* V41, has also been shown to inhibit *Listeria monocytogenes* for up to 4 weeks of vacuum storage at 4°C and 8°C (< 50 CFU/g) (Brillet et al., 2004) This has been credited to the class IIa bacteriocin, divercin V41 (Métivier et al., 1998) whose safety and acceptability standards were presented by (Brillet et al., 2005). (Brillet et al., 2005) did not record any spoiling capacity for *Carnobacterium sp.* and the microorganism was not shown to produce any off odour in cold smoked meat in the 14-day period. The tasting panel however recorded a slight off taste at very low levels in the samples with high *C. divergens* V41 although the samples did not contain significant bacterial counts. The sensory parameters are regarded as so low that it would probably be undetected by an untrained consumer palate (Brillet et al., 2005).

It could be possible that the other strains that did not produce zones did not have potential bacteriocins or that the potential indicators used were just not the right ones. A suitable strain may not reach the required density if the media and required conditions are not conducive. This could mean that bacteriocin is being produced in very minimal quantities resulting in failure in observing the activity. It is imperative to ensure that the growth conditions are optimal. Choice of indicator strain is also very critical in terms of significance. Moreover, given that different strains even within the same species can vary in terms of antimicrobial sensitivity, initial screening of multiple strains should be done to ascertain the best results and reduce chances of an antimicrobial being overlooked. The indicator strain should also have a high degree of sensitivity to detect activity in low concentrations (Twomey et al., 2021). These could be the possible reasons for lack of activity on many of the screened cultures and explain why out of a large starting bank of 1000 isolates, only 1% showed activity. The fact that a particular bacterium is from a different niche could also cause its potential antimicrobial activity to be missed (Pircalabioru et al., 2021).

Declarations

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Conflict of Interest

The authors declare existence of no conflict of interest whatsoever.

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