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OPTIMIZATION OF FERMENTATION CONDITIONS FOR THE PRODUCTION OF BACTERIOCIN "FERMENTATE"

by
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14. ABSTRACT

This report summarizes research to evaluate novel techniques to improve preservation of and to incorporate those best approaches to improve quality and stability of combat operational rations. The objectives were to optimize the production of bacteriocins (BAC), i.e., nisin and pediocin, using various unusual food-grade substrates (e.g. whey, soy milk, peanut milk, etc) for production and incorporation into military ration items. BAC are an abundant and diverse group of ribosomally synthesized antimicrobial peptides produced by bacteria. They are generally recognized as "natural" compounds able to influence the safety and quality of foods. Of the several substrates evaluated, whey was the best producer of both nisin and pediocin. Whey + yeast extract was the best performing whey fermentation media. The nisin producer strain *Lactococcus. lactis* ssp. *lactis* was shown to be the most active BAC producer (i.e., 24,000-32,000 activity units/gm of dried fermentate when fermented on the whey media). *Pediococcus acidilactici* was found to be the most active producer of pediocin, also fermented on the whey media. This research also showed that nisin and pediocin fermentates clearly have promise as food preservatives; however, their effectiveness will depend on the food system in which they are used and the level of protection expected. Microbial fermentates tended to be more efficacious when used in conjunction with other food preservatives.

15. SUBJECT TERMS

SOY	QUALITY	SUBSTRATES	FOOD QUALITY	NATURAL PRODUCTS
WHEY	PEDIOCIN	FERMENTATE	FERMENTATION	FOOD PRESERVATION
NISIN	BACTERIA	FOOD SAFETY	CULTURE MEDIA	FERMENTATION BROTH
SAFETY	INHIBITION	BACTERIOCINS	LACTOBACILLUS	ANTIMICROBIAL AGENTS
PEPTIDES	INCUBATION	PEANUT MILK	MILITARY RATIONS	

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PREFACE

This report summarizes research conducted at the US Army Natick Soldier Research, Development and Engineering Center (NSRDEC), between October 2010 and November 2013 (under program element number 622786 and project numbers AH-98 and BB-12-42) to evaluate novel techniques to improve preservation of and to incorporate those best approaches to improve quality and stability of combat operational rations. The overall goals of the research were to (1) optimize the production of bacteriocins from food-grade media/substrate for incorporation into military ration items and (2) to define and document the parameters for optimized pilot-scale fermentation of bacteriocins.

The authors would like to thank Dr. Bibek Ray, University of Wyoming, for providing the strain of *Pediococcus acidilactici* that was used to produce pediocin; Mr. Ken Racicot, Performance Nutrition Team (PNT), Combat Feeding Directorate (CFD), NSRDEC, for assisting with the drafting of the original fermentation research proposal; and Ms. Michelle Richardson and Dr. Tom Yang, Food Engineering and Analysis Team (FEAT), NSRDEC, for providing an insightful and critical review of the manuscript.

OPTIMIZATION OF FERMENTATION CONDITIONS FOR THE PRODUCTION OF BACTERIOCIN "FERMENTATE"

1. INTRODUCTION

This report summarizes research to evaluate the potential for use of two natural bacteriocins (BAC), nisin and pediocin, in combat operational rations with the aim of improving preservation techniques of the rations. The research was performed from October 2010 to November 2013 by the Natick Soldier Research, Development, and Engineering Center (NSRDEC). The overall goals were (1) to optimize the production of BAC from food-grade media/substrate for incorporation into military ration items and (2) to define and document the parameters for optimized pilot-scale fermentation of BAC. The specific objectives were:

(1) Determine optimal food-grade media/substrate for the growth of nisin and pediocin.
(2) Determine the minimum inhibitory concentration (MIC) for nisin and pediocin using *Staphylococcus aureus* and *Listeria monocytogenes*;
(3) Determine the effect of nisin and pediocin fermentates on the final pH of growth medium.
(4) Use this data,to recommend potential application of BAC as a novel technique to improve preservation, quality and stability of operational rations.

Bacteriocins (BAC) are an abundant and diverse group of ribosomally synthesized antimicrobial peptides produced by bacteria and archaea (Dobson et al., 2012). They are produced by all major lineages of bacteria and archaea and constitute a heterogeneous group of peptides with respect to size, mode of action, antimicrobial potency, immunity mechanisms, and target cell receptors (Gillor et al., 2008).

The use of microorganisms and their natural products for the preservation of foods is referred to as biopreservation and has been a common practice for centuries (Ross et al., 2002). BAC produced by lactic acid bacteria (LAB) offer several desirable properties that make them suitable for food preservation: (1) they are generally recognized as safe (GRAS), (2) they are rendered inactive by digestive proteases, (3) they have little influence on gut microbiology, (4) they are pH and heat tolerant, and (5) they have a relatively broad antimicrobial spectrum against many food-borne pathogens and spoilage bacteria.

A review by Thomas et al. (2000) found that the application of BAC in food preservation can have several benefits, including extended shelf life of foods, decreased risk of food pathogens, reduced application of chemical preservatives, and better preservation of labile nutrients through less severe heat treatments.

Food scientists are continually evaluating novel techniques to improve food preservation and to incorporate those best approaches where appropriate to improve the quality and stability of operational rations. Also there is an ongoing attempt , where possible, to minimize the use of synthetic additives and expand the use of proven, naturally occurring BAC as preservation hurdles in combat ration components and to expand the current types of military unique intermediate moisture (IM) ration components that are being transitioned to the Meal, Ready-to-Eat™ (MRE™).

2. METHODS AND MATERIALS

Various fermentation organisms were acquired to grow the two BAC chosen for study, nisin and pediocin. They were acquired from the Northern Regional Research Lab (NRRL), US Department of Agriculture (USDA), Peoria, IL: the American Type Culture Collection (ATCC), Rockville, MD; and Dr. Bibek Ray, University of Wyoming, Department of Animal Science. Nisin-producing strains of *Lactococcus (L.) lactis* ssp. *lactis* (B-23802, B-2356, and B-4449) and pediocin-producing strains of *Pediococcus (P.) acidilactici* (B-23864) and *P. pentosaceus* (B-14009) were obtained from NRRL. Nisin-producing *L. lactis* ssp. *lactis* 11454 and pediocin-producing *P. pentosaceus* 43200 were obtained from ATCC. *Lactobacillus* (*L.) plantarum* NCDO 955, which was used as an indicator organism, and a strain of pediocin-producing *P. acidilactici* were obtained from Dr. Ray.

Preliminary assays were conducted to determine BAC production capability and activity of each strain. Further activity assays were conducted using the four strains that produced BAC. Preliminary (shake flask) bench fermentation tests were then conducted using various substrates to ferment the two nisin-producing strains and one of the two pediocin-producing strains. Once the best substrate was identified, the fermentation process was scaled up using the pediocin-producing strain and one of the nisin-producing strains. The MIC of those two fermentates were then determined in a model food system, as were the effects of those fermentates on the final pH of the growth media. The MIC of the nisin fermentate was also determined in real food.

2.1 BAC Activity Assay

All of the BAC producing bacteria were screened with the procedures described by Biswas et al. (1991) on trypticase [1%, Difco], glucose [1%, Sigma], and yeast extract [1%, Difco] (TGE) broth for their ability to produce BAC. In addition to TGE, the TGE broth consisted of Tween 80 (0.02%, Fisher Scientific), Mn^{2+} (0.033 mM, Fisher Scientific), and Mg^{2+} (0.02 mM, Fisher Scientific) (pH 6.5) (Biswas et al., 1991). The growth conditions for each strain were 20-24 h at 35 °C (Table 1).

Table 1. Bacterial strains and growth conditions

Strain	Bacteriocin produced	Growth medium	Growth conditions	Source
L. lactis* ssp. *lactis				
11454	nisin	TGE	35 °C, 20 h	ATCC
B-23802	nisin	TGE	35 °C, 20 h	NRRL
B-2356	nisin	TGE	35 °C, 20 h	NRRL
B-4449	nisin	TGE	35 °C, 20 h	NRRL
P. acidilactici				
B-23864	pediocin AcH	TGE	35 °C, 24 h	NRRL
	pediocin AcH	TGE	35 °C, 20 h	U. Wyoming
P. pentosaceus				
43200	pediocin AcH	TGE	35 °C, 20 h	ATCC
B-14009	pediocin AcH	TGE	35 °C, 20 h	NRRL
L. plantarum				
NCDO 955	None	TGE	30 °C, 24 h	U. Wyoming

In the subsequent assays using only the four positive producers, BAC activity was determined by methods described by Biswas et al. (1991) and Bhunia et al. (1991). Serial twofold dilutions of the culture (heat inactivated, 80 °C for 10 min) were made in sterile

deionized water. From each dilution, 5 µL was delivered to a de Man, Rogosa, and Sharp (MRS), Difco, soft agar overlay (0.75%) seeded with *L. plantarum* NCDO 955 (sensitive indicator strain). Assay plates were incubated at 37 ^{o}C for 18-24 h. One activity unit (AU) was defined as 5 µL of the highest dilution of heated inactivated culture that yielded a definite zone of growth inhibition on the indicator lawn. The titer was expressed as the reciprocal of the highest dilution showing inhibition (Gonzalez and Kunka, 1987; Pucci et al., 1988; Ray and Daeschel, 1992).

2.2 Bench Fermentation Experiments.

2.2.1 Substrate Shake Flask Evaluations. Several types of substrates were selected, prepared, and evaluated in shake flasks for efficacy of the selected bacteria to utilize different substrates for BAC production during fermentation based on their availability, inexpensive cost, ability to support lactic bacterial fermentation and BAC production, and potential to enhance ration variety. The substrates chosen were the commercial TGE broth used in the initial assays combined with the MRS used in the subsequent assays and several food-grade substrates: whey broth, whey media (whey + yeast extract), whey + basal salts media, soy protein isolate (SPI) + lactose, soymilk (legume), and peanut milk (legume). Testing involved placing 250 mL of each substrate in a 500 mL Erlenmyer flask, sterilized for 15 min at 121 ^{o}C, inoculated at 5% (v/v) with an 18-24 h culture of *P. acidilactici* or *L. lactis* ssp. *lactis*. Prior to use, *P. acidilactici* or *L. lactis* ssp. *lactis* was activated in food-grade growth media equivalent to MRS. After inoculation, broth cultures were incubated for 18-24 h at 30 ^{o}C for the *P. acidilactici*, and 35 ^{o}C for the *L. lactis* ssp. *lactis*,. After growth, both cultures were freeze dried. To test for activity after drying, appropriate amounts of the dried powder were weighed (e.g., 1 g), rehydrated in 9 mL of deionized water, and tested for BAC activity (previously described).

The whey broth.consisted of dehydrated whey powder (Hilmar 8200) that was rehydrated by suspending 10% (w/v) whey protein concentrate (WPC) in deionized H_2O. After mixing, pH was adjusted to 4.5 with 5 N HCl (Fisher Scientific) and heated at 121 ^{o}C for 15 min to denature the proteins. Denatured proteins were removed by filtration through double layers of cheese cloth or centrifugation (12,000 x g for 15 min). The supernatants were adjusted to pH 6.3 and sterilized at 121 ^{o}C for 15 min (Guerra et al., 2001).

The whey media consisted of 10% whey powder (Hilmar 8200) + 1% Yeast extract (Difco, pH 6.35) per L of deionized water.

The whey + basal salts media contained 25% (w/v) whey (Hilmar 8200), 0.2% (w/v) $CaCl_2$, 0.05% (w/v) KCl, 0.05% (w/v) $MgSO_4.7H_2O$, 0.5% (w/v) NH_4NO_3, 0.001% (w/v) $FeSO_4.7H_2O$, 0.001% (w/v) $MnSO_4.H_20$ in 1 L of deionized water. Upon reconstitution, 200 mL aliquots of the suspension were dispensed into glass bottles and autoclaved at 121 ^{o}C for 15 min. All salts listed above were purchased from Fisher Chemical Co.

The SPI + lactose broth was made by adding 40 g of SPI (Archer Daniels Midlands Co) and 20 g of lactose into 1L of deionized water. After rehydration, 200 mL aliquots were dispensed into glass bottles and autoclaved at 121^{o}C for 15 min. After allowing media to cool at room temperature, the pH was adjusted to 6.7 with filter sterilized 5 M sodium hydroxide (Ding and Shah, 2010).

The soymilk was prepared using a procedure described in “The Book of Tofu” by Shurtleff and Aoyagi (1975). One cup (8 oz) of soybeans (Archer Daniels Midland Co) was washed with tap water and drained three times, soaked in 2 qt of tap water for 10 h then rinsed and drained twice using 1 qt of tap water. Subsequently, beans were combined with 2 2/3 cups of tap water in a Waring Blender jar and pureed at high speed for 3 min. Soybean puree was

added to a cooking pot containing boiling water (1/2 cup) and continued to be heated while being constantly stirred with a wooden spoon to prevent sticking. When foam suddenly rose, the heat was turned off and the contents were poured into a pressing sack (cheese cloth). Then, the hot sack was placed in a colander and allowed to drain. The remaining soymilk was pressed using a glass jar. Finally, the expressed soymilk was poured into a cooking pot and cooked for 5-7 min over medium-high heat with constant stirring to prevent sticking. The resulting soymilk was sterilized at 121 ^{o}C for 15 min and reserved for fermentation substrate.The peanut milk was prepared according to the procedure outlined by Lee and Beuchat (1992). Raw peanuts (Fisher Chef's Naturals) were locally purchased, soaked in tap water (2:1, water: peanuts,) containing 1% (w/v) $NaHCO_3$ for 18 h at 21-23 ^{o}C. After draining, seeds were washed with fresh tap water, then combined with water (2:1, water: peanuts), placed in glass jars, sealed and steamed (100 ^{o}C) for 10 min. After draining and washing with tap water, peanuts were mixed with distilled water (5:1, water: peanuts), ground in a blender (Waring) and filtered through cheese cloth. The filtered peanut milk was homogenized, using a Kinematica Polytron Lab Homogenizer Mixer, Model PT 10/35, (Aaron Equipment Co., 735 E. Green St, Bensenville, IL). Peanut milk was then sterilized at 121 ^{o}C for 15 min and stored at 25 ^{o}C until used.

2.2.2 Scaled-up Experiments/BAC Diffusion Assays. Once the best substrate (whey + yeast extract) was identified, the fermentation process was scaled up. *P. acidilactici* and *L. lactis* ssp. *lactis* were prepared for fermentor production of pediocin and nisin fermentates, respectively, by activating cells in whey + yeast extract, a food-grade growth media equivalent to MRS (Sawatari et al., 2006). Fermentations were carried out in a 7.5 L BioFlo 110 Modular Benchtop Fermentor (New Brunswick Scientific Co., Edison, NJ). The whey + yeast extract media was prepared as previously described, and 4-5 L was dispensed in the fermentation vessel and sterilized (Tomy High-Pressure Steam Sterilizer, model ES-315, Tomy Seiko Co., LTD) at121 ^{o}C for 1.5 h. After cooling to ambient temperature, the media was inoculated at 1% (v/v) with the following operating parameters: 50 rpm agitation speed, 35^{o}C and pH 5.6 - 5.9. The fermentation process ended after 24 h incubation.

To enhance the production obtained, further fermentation efforts were performed using several substrate combinations to optimize the inoculum level, which was set at 1.0% (v/v). After fermentation, all BAC-laden broths, (i.e. MRS, TGE, whey + yeast extract, soymilk, peanut milk and peanut milk + soymilk + glucose) were heated with flowing steam (~100 ^{o}C for 20 min) to kill viable cells, freeze dried, and tested for BAC activity, using *L. plantarum* NCDO 955 as the BAC-sensitive indicator organism for both nisin and pediocin fermentates. The AU/gm of dried BAC-laden fermentate was determined by a well diffusion assay (Tagg and McGiven, 1971). One mL of an overnight grown sample of *L. plantarum* (TGE broth, 37 ^{o}C) was inoculated on 100 mL of sterile TGE agar, and 10-15 mL of the inoculated agar was placed on sterile petri plates (Biswas et al., 1991, and Bhunia et al., 1991). Plates were allowed to solidify for several hours under refrigeration. Wells (6 mm in diameter) were bored in the agar, and 20 µL of the reconstituted fermentate (1 g/10 mL) was dispensed into the wells. Plates were then incubated at 37 ^{o}C for 24 h, and the diameters of the inhibition zones ($\geq$ 2 mm) around the wells were measured with a caliper (Mitutoyo Corp., Minato-Tu, Tokyo, Japan).

2.3 MIC.

The MIC is the lowest concentration of BAC fermentate (antimicrobial) that will inhibit the visible growth of a microorganism after overnight incubation. It was necessary to establish this concentration to accurately assess the true antimicrobial potential and use of the nisin and

pediocin fermentates in food. The MICs for nisin and pediocin fermentates were determined using two strains each of *Staphylococcus (S.) aureus* and *Listeria (L.) monocytogenes*.

Dried fermentates were rehydrated and diluted in the model food system brain heart infusion broth (BHIB) to concentrations of 5, 10, 15, and 20% (w/v). The rehydrated BHIB containing the fermentates was inoculated with *S. aureus* (a mixed strain of 8095 and 27664) or *L. monocytogenes* (a mixed strain of 43258 and 1411) to give a cell concentration of 10^3-10^4 CFU/mL. Immediately after preparation of the BHIB suspensions, 200 µL samples (quadrupled) were dispensed into wells of a 100-well sterile microtiter plate, placed in a Bioscreen C turbidometer (Version 2.6; Oy Growth Curves AB Ltd., Helsinki, Finland) and incubated at 30 ^{o}C for 48-72 h. Optical density at 420-580 nm was measured, and readings for each well were taken every hour for 48-72 h. Growth was monitored using a Bioscreen instrument.

Dried nisin fermentate was also rehydrated and diluted in real food (i.e., sterile whole milk) and used to evaluate the antimicrobial effectiveness of nisin fermentates produced in the clean room by *L. lactis* ssp. *lactis.* Whole milk was treated with concentrations of 1%, 5%, and 10% of the dried fermentate and inoculated with 1000 to 10000 cfu /mL of a 1:1 mixture of *L. monocytogenes* strains1411 and 43258 and incubated at 30 ^{o}C.

2.3.1 Enumeration of *S. aureus* in BHIB. A mixed strain of *S. aureus* 8095 and 27664 was grown at 30 ^{o}C in BHIB with different concentrations of nisin fermentate (i.e.,1, 5, and 10% w/v), and 1 mL aliquots of the BHIB + fermentate solution were removed at 0, 3, 5, 15, 18, 21, and 24 h. The aliquots were serially diluted in 0.1% (w/v) peptone water (Difco). Subsequent serial dilutions were vortexed for 20-30 s before spread plating (0.1 mL aliquots) on brain heart infusion agar (BHIA). Plates were incubated aerobically at 30 ^{o}C for 24 h. All plates were done in duplicates.

2.3.2 Enumeration of *L. monocytogenes* in BHIB and Milk Containing Fermentate. The mixed strain of *L. monocytogenes* 1411 and 43258 was grown at 30^{o}C in whole milk (parmalat) with different concentrations of nisin fermentate (i.e., 0, 5, and 10%, w/v), and 1 mL aliquots of the BHIB + fermentate solution were removed at 0, 3, 5, 15, 18, 21, and 24 h. The aliquots were serially diluted in 0.1% (w/v) peptone water (Difco). Subsequent serial dilutions were vortexed for 20-30 s before spread plating (0.1 mL aliquots) on BHIA. Plates were incubated aerobically at 30^{o}C for 24-48 h. All plates were done in duplicates.

3. RESULTS AND DISCUSSION

Two positive nisin-producing strains of *L. lactis* ssp. *lactis* (B-23802 and11454**)** and two pediocin-producing strains of *Pediococcus* (*P. acidilactici* from U. Wyoming and *P. pentosaceus* 43200) were identified as positive producers during the initial assay of selected candidates.

Howewer, in the subsequent assays, the *P. pentosaceus* 43200 was shown not to be a reliable pediocin fermentate, since it did not produce BAC on any of the test substrates, e.g. MRS, TGE, whey. It was speculated that the organism had lost its ability to produce BAC due to the loss of its 13.6 megadalton (MDa) plasmid, which controlled BAC production. As a result of this problem, ATCC scientists were contacted. They reasoned that, in order for the strain to produce pediocin, a growing colony of *P. pentosaceus* was needed to demonstrate inhibition of the susceptible Gram positive organism (e.g., *Listeria* sp.). Using ATCC scientists' procedures, an experiment was performed that had previously demonstrated production of pediocin (Fleming et al. 1975). However, in the ATCC experiment, pediocin-production by *P. pentosaceus* was too limiting to be considered for use as an additive in ration preservation. Therefore, attempts to use *P. pentosaceus* as a pediocin producer were abandoned.

Thus, only three bacteria (the two nisin-producing strains of *L. lactis* ssp. *lactis* and the pediocin-producing strain of *P. acidilactici* from U. Wyoming) were evaluated for their ability to ferment different substrates and produce BAC in shake flask bench experiments. As shown in Table 2, the *L. lactis* ssp. *lactis* strain 11454 was clearly the better nisin fermentate producer. Activity was attained with strain 11454 when cultured in all six substrates, and high nisin activity, (i.e., 24-32 $x10^3$ AU/mL) was attained with four of the six substrates (i.e., the commercial TGE/MRS and the three whey-based media). In contrast, *L. lactis* ssp. *lactis* strain B-23802 demonstrated no ability to produce nisin on the food-grade substrates and only an activity range of 64-128 $x10^2$ units/mL on the commercial media TGE/MRS. The *P. acidilactici* demonstrated an ability to produce adequate levels of pediocin for only two of the six fermentation substrates: an average activity value of 12000 AU/mL for in the TGE a/MRS broth and only 1500 AU/mL in the whey + yeast extract media.

Table 2. Bacteriocin production using different substrates in shake flask experiments

Substrate	*L. lactis* ssp.*lactis* (nisin)		*P. acidilactici* (pediocin)
	B-23802 (AU/mL)	11454 (AU/mL)	U of WY (AU/mL)
TGE/MRS	6,400-12,800	24,000-32,000	8,000-16,000
Whey	NDA	24,000-32,000	NDA
Whey + Yeast Extract	NDA	24,000-32,000	1,000-2000
Whey + Basal Salts	NDA	24,000-32,000	NDA
SPI + Lactose	NDA	4,000-8,000	NDA
Soymilk	NDA	1,000-2,000	NDA

AU = The reciprocal of the highest dilution factor that shows inhibition of the indicator strain
NDA = No detectable AUs/mL

Because high levels of nisin were produced in the whey + yeast extract media and it was the only food-grade substrate that supported growth of pediocin in the shake flask tests, it was chosen as a basic fermentation medium for continued media optimization of nisin and pediocin in scaled-up fermentation tests using the fermentor, which were performed using 4-5 L of whey

+ yeast extract media at 35 ^{o}C with a pH 5.6-5.9 and 50 rpm agitation. Strain 11454 was chosen as the nisin producer, since it vastly outproduced the B-23802 in the shake flask tests.

Results from the *P. acidilactici* fermentation on whey media + yeast extract indicated that pediocin levels produced in the fermentor were comparable to the activity levels produced during shake flask bench experiments, i.e., $\leq 1.5 \times 10^3$ AU/mL. However, this activity level was low and would require larger quantities of the dried pediocin fermentate to reach an effective level to deliver enough pediocin to inhibit the targeted organism (pathogenic *L. monocytogenes* strains) in a food product. Similar to pediocin production, production of nisin by *L. lactis* ssp. *lactis* 11454 in whey media + yeast extract was as efficient (i.e., $\geq 2.4 \times 10^4$ AU/mL) in the fermentor as in the shake flask. Additionally, when the the whey protein concentrate was reduced by 50% (while the yeast extract was maintained at 1%), there was no observable difference in the activity level produced.

It was essential to demonstrate the feasibility of producing BAC-laden fermentates in designing the larger scale fermentor production effort. The AU/gm of dried BAC-laden fermentate (using the various BAC-laden broths MRS, TGE, whey + yeast extract, soymilk, peanut milk and peanut milk + soymilk + glucose) was determined by a well diffusion assay (Tagg and McGiven, 1971). The results from the BAC diffusion assay were the same as the results from the shake flask tests and initial fermentor tests: 24-32 x 10^3 AU/gm dried fermentate for *L. lactis* ssp. *lactis* and 1.0-2.0 x 10^3 AU/gm dried fermentate for *P. acidilactici* fermentate.

The MIC (i.e., the lowest concentration of BAC fermentate that will inhibit the visible growth of a microorganism after overnight incubation) was then determined to establish the antimicrobial effectiveness of the fermentates when used in food. The results obtained for the pediocin fermentate using the model food system BHIB were 10% (w/v) and 15% (w/v) for *L. monocytogenes* strains 1411 (pH 6.1) and 43258 (pH 5.9), respectively. The nisin fermentate MIC results obtained for *S. aureus* were slightly better; i.e., MIC 5% (w/v) and 10% (w/v) for strains 8095 (pH 5.4) and 27664 (pH 5.0), respectively. It was also observed that the concentration of the fermentates influenced both the pH and Aw of the BHIB. The pH of the pediocin fermentate (7.34-5.80) tended to be slightly higher than the nisin fermentate pH (7.34-4.75) (Figure 1). Similarly, the Aw of the pediocin fermentate tended to be higher than the nisin fermentate in response to increases in the fermentate concentration (Figure 2). As a result, certain problems might be anticipated with the application of the fermentate to a food matrix; for example, limited or restricted use may be encountered when used in different food matrices.

Thus, several concentrations of both fermentates were added to the BHIB containing the targeted pathogens (*L. monocytogenes* and *S. aureus*), and growth was monitored. The results for growth of *S. aureus* 27664 and 8095 are displayed in Figures 3 and 4, respectively, at different concentrations of fermentate and pH values. Nisin fermentate of 1400 AU/mL (5%) resulted in growth inhibition for 28-32 h at 30 ^{o}C, while 2800 AU/mL (10%) resulted in no growth during 72 h of incubation. For the *L. monocytogenes* strains, higher levels of pediocin fermentate were required to affect a similar level of inhibition. For example, between 10% (1200 AU/mL) and 15% (1800 AU/mL) of pediocin was necessary to effect 100% inhibition of strain 1411 after 24 h of incubation at 30 ^{o}C. *L. monocytogenes* 43258 was apparently more sensitive than 1411 to pediocin fermentate; 1200 AU/mL (10%, w/v) was sufficient to inhibit growth for 24 h at 30 ^{o}C (Figures 5 and 6). Both *L. monocytogenes* strains (1411 and 43258) in BHIB were inhibited at 5, 10, and 15% (w/v) levels of nisin fermentate (Figures 7 and 8). The antimicrobial effectiveness of pediocin against *S. aureus* was less evident (data not shown).

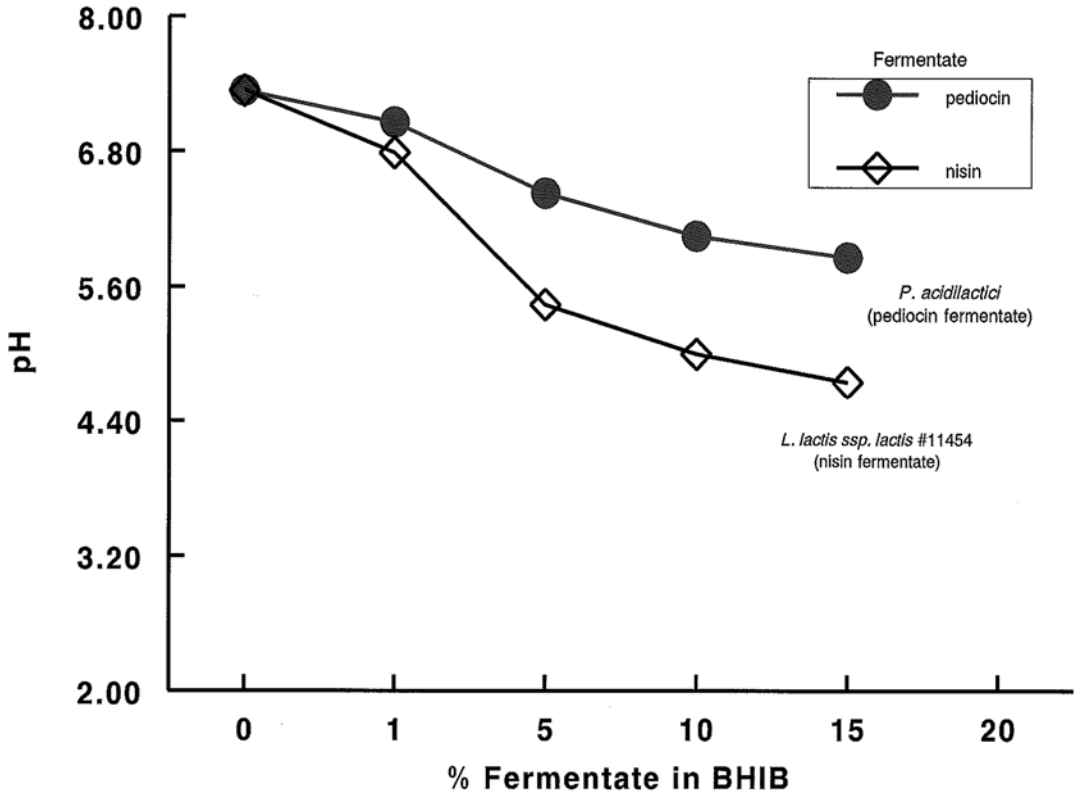

Figure 1. Comparison of influence of pediocin and nisin fermentates (%) on final pH of BHIB

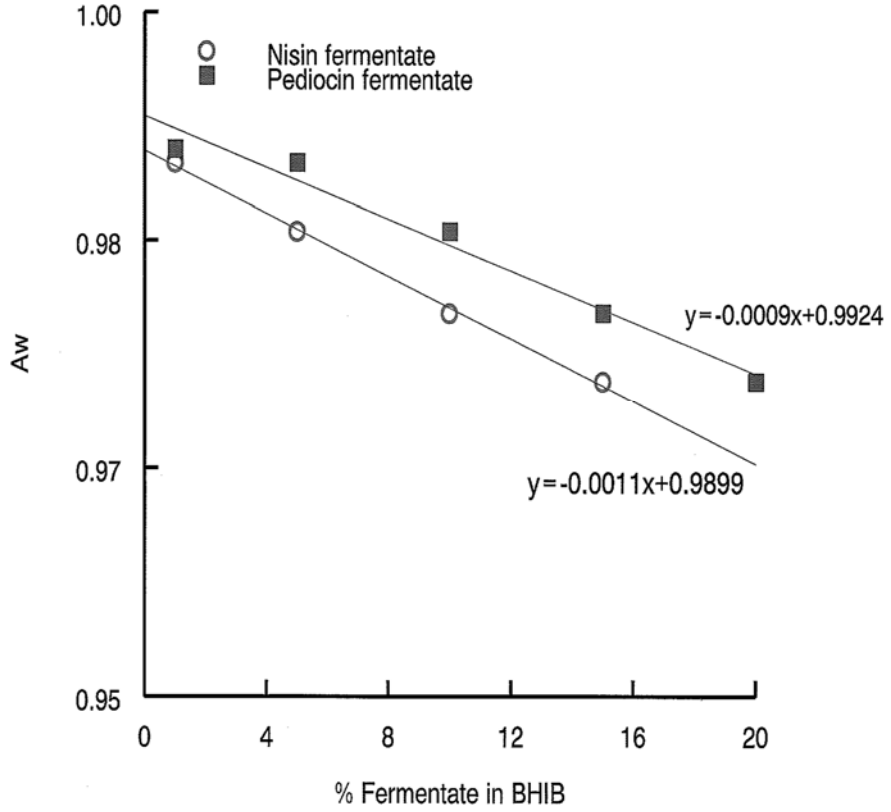

Figure 2. Comparison of influence of pediocin and nisin fermentates (%) on Aw in BHIB

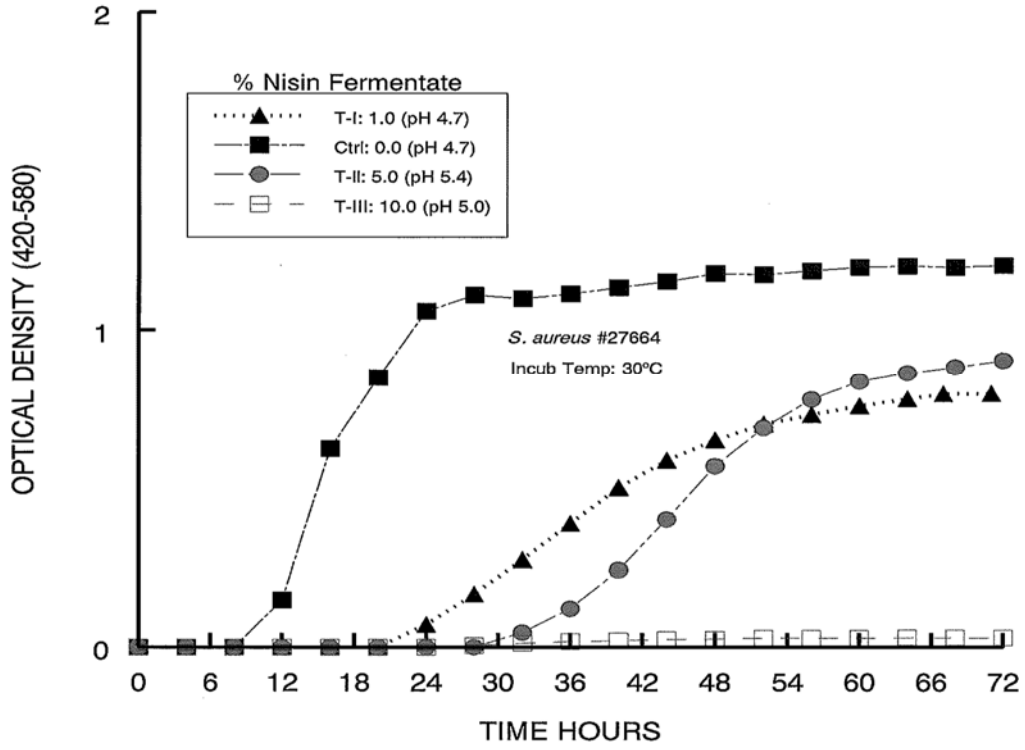

Figure 3. Turbidity of BHIB cultures inoculated with *S. aureus* 27664 plus nisin fermentate at different concentrations (%) and incubated at 30 °C and different pH values

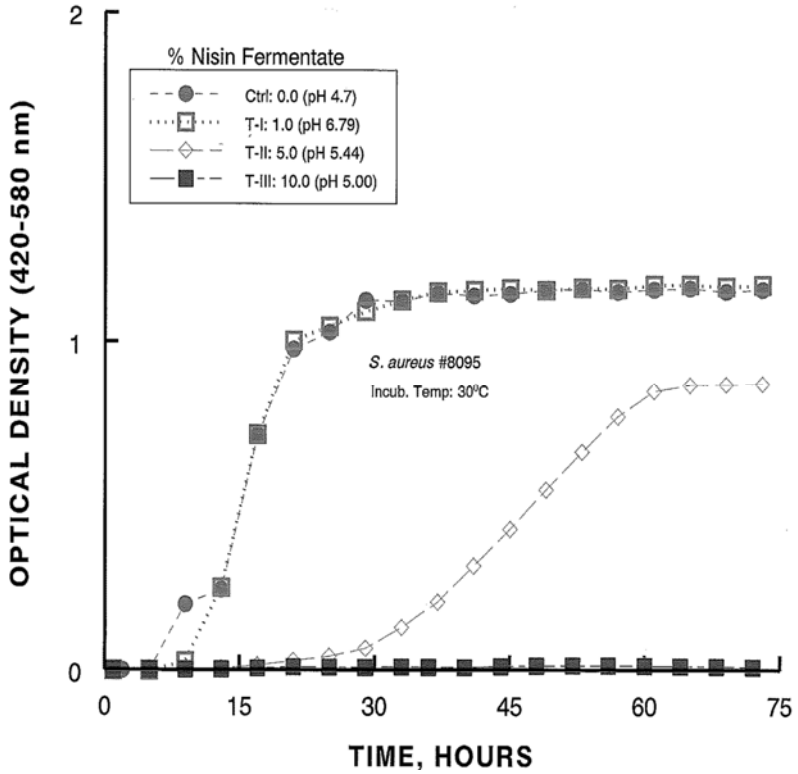

Figure 4. Turbidity of BHIB cultures inoculated with *S. aureus* 8095 plus nisin fermentate at different concentrations (%) and incubated at 30 °C and different pH values

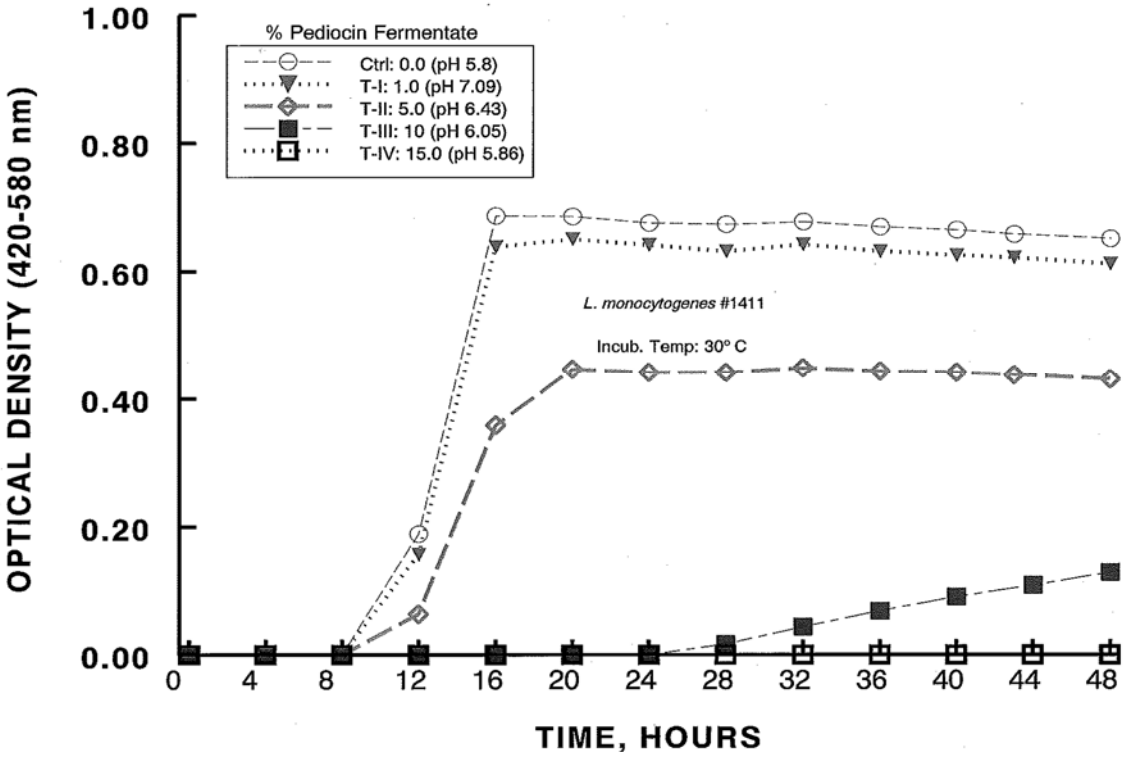

Figure 5. Turbidity of BHIB cultures inoculated with *L. monocytogenes* 1411 plus pediocin fermentate at different concentrations (%) and incubated at 30 °C and different pH values

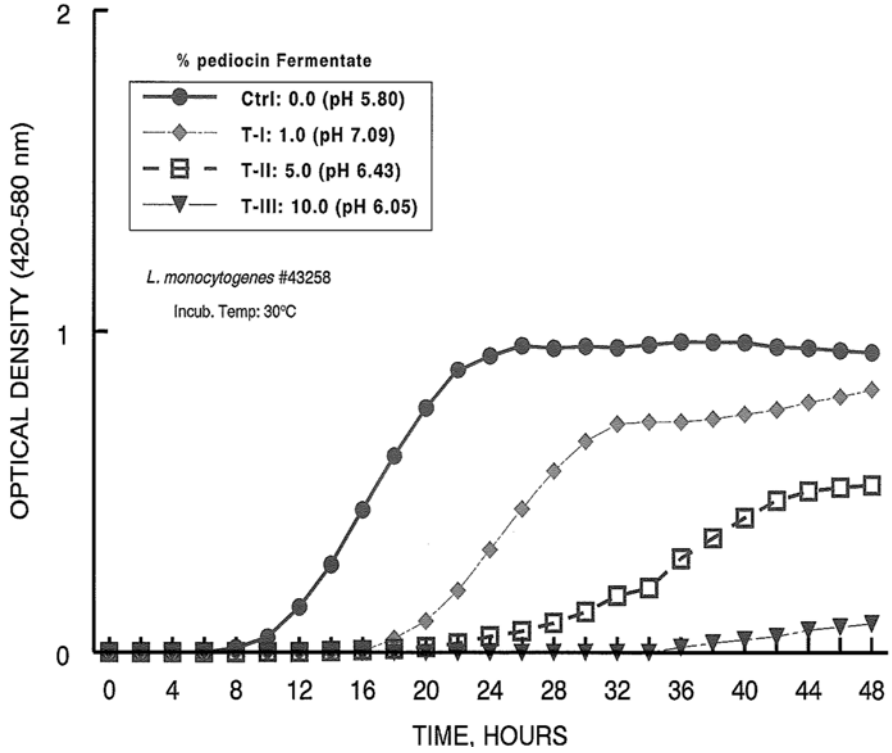

Figure 6. Turbidity of BHIB cultures inoculated with *L. monocytogenes* 43258 plus pediocin fermentate at different concentrations (%) and incubated at 30 °C and different pH values

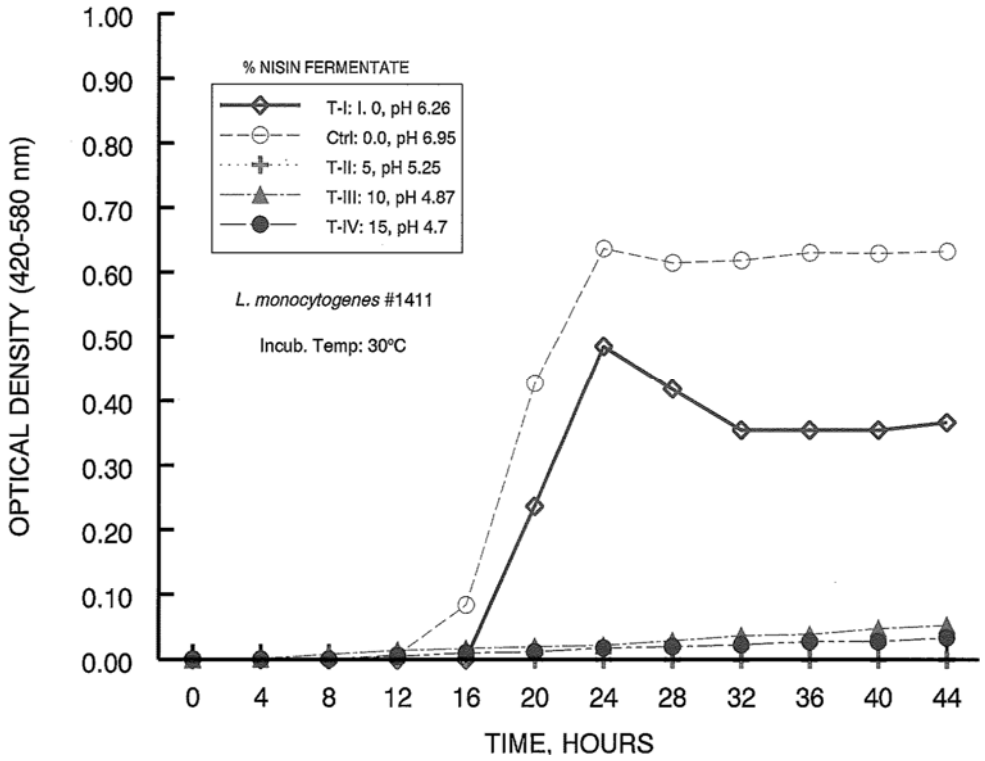

Figure 7. Turbidity of BHIB cultures inoculated with *L. monocytogenes* 1411 plus nisin fermentate at different concentrations (%) and incubated at 30 °C and different pH values

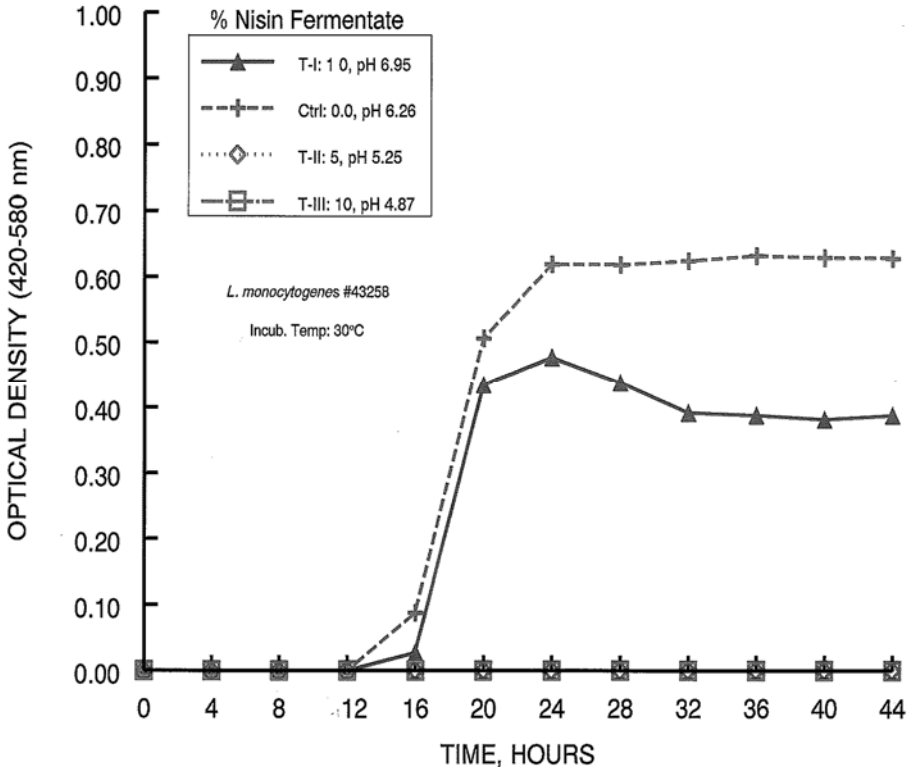

Figure 8. Turbidity of BHIB cultures inoculated with *L. monocytogenes* 43258 plus nisin fermentate at different concentrations (%) and incubated at 30 °C and different pH values

Results of the evaluation of the real food antimicrobial effectiveness of nisin, produced in the clean room and inoculated with 1000 to 10000 cfu /mL of a 1:1 mixture of strains 1411 and 43258 in sterile whole milk and incubated at 30 ºC, indicated that nisin fermentate concentrations greater than ≥ 5% inhibited growth of *L. monocytogenes* (Figure 9).

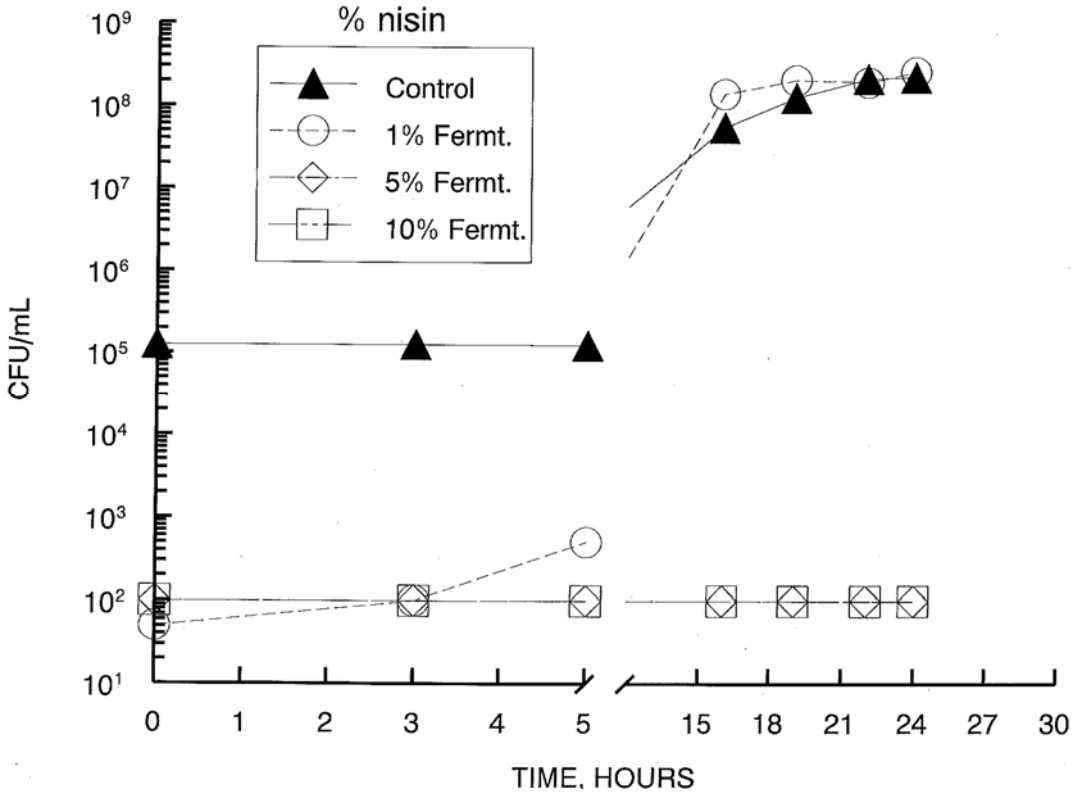

Figure 9. Growth of a 1:1 mixture of *L. monocytogenes* 1411 and 43258 in sterile whole milk containing 0, 1%, 5%, and 10% nisin fermentate

4. CONCLUSIONS

Results from the study described in this report demonstrate the potential for using the fermentation broths of two GRAS bacteria (i.e., *L. lactis* ssp. *Lactis* and *P. acidilactici*) to extend the keeping quality of food. Both fermentation broths were effective in limiting microbial activity in a model food system (BHIB), and the *L. lactis* ssp. *Lactis* broth was effective in limiting growth in a real food (sterile whole milk).

When nisin fermentate was evaluated in BHIB at an activity level of 1400 AU/mL (5% dried fermentate), *S. aureus* (strains 27664 and 8095) growth was inhibited for 28-32 h at 30 ^{o}C and pH 5.4. When evaluated at 2800 AU/mL (10% dried fermentate) in BHIB, nisin inhibited *S. aureus* for > 72 h at 30 ^{o}C and pH 5.

For *L. monocytogenes* (strains 1411 and 43258), higher concentrations of pediocin fermentate were required to effect a similar level of inhibition. For example, between 10% (1200 AU/mL) and 15% (1800 AU/mL) of dried fermentate was necessary to effect 100% inhibition of strain 1411 in BHIB (pH 5.9-6.0) for 24 h at 30 ^{o}C. Strain 43258 proved to be more sensitive to the pediocin fermentate than strain 1411. The addition of 1200 AU/mL of 10% dried pediocin blocked growth for 24 h at 30 ^{o}C and pH 6.05.

When nisin was evaluated in whole milk (1%, 5% and 10% dried fermentate), growth inhibition was observed at concentrations $\geq$ 5% at 30 ^{o}C.

Clearly, the two fermentates that were evaluated in this research show promise as food preservatives; however, their effectiveness will depend on the food system in which they are used and the level of protection expected. Microbial fermentates tended to be more efficacious when used in conjunction with other food preservatives.

This document reports research undertaken at the U.S. Army Natick Soldier Research, Development and Engineering Center, Natick, MA, and has been assigned No. NATICK/TR- 15/018 in a series of reports approved for publication.

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