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Deploying Novel Forms of Nisin to Control Listeria Monocytogenes in Food Industry

Teboho Lekatsa*, Mothibe Kebitsamang Joseph, `Mamakase Sello

Department of Nutrition, Faculty of Health Sciences, National University of Lesotho, Roma 180, Lesotho.

***Corresponding author:** Phone: (+266) 6305 1430; E-mail: tk.leekatsa@nul.ls

Abstract: There is growing consumer awareness of the link between diet and health. Consumers are more concerned about the synthetic chemicals used as preservatives in food now-a-days, and there is resulting trend towards less processed food. These untreated foods can harbour dangerous pathogens which can multiply under refrigeration and without oxygen. Lantibiotics are post-translationally modified antimicrobial peptides, of which nisin A is the most extensively studied example. Nisin is a natural, toxicologically safe, antibacterial food preservative. Here we address this issue by assessing the ability of nisin A and nisin V to control Listeria monocytogenes EGDe in two commercially available milk products. The efficacy of the nisin peptides in chocolate milk resulted in significant reduction (over 1 log) in listerial numbers for nisin V after 24 hrs. In cottage cheese, after 48 and 72 hours no listerial cells were detected in the nisin V containing cottage cheese sample while low cell numbers (7 X 10^2) were detected in the nisin A samples This analysis revealed that nisin V was more effective than Nisin A with respect to being used as anti-Listeria food preservative. Further studies will be essential to find the optimum pH and sodium chloride conditions to control Listeria in particular target food products.

Keywords: Nisin, anti-microbial activity, *Listeria monocytogenes,* natural preservative, Lanibiotics

1. Introduction

Nisin (figure 1) is a low molecular weight antimicrobial protein produced by certain strains of *Lactococcus lactis* [1] . It is a member of a large group of bacterially produced peptides or bacteriocins that are active against other bacteria and to which the producer has a specific immunity mechanism. Production of bacteriocins by lactic acid bacteria (LAB) have been the focus of much attention as a result of their generally regarded as safe (GRAS) status, potential commercial applications and the high prevalence and pivotal roles that this group of b Though the LAB bacteriocins represent a heterogeneous group of molecules, two major groups have been defined; i.e. those that are modified to incorporate unusual residues including lanthionine and methyl lanthionine (class I or lantibiotics) and those that are unmodified (class II). Lantibiotics are produced by, and act mainly against Gram-positive bacteria, including nosocomial pathogens such as MRSA, *enterococci* and *Clostridium difficile*, but also food spoilage and pathogenic bacteria such as *Bacillus cereus, Staphylococcus aureus* and *Listeria monocytogenes* plays in food applications [2]. The consumption of food that has been formulated with chemical preservatives has heightened consumer concern and created a demand for more “natural” and “minimally processed” food. Hence, there has been a great interest in bacteriocins as potentially safe and natural food preservatives and additives. . While research into bacteriocins has been underway for decades, it is only recently that genetic engineering investigations have led to the discovery of improved lantibiotic peptides including nisin [3], mersacidin [4] and nukacin ISK-1[5]. Importantly, in the case of nisin, a number of derivatives were found to have increased activity against Gram-positive pathogens including *Listeria monocytogenes* and *Staphylococcus aureus* [3]. This is particularly important as *L. monocytogenes* is the causative agent of listeriosis, one of the most significant food borne diseases in industrialized countries. Understandably, the food manufacturing industry is very concerned with *L. monocytogenes* because of its potential for disease and its capacity to survive and grow in a wide variety of food substrates and enviromental conditions, including refrigeration.
Worryingly, increases in the incidence of Listiriosis were observerd in many EU member states in recent years [5]. Many of these is because *L. monocytogenes* can survive in stressful enviroments, including refrigeration temperatures, low pH and high salt concentrations [6].

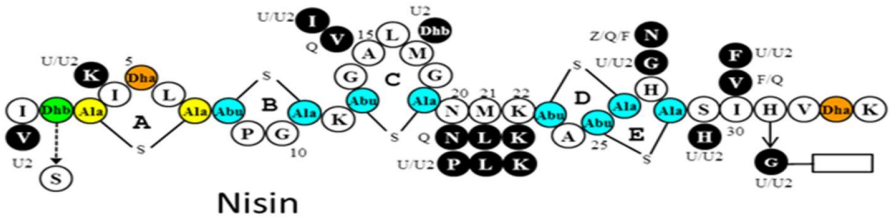

Figure 1. Structure of nisin A. Ala-S-Ala, Lanthionine; Abu-S-Ala, ß-methyllanthione; Dha, dehydroalanine; Dhb, dehydrobutyrine. (ß-methyl)lanthionine rings are labelled A-E. (Kuipers O.P et al. Biol. Chem. 1995; 270: 27299-27304).

2. Materials and Methods

Bacterial strains and growth conditions:

L. lactis strains were grown in M17 broth supplemented with 0.5% glucose (GM17) or GM17 agar at 30˚C. *S. aureus* and *Listeria* strains were grown in Brain Heart Infusion (BHI) or BHI agar at 37˚C. Antibiotics were used where indicated at the following concentrations: kanamycin 80µg ml^{-1}on *S. aureus* and chloramphenicol 2.25µg ml^{-1}, on *Listeria monocytogenes*.

Nisin Purification:

Lactobacilus lactis NZ9700 or the mutant Nisin strain of interest was sub cultured twice in GM17 broth at 1% at 30°C before use. Two litres of modified TY broth were inoculated with the culture at 0.5% and incubated at 30°C overnight. The culture was centrifuged at 7,000 rpm for 15 min. The cell pellet was resuspended in 300 ml of 70% isopropanol 0.1% TFA and stirred at room temperature for approximately 3h. The cell debris was removed by centrifugation at 7,000 rpm for 15 min and the supernatant retained. The isopropanol was evaporated using a rotary evaporator (Buchi) and the sample pH adjusted to 4 before applying to a 10g (60ml) Varian C-18 Bond Elute Column (Varian, Harbor City, CA) pre-equilibrated with methanol and water. The columns were washed with 100 ml of 20% ethanol and the inhibitory activity was eluted in 100 ml of 70% IPA 0.1% TFA. 15 ml aliquots were concentrated to 2 ml through 0µg ml^{-1}on *S. aureus* and chloramphenicol 2.25µg ml^{-1}, on *Listeria monocytogenes*.

3. Results and Discussion

Determination of Salt and pH limits of L. monocytogenes:

High levels of this bacteriocin have been shown to completely eliminate *L. monocytogenes* in soft cheeses within periods as short as 24 h [7]. Thus, to assess potential combinatorial effects using nisin V and nisin A in a food setting, initial investigations on the growth and survival of *L. monocytogenes* EGDe and *S. aureus* Xen 29 at different pH levels and salt concentrations were assessed. The bacteria were first plated on BHI agar at a range of different concentrations of salt (2, 4, 6, 8 and 10%). The maximum salt concentration that permitted *L. monocytogenes* growth after 24hr

Table 2. Results of agarose-based deferred antagonism assays against S. aureus Xen29 with purified nisin A and nisin V under various conditions

Chemical and Physical test parameters	Nisin WT (zone diameter in mm)	Nisin V (zone diameter in mm)	%WT
NaCl (%)			
0	11.58667 ±0.18	14.36333 ±0.14	124.1
2	7.37 ±0.38	10.29 ±0.03	139.6
4	7.066667 ±0.65	10.17333 ±0.53	144.1
6	9.876667 ±0.05	12.57333 ±0.24	127.3
Temp (˚C)			
4	N/G	N/G	
RT	13.47 ±0.35	14.83667 ±0.02	110.1
30	14.61667 ±003	14.67333 ±0.01	100.1
37	14.10667 ±0.14	14.47333 ±0.02	102.6
pH			
4.5	11.93 ±0.03	12.85333 ±0.24	107.7
7.5	12.73667 ±0.21	14.72333 ±0.22	115.6
8	13.03 ±0.22	14.95333 ±0.45	115.6
10	14.54667 ±0.49	15.62 ±0.14	107.4

Table 1. Effect of NaCl and pH on the growth of *L. monocytogenes* EGDe and *S. aureus* Xen 29

	NaCl concentration					pH		
	2%	4%	6%	8%	10%	4.5	7.5	10
L. monocytogenes EGDe	√	√	√	X	X	✓	✓	x
S. aureus Xen29	√	√	√	√	√	✓	✓	✓

incubation at 37˚C was 6% (Table 1). This is in agreement with previous studies that indicate higher a concentration of salts limits the growth of the bacteria [8].

Assessment of nisin V and nisin A in combination with NaCl or pH via agarose assays:

Results of deferred antagonism assays with *L. monocytogenes* EGDe across NaCl concentrations ranging from 0% to 6% indicated that nisin V exhibited its greatest inhibition (147.5%) when compared to nisin A at 2% sodium chloride (Table 2).

4. Conclusions

Listeria monocytogenes continues to be a concern to food industries because of its ability to grow at refrigeration temperatures. It is causative agent of Listeriosis, one of the significant food borne disease for humans. Thus, the novel approaches used to prevent the contamination of this pathogen in food is the use of bacteriocin-producing lactic acid bacteria. In this study we demonstrated that, with added hurdles it shows significant antimicrobial activity over nisin A with respect to controlling *Listeria monocytogenes*. In recent years, there has been a particular interest on the potency of nisin V against Gram-positive pathogens including *L. monocytogenes* [9,11]. In this study we demonstrated that, with added hurdles it shows significant antimicrobial activity over nisin A with respect to controlling *Listeria monocytogenes*. Further studies will be essential to find the optimum pH and sodium chloride conditions to control Listeria in particular target food products. Notably, these studies employed a high initial inoculum (1 x 10^6 cfu ml^{-1}) which is much higher than would be expected in a food processing plant (~20 cfu/g) and were carried out at room temperature, a temperature that would not be part of normal manufacturing processes.

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