TECHNICAL REPORT
NATICK/TR-14/020

AD _____

NISIN MIGRATION IN SHELF STABLE, TUNA-FILLED TORTILLAS DURING STORAGE

by
Anthony Sikes
Claire Lee
Michelle Richardson
and
Melvin Carter

September 2014

Final Report
October 2011 – November 2012

U.S. Army Natick Soldier Research, Development and Engineering Center
Natick, Massachusetts 01760-5000

DISCLAIMERS

The findings contained in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Citation of trade names in this report does not constitute an official endorsement or approval of the use of such items.

DESTRUCTION NOTICE

For Classified Documents:

Follow the procedures in DoD 5200.22-M, Industrial Security Manual, Section II-19 or DoD 5200.1-R, Information Security Program Regulation, Chapter IX.

For Unclassified/Limited Distribution Documents:

Destroy by any method that prevents disclosure of contents or reconstruction of the document.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
22-09-14	Final	October 2011 - November 2012

4. TITLE AND SUBTITLE

NISIN MIGRATION IN SHELF STABLE, TUNA-FILLED TORTILLAS DURING STORAGE

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Anthony Sikes, Claire Lee, Michelle Richardson and Melvin Carter

5d. PROJECT NUMBER
JSN-04-17

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

U.S. Army Natick Soldier Research, Development and Engineering Center
ATTN: RDNS-SEC-O
Kansas St., Natick, MA 01760-5000

8. PERFORMING ORGANIZATION REPORT NUMBER

NATICK/TR-14/020

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This report summarizes a 1-year study, completed in November 2012, of the effectiveness of nisin as a bacteriocin in tuna-filled tortillas during storage, as part of the Next Generation Hurdle Technologies (NGHT) Program. This Department of Defense, Combat Feeding Directorate Program provides not only the foundation for the development of innovative intermediate moisture (IM), eat-on-the-move ration components, but also an opportunity to improve existing IM ration components, which rely on controlling both the product's water activity (a_w) and pH. The NGHT Program lowers these boundaries, i.e., increases pH and a_w, of existing hurdles, using food-grade bioactive compounds to yield a higher quality ration component without compromising food safety. Ham, chicken, and tuna spread wraps were formulated with nisin because it is generally regarded as safe and as the most effective bacteriocin with possible use at lower concentrations when used in conjunction with other bacteriocins. However, nisin is only added to the tuna filling, not to the wrap. Thus there is concern that the nisin may migrate from the filling to a tortilla wrap during storage, thereby diluting bacteriocin concentration in the tuna, reducing the total bacteriocin activity and increasing the possibility of microbial/pathogen growth. The purpose of this study was to determine if nisin migrates from the tuna filling to the wrap during storage at 25 ^{o}C.

15. SUBJECT TERMS

NISIN	STABILITY	BACTERIOCINS	MICROORGANISMS
TUNA	BACTERIA	FOOD SAFETY	MICROBIAL CONTROL
WATER	PH FACTOR	TEMPERATURE	FOOD PRESERVATION
SAFETY	MOISTURE	FOOD STORAGE	HURDLE TECHNOLOGY
RATIONS	SHELF LIFE	ACCEPTABILITY	MICROCOCCUS LUTEUS
STORAGE	INHIBITION	MILITARY RATIONS	INTERMEDIATE MOISTURE FOODS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Claire Lee
U	U	U	SAR	20	**19b. TELEPHONE NUMBER** *(include area code)* 508-233-4696

This page intentionally left blank

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

PREFACE

This report summarizes research conducted at the U.S. Army Natick Soldier Research, Development and Engineering Center (NSRDEC), between October 2011 and November 2012 to explore/incorporate new bioactive hurdle technologies that will improve the quality and safety of intermediate moisture military ration components. The work effort was completed under JSN 04-17, the Next Generation Hurdle Technologies (NGHT), Department of Defense (DoD), Combat Feeding Directorate (CFD) Program.

THIS PAGE INTENTIONALLY LEFT BLANK

NISIN MIGRATION IN SHELF STABLE, TUNA-FILLED TORTILLAS DURING STORAGE

1. INTRODUCTION

This report summarizes a 1-year study completed in November 2012 at the Natick Soldier Research, Development and Engineering Center (NSRDEC) as part of the Department of Defense (DoD), Combat Feeding Directorate (CFD), Next Generation Hurdle Technologies (NGHT) Program. The purpose of the study was to determine if nisin, an important bacteriocin in intermediate moisture (IM) military ration foods, migrates from the tuna filling in tuna tortillas to the tortilla wrap during storage at 25 °C, thereby diluting its concentration in the tuna, reducing the total bacteriocin activity, and increasing the possibility for microbial/pathogen growth.

Warfighters are highly mobile, have very little time to stop and prepare food, and consume it in theater. Because some IM foods can directly feed a faster, lighter mobilized military, they are an improvement for military field feeding, as summarized below:

(1) They have an extended shelf life, i.e., they are microbiologically stable and do not require refrigeration.

(2) They are ready to eat, i.e., they require very little or no preparation.

(3) They are low in moisture, giving a reduced weight and volume.

(4) They are pliable, allowing them to be molded for packaging, transportation, and storage.

The military has been exploiting IM technology for over 40 years (Taoukis, P.S, and Richardson, M. 2007; Richardson, M., 2012). IM food technology and variety have been expanded by developing innovative shelf-stable bakery (Leung, 1986 and Taub, 2003), meat (Pavey, 1972; Powers, 1981; Yang, 1997 and Richardson, 1995), seafood (Dymsza, 1979), eggs (Richardson, 2008 and Peaco, 2010), and multi-component ration items (Powers, 1999, Peaco 2010, and Feeherry 2009).

The NGHT Program provides not only the foundation for the development of innovative IM, eat-on-the-move ration components, but also an opportunity to improve existing IM ration components. Sustainability is supported by providing a mechanism to develop more IM entrée items, expanding component variety, and ultimately increasing menu options. The current IM components rely on controlling both the product's water activity (a_w) and pH. The existing shelf stable sandwiches were developed to have a low pH (4.6-4.8) and an intermediate a_w (0.84-0.88) to ensure microbial stability.

Existing hurdles used to develop the IM components include:

(1) Water activity, which is controlled by incorporating various humectants such as salt, glycerol, and rice syrup into the formulation.

(2) pH, which is controlled by choosing foods that are naturally acidic or by incorporating food grade acids (sorbic, ascorbic, citric, acetic).

(3) Incorporation of Preservatives, such as nitrites and antioxidants.

(4) Thermal pasteurization by baking.

(5) Appropriate Packaging, such as the use of high barrier films.

(6)) Incorporation of Oxygen scavenging sachets.

The NGHT Program pushes these boundaries, pH and a_w, of existing hurdles by adding food-grade bioactive compounds to yield a higher quality ration component without compromising food safety. This program embodies the hurdle approach for development of safe, stable and highly acceptable food products.

Under the NGHT effort, ham, chicken, and tuna spread filled tortillas were formulated with nisin and evaluated for microbiological stability and then sensory acceptability. Nisin is generally regarded as safe (GRAS) and produced by certain strains of *Lactococcus lactis* subsp. lactis (Cheigh, 2005 and Liu, 2005). Nisin inhibits cells by forming pores in the bacterial membrane (Breukink, 1999). Nisin has demonstrated antimicrobial activity against a wide range of spores and Gram-positive bacteria, including *Staphylococcus*, *Clostridium*, *Bacillus* and *Listeria* (Abdollahzadeh, 2013; Muller, 2008; Pinto, 2011; Rajkovic, 2005; Richardson, 2011; Wijnker 2011).

Previous studies showed nisin was the most effective of the bacteriocins evaluated with possible use at lower concentrations when used in conjunction with other bacteriocins. From a previous inoculated pack study, it was determined that the tortilla weight has to be accounted for when incorporating nisin into multi-component items (M. Carter, NSRDEC. Personal communications, 2012). Because nisin is only added to the filling, and not to the wrap, the bacteriocin concentration may become diluted due to migration from the filling to the tortilla wrap.

2. **MATERIALS AND METHODS**

2.1 BACTERIAL STRAINS, MEDIA, AND BUFFER

The nisin-sensitive test organism, *Micrococcus luteus* ATCC® 10240, was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). The procedure outlined by ATCC (Gherna, Nieman and Pienta, 1985) was used to prepare and activate the culture for the nisin assay. *M. luteus* was cultured in tryptic soy agar (TSA, Difco) or tryptic soy broth (TSB, Difco). Working cultures of *M. luteus* were maintained on slants of TSA at 4 ^{o}C.

Media used included TSA (Difco) supplemented with 0.5 percent yeast extract (Difco), TSB-YE; Tryptic soy broth (TSB, Difco); and 0.1 percent sterile peptone water (Difco). The diluent used for the test organism was Butterfield's Phosphate Buffer pH 7.2.

The nisin concentration was determined by following modified methods of Tramer and Fowler, 1964. The standard assay medium (SAM) for the well diffusion assay contained the following components (g/L): 10 g bacteriological peptone (Difco), 3 g beef extract (BBL), 3 g sodium chloride (Mallinckrodt), 1.5 g yeast extract (Difco), 1 g brown sugar, 10 g bacto agar (Difco), and 1% tween 20 (Sigma Chemicals, St. Louis, Mo). All ingredients were suspended and dissolved in boiling deionized water and then autoclaved at 121 °C for 15 min.

2.2 INOCULUM PREPARATION FOR NISIN BIOASSAY

After activation on TSA slants, a loop full of *M. luteus* was inoculated into TSB (10 mL) and incubated at 37 ^{o}C for 24 h. From this culture, 0.1 mL was spread on SAM agar plates and incubated overnight. Subsequently, the growth was washed off several SAM plates with sterile half-strength Ringer's solution (10 mL). Cells were pooled and centrifuged (1000 x g) for 10 min. The supernatant was decanted, and the pellet was re-suspended in an equal volume (10 mL) of Ringer's solution. Finally, cell density was adjusted (half-strength Ringer's solution) to approximately $10^8 – 10^9$ colony forming units (CFU)/mL, using previously prepared standard growth curves (optical density$_{600mm}$ vs Log$_{10}$ cell numbers). SAM assay plates were prepared by melting and cooling agar at 45 ^{o}C and seeding with *M. luteus* to a final approximate concentration of 10^7 cfu/mL. 20 mL of seeded agar was dispensed into sterile disposable Petri dishes with grids (100 x 100 x 15 mm, Fisher Co.). The assay plates were refrigerated (4 ^{o}C) for 2-4 h to allow thorough solidification. Four to six test wells were cut (6-mm core borer) per SAM assay plate (G.G. Fowler, et al., 1975 and Pongtharangkul and Demirei, 2004). Using a glass tube (~6 mm), agar plugs were removed from the wells with slight suction.

A stock solution of nisin was prepared by dissolving 100 mg of commercial nisin (10^6 international units/g or mL^{-1}; Sigma Chemical Co., St. Louis, MO.) in 80 mL of 0.02 N HCl and heated in a boiling water bath at 98 ^{o}C $\pm$ 5 ^{o}C) for 5 min. The solution was rapidly cooled to 20 ^{o}C, allowed to stand for 2 h, and then diluted with 100 mL 0.02 N HCl. The solution contained 1000 IU nisin/mL^{-1}. For the assay, the nisin standard solution was diluted to the desired levels (1.25, 2.5, 5, 10, and 20 IU/mL^{-1}) using Extract C.

New standards were prepared each time an assay was performed (G.G. Fowler, et al., 1975). Dilutions (i.e., 1/3, 1/7 and 1/15) of Extracts A, AX and B, respectively, were prepared using Extract C. The assay plates were incubated for 18-20 h at 35 ^{o}C and the diameters of zones of

inhibition were measured in triplicate with a Digimatic caliper (Mitutoyo Corporation, Tokyo, Japan). When the log of the nisin concentrations was plotted against the linear zone diameters, a standard curve was obtained (Figure 1). It was then used to calculate the concentrations of the unknowns from the regression equation of the straight line (Figure 1). Results were expressed as IU/mL of extract.

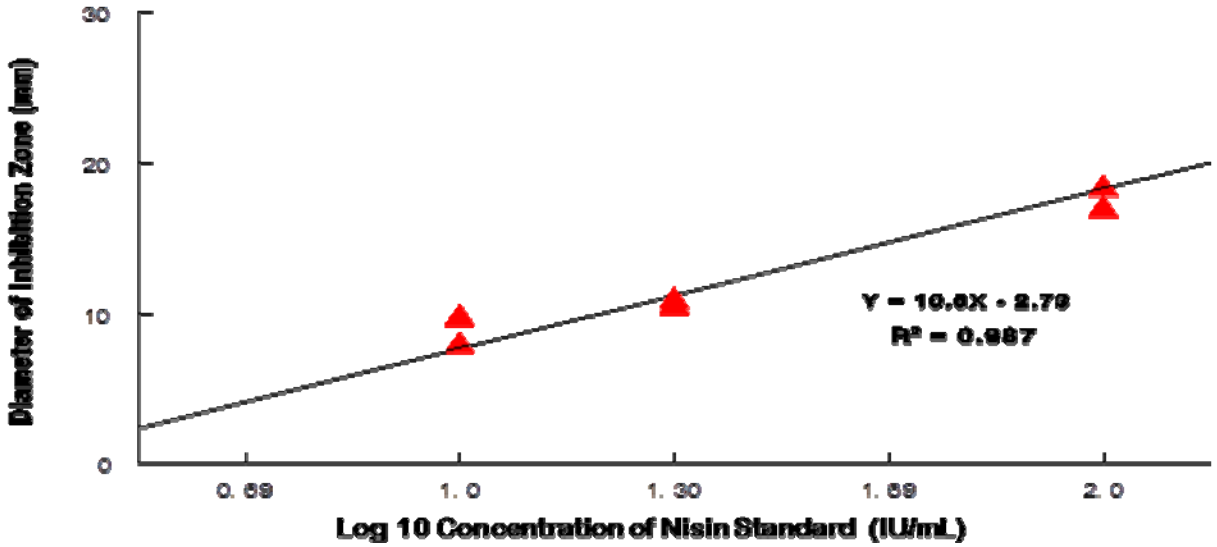


Figure 1. Zones of *M. luteus* inhibition produced by different concentrations of nisin in 0.02N HCl acid

2.3 INGREDIENTS AND PROCESSING

The ingredients used in the tuna filling formulation were: solid white tuna in water, Bumble Bee Foods, LLC (San Diego, CA); mayonnaise, Kraft Foods Group Inc. (Northfield, IL); sweet pickle relish, Pinnacle Foods (Peoria, IL); glycerol, KIC Chemicals, (Amonk, NY); Dijon mustard, Kraft Foods Group Inc. (Northfield, IL); tuna flavor, 19555SD, David Michael & Co (Philadelphia, PA); salt, Morton Salt Inc., (Chicago, IL); onion powder and ground white pepper, McCormick (Sparks, MD); cultured sugar, PuraQ® VERDAD® RV75 Purac America (Lincolnshire, IL); sodium acid sulfate (SAS), pHase™, Jones-Hamilton Co. (Walbridge, OH); nisin, Nisaplin (Danisco Madison, WI); dehydrated celery and green and white onions, Anthony Silva International, (Momence, IL); and tortillas (PCR-T-008, tortillas, packaged in a flexible pouch, shelf stable). The percentages used in each of the filling ingredients are listed in Table 1.

Samples were commercially produced by Peppercorn Food Service (Boston, MA). Liquid ingredients (mayonnaise, relish, glycerol Dijon mustard, and Verdad) were thoroughly mixed together to form a paste. The dry ingredients (salt, onion powder, white pepper, Phase™ SAS, and nisin) were blended together. The dehydrated celery, green onions, and white onions were blended. Free liquid was removed from the tuna using a sieve. The tuna was broken into ¼-½ inch pieces using a Hobart mixer with a paddle attachment on medium speed. The paste pre-blend was added and mixed on low speed until the mix was uniform. The dry ingredient pre-

blend was then added until the mix was uniform. The dehydrated vegetable mixture was added and mixed for approximately one min.

The wraps consisted of 40 g of tuna filling (F) and a 33 g, 6-inch tortilla (W). The tuna filling was spread evenly over the tortilla surface. The ends were folded in and the filled tortilla was then rolled and placed with the rolled end down on a baking pan lined with parchment paper. The tuna wraps were baked at 275 °F-300 °F to an internal temperature of 185 °F-195 °F. They were cooled between 100 °F and 120 °F, and two filled tortillas were then packaged with 100 cc oxygen scavengers (Multisorb, Albany, NY) in pre-formed tri-laminated pouches.

Table 1 Tuna filling formulation

Ingredient	Percent
Solid White Tuna	60.00
Mayonnaise	24.31
Sweet Pickle Relish	5.50
Glycerol	4.00
Dijon Mustard	1.75
Tuna Flavor	1.00
Salt	0.85
Onion Powder	0.75
Cultured Sugar	0.50
Ground White Pepper	0.50
Dehydrated Celery	0.30
Dehydrated Green and White Onion	0.30
Sodium Acid Sulphate	0.20
Nisin	0.04
TOTAL	**100**

2.4 TUNA WRAP STORAGE

The tuna wraps were stored for 6 months at 25 °C and withdrawn for nisin extraction in triplicate at 0, 2, 4, and 6 months.

2.5 PREPARATION OF TUNA WRAP EXTRACTS

Three pouches of tuna filled tortilla wraps were removed from storage (25 ^{o}C) on the designated pull days and brought to the lab for nisin extraction analysis. The pouches were opened, and the filling and tortilla wrap were separated. A composite of both the tortilla wrap and tuna fillings was prepared from the three pouches. To prepare the samples (tuna and wraps) for the nisin extraction, 40 g of each composite sample was weighed out in a 500 mL beaker or stomacher bag and dispersed in 0.02N HCl (160 mL). The subsequent procedures used to obtain sample extracts followed the methods described by Fowler, Jarvis, and Tramer (1975). The extraction procedures resulted in four extracts each from the wrap and the filling: A, AX, B, and C. Extract A was the filtrate that results from the initial 0.02 N HCl extraction procedure. Extract AX was derived by heating 20 mL of Extract A in a boiling water bath for 5 min. Extract B was obtained

through adjustment of 60 mL of Extract A to pH 11.0 with 5N NaOH, heated for 30 min to 60 ^{o}C and cooled to 20 ^{o}C before re-acidifying with 5N HCl to pH 2.0. Finally, Extract C was formed by adding 120 mL of 0.02 N HCl to 40 mL of Extract B. Extract C was used as a nisin free diluent for both test and standard samples. Figure 2 is a diagram of the nisin extraction procedure.

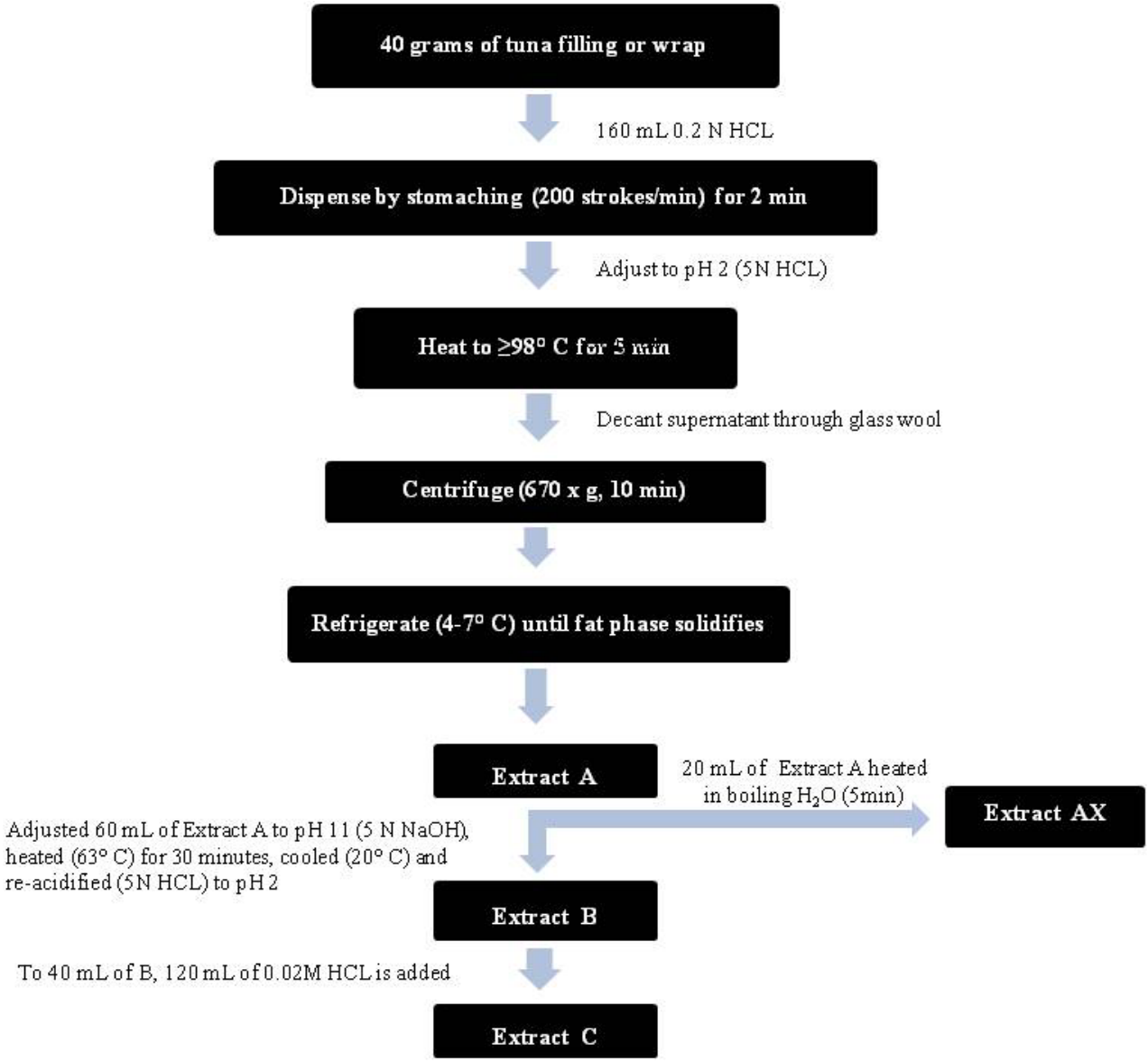


Figure 2. Extraction procedures for tuna filling and tortilla wrap

2.6 WATER ACTIVITY (a_w) AND pH ANALYSIS

Water activity (a_w) measurements were obtained using an Aqua Lab Model 3TE (Decagon Devices Inc., Pullman, WA). Plastic disposable cups were half filled with either ground tuna or ground tortilla wrap. The cups were then inserted into the instrument for a_w measurements at 25 °C.

The pH measurements of the tuna filling and ground tortilla wrap were mixed with distilled deionized water (1:1 w/v) at 25 °C. An Orion 3 Star pH meter (Thermo, Electron Corp., Beverly, MA) with a combined flat surface pH electrode (Thermo Scientific) was used to measure the pH of the samples.

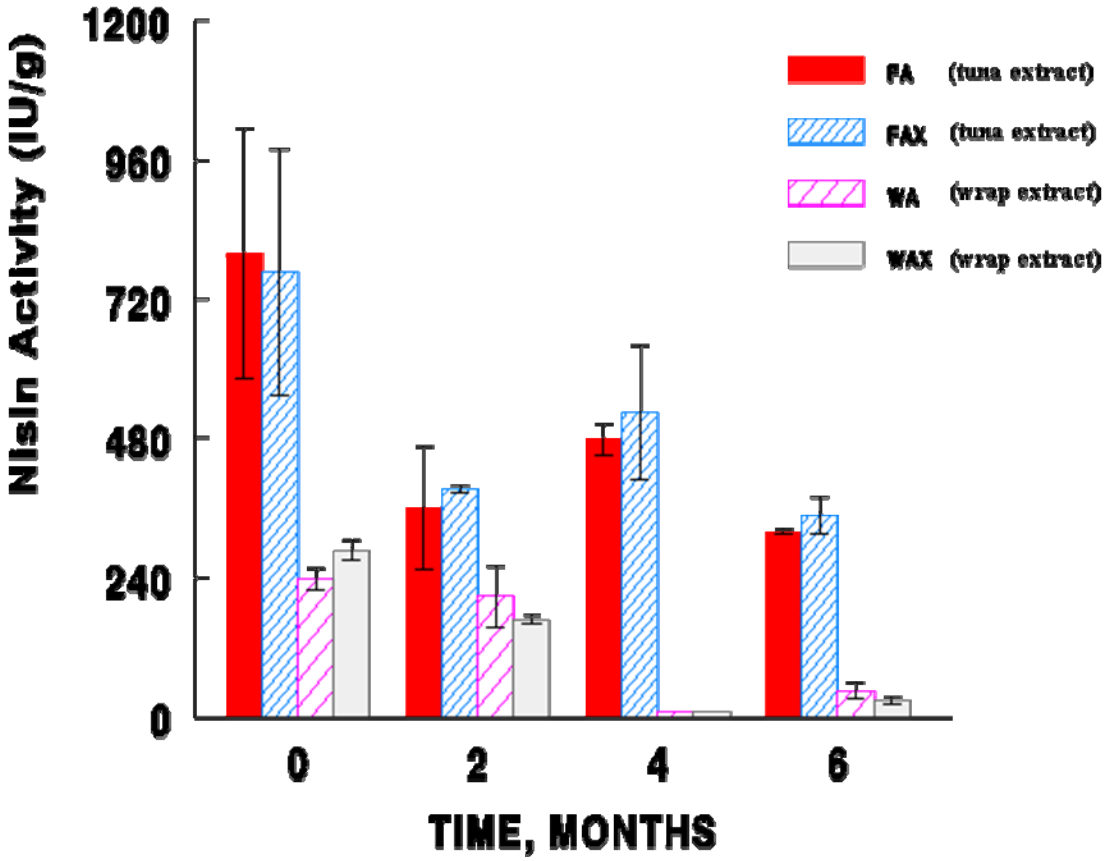
3. **RESULTS AND DISCUSSION**

The initial pH of the tuna filling was 5.00 and the wrap 5.17. The initial a_w of the tuna filling and wrap were 0.931 and 0.864, respectively, while the a_w of the wrap at the filling-wrap interface was 0.927. Because of the major differences in the initial a_w of the components, it is obvious that moisture migration did not take place prior to the analysis and storage of the tuna wraps.

Since nisin is stable to heat at pH 2, identical zone diameters should be obtained. When the dilutions of tuna extract FA and FAX were compared, very little difference was observed between the two initially or after the 6 months of storage at 25 ^{o}C (Figure 3). The initial nisin activity for Extract FA and FAX was 799 and 765 IU/g, respectively. After 6 months of storage at 25 ^{o}C, the nisin levels in Extract FA decreased to 319 IU/g (a 60% reduction) and Extract FAX to 348 IU/g (a 55% reduction) as shown in Figure 3. Further, it was observed that when $\log_{10}$ of the nisin concentration of FA and FAX was plotted against the zone diameter, a straight line relationship resulted (not shown). This was expected, since the zone diameters of the extracts (FA and FAX) were similar (Fowler et al., 1975).

Relative to the filling extracts, low nisin activity was found in the wrap extracts (WA and WAX). Initial nisin activity levels for WA and WAX were 238 and 287 IU/g, respectively. During the subsequent storage for 6 months, nisin levels in the wraps never exceeded the initial levels, but decreased to a final level of 45 (WA) and 29 (WAX) IU/g. Since nisin was only added to the filling, it is assumed that the small amount of nisin activity found in the wraps at time zero (WA: 238 IU/g and WAX: 287 IU/g) could be due to the residual nisin at the filling-wrap interface (the common boundary where the filling and wrap touch) or limited migration that took place during baking. Similar to the filling, the nisin activity in the wraps also decreased during storage (at 25 ^{o}C; WA: an 81% reduction; and WAX: a 90% reduction). The decreased activity in both the filling and tortilla was expected. According to Aasen (2003) nisin is ultimately exhausted from the foods due to its interactions with the food components. Houlihan (2007) reported that both calcium and magnesium decreased nisin activity. Jung (1992) used a biological activity assay that indicated that initial nisin activity decreased by 33 to 88% when it was added to milk with various fat concentrations.

If active nisin migration were occurring between the filling and wrap during storage (at 25 ^{o}C), nisin levels would be higher especially after 2 months of storage at 25 ^{o}C. However, the nisin levels observed in the wraps (WA and WAX) after 2 months decreased from an initial 238 IU/g to 207 IU/g for WA and from 287 IU/g to 167 IU/g for WAX. During the next 4 months of storage (25 ^{o}C), WA and WAX nisin levels continued to decrease to a final level (after 6 months) of 45 and 29 IU/g, respectively. If active nisin migration were occurring in the samples during storage at 25 ^{o}C, the nisin activity among the two samples (filling and wrap) would show less disparity during and after storage at 25 ^{o}C.



Error bars represent standard deviations of the means of data from triplicate samples.

Figure 3.The assay of nisin against *Micrococcus luteus* ATCC 10240 in tuna enrobed in a tortilla wrap and stored at 25 ^{o}C.

4. **CONCLUSIONS**

The purpose of this study was to determine the likelihood that nisin will migrate from nisin-loaded tuna filling (400 ppm) to a tortilla wrap using a biological assay procedure to measure nisin levels. The significance of nisin bleeding/migration from the filling to wrap is that it could reduce nisin's overall antimicrobial effectiveness and thereby increase the possibility of microbial/pathogen growth during storage. When nisin levels were measured initially and during storage, results showed a decrease in activity levels and no indication of migration. This was indicated by the small quantities of nisin initially found in the wraps and the decrease in activity over 6 months of storage at 25 ^{o}C.

The results of this study are positive but further studies will have to be done to determine if nisin migration holds true to the variety of tortilla wrapped products that may be developed and incorporated into military rations. The efficacy and activity of nisin in a food product may be affected by many factors, such as the composition of the food matrix, pH, binding of nisin by fat or protein from the food matrix, temperature, and other variables that may come into play in the food product (Tramer and Fowler, 1964).

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- 14/020 in a series of reports approved for publication.

5. **REFERENCES**

Aasen, I. M., Markussen, S., Moretro, T., Katla, T., Axelsson, L., and Naterstad, K. (2003). Interactions of the bacteriocins sakacin P and nisin with food constituents. International Journal of Food Microbiology, 87, 35-43.

Abdollahzadeh, E., Rezaei, M., and Hosseini, H. 2013. Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control *Listeria monocytogenes* inoculated in minced fish meat. Food Control, Volume 35:177-183.

Breukink, E., Kruijff, B. 1999. The lantibiotic nisin, a special case or not? BBA Biomembranes volume 1462:223-234

Carter, M., personal communication, 2012

Cheigh, C. and Pyun, Y. 2005. Nisin biosynthesis and its properties. Biotechnology Letters, 27, 1641-1648.

Dymsza, H.A. and Silverman, G. 1979. Improving the acceptability of intermediate-moisture fish. *Food Technol*. 23:52-53.

Feeherry, F., Ross, E., and Doona C. 2009. Comparison of *Staphylococcus aureus* survival in filled French toast sandwiches at three years versus an accelerated microbiological challenge test protocol. Published abstract. Institute of Food Technologists Annual Meeting.

Fowler, G.G., Jarvis, B. and Tramer, J. 1975. The assay of nisin in foods, p. 91- 105. *In* R. G. Board and D. C. Lovelock (ed), Some methods for microbiological assay. Academic Press, United Kingdom.

Gherna, T., Nieman, W. and Pienta, P. (ed), 1985. ATCC Catalogue of Bacteria, Phages and rDNA Vectors, 16th ed. ATCC, Rockville, Maryland.

Houlihan, A. J. and Russell, J.B. 2007. The effect of calcium and magnesium on the activity of bovicin HC5 and nisin. Current Microbiol 53(5):365-9.

Jung, D., Bodyfelt, F., and Daeschel, M. 1992. Influence of fat and emulsifiers on the efficacy of nisin in inhibiting *listeria monocytogenes* in fluid milk. Journal of Dairy Science Volume 75:387-393

Leung, H.K. 1986. Bread quality: Effects of water binding ingredients. Res. Dev. Assoc.Annual.Meeting Rep., U.S. Army Natick Lab., Natick, MA. p.p. 43-48.

Liu, X., Chung, Y., Yang, S., and Yousef, A. 2005. Continuous nisin production in laboratory media and whey permeate by immobilized *Lactococcus lactis.* Process Biochemistry 40:13-24.

Muller, W., Sikes, A., Lee, C. and Richardson, M. 2008. The evaluation of novel bioactive Ingredients for combat ration intermediate moisture (IM) products to assure the microbiological safety. Technical Report Natick/TR-09/003. U.S. Army NSRDEC.

Pavey, R.L. 1972. Controlling the amount of internal aqueous solution in intermediate moisture foods. Contract No. DAAG-17-70-C-0077, Swift & Company Research and Development Center, Illinois. Technical Report 73-17-FL, 1972 (AD 757 766). U.S. Army Natick, Laboratories, Natick, MA 01760.

Peaco, S, Harte, J., Rubino, M. and Richardson, M. 2010. The effects of trehalose on moisture migration and texture in shelf stable egg patties. Published abstract. Institute of Food Technologists Annual Meeting.

Pinto M., Carvalho A., Pires A., Souza A., Fonseca da Silva P., Sobral D., Jacinto de Paula J., and Santos, A. 2011. The effects of nisin on Staphylococcus aureus count and the physicochemical properties of Traditional Minas Serro cheese. International Dairy Journal 21: 90-96.

Pongtharangkul, T. and Demirei, A. 2004. Evaluation of agar diffusion bioassay for nisin quantification. Appl. Microbiol Biotechnol. 65: 268-272.

Powers, E.M., Briggs, J., DeFao, A., Lee, C., Racicot, K., Richardson, M., Senecal, A., and Wong, C. 1999. Effect of water activity on the microbiological stability of mobility-enhancing ration components. Technical Report Natick/TR-00/003. U.S. Army Natick Soldier Center, Natick, Massachusetts.

Powers, E.M., Berkowitz, and Walker, G.C. 1981. Bacteriology, water activity, and moisture/salt ratio of six brands of precooked canned bacon. *J. Food Prot.* 44:447-449.

Rajkovic, A., Uyttendaele, M., Courtens, T., and Debevere J. 2005. Antimicrobial effect of nisin and carvacrol and competition between *Bacillus cereus* and *Bacillus circulans* in vacuum-packed potato puree. Food Microbiology, Volume 22:189-197.

Richardson, M., Briggs, J., Senecal, A., Dunne, P., and Lee, C.1995. Development of intermediate moisture meats for use in military shelf-stable sandwiches. Proceedings of the 41st Annual International Congress of Meat Science and Technology.

Richardson, M., and Sikes, A. 2008. Development of an intermediate moisture egg (IME) using hurdle technology. Institute of Food Technologists Annual Meeting.

Richardson, M., Sikes, A., Lee, C., and Walker, S. 2011. Inoculated pack study of an intermediate moisture egg patty. International Congress on Engineering and Food 11, Proceedings.

Richardson, M. 2012. Intermediate moisture technologies for rations. *In* A.H. Barrett and A.V. Cardello (ed.), pp. 225-255, Military Food Engineering and ration Technologies. Destech Publishing, Lancaster, PA.

Taoukis, P.S. and Richardson, M. 2007. Principles of intermediate-moisture foods and related technology. *In* G. Barbosa-Cánovas, A.J. Fontana Jr., S.J. Schmidt and T.P. Labuza (ed.), pp. 273-312, Water Activity in Foods. Blackwell Publishing, Ames, Iowa.

Tramer, J. and Fowler, G.G. 1964. Estimation for nisin in foods. J. Sci. Fd. Agric., 15: 522-528.

Taub, I.A., Feeherry, F.E., Ross, E.W., Kustin, K., and Doona, C.J. 2003. A quasi-chemical kinetics model for the growth and death of *Staphylococcus aureus* in intermediate moisture bread. J. of Food Sci. 68(8):2530-2537.

Yang, A.P.P. and Yaub, I.A. 1997. Preparation of intermediate moisture sandwich meats. Published abstract. Institute of Food Technologists Annual Meeting.

Wijnker, J., Weerts E., Breukink E., Houben J., and Lipman L. 2011. Reduction of *Clostridium sporogenes* spore outgrowth in natural sausage casings using nisin. Food Microbiology Volume 28:974–979.