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THE EVALUATION OF NOVEL BIOACTIVE INGREDIENTS FOR COMBAT RATION INTERMEDIATE MOISTURE (IM) PRODUCTS TO ASSURE THE MICROBIOLOGICAL SAFETY

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14. ABSTRACT

Military intermediate moisture (IM) sandwiches have a shelf life requirement of two years at 80° F. The stability of IM sandwiches is maintained using Hurdle technology, which typically provides a pH below 5.5 and a water activity below 0.89. These levels are effective for microbial stability; however, they can have an effect on the sensory quality of the product and can limit the development of new products. This report describes a series of challenge studies to raise both levels. Two IM products, the Italian pocket sandwich (IPS) and the bacon cheddar sandwich (BCS), were reformulated to a pH above 6 and a water activity above 0.9. Various bioactive ingredients (BIs) were then added to the reformulations (as an additional hurdle), in the form of Purasal®P (lactate/diacetate), ε-polylysine, or Nisaplin® (nisin), to provide microbial stability. The studies were conducted on the reformulated products with five different strains of *Staphylococcus. aureus*, which would be the most likely microbial contaminant in these products. The studies were carried out for 180 days at 25° C. For the IPS the three BIs were incorporated into both the bread and the filling. It was determined that 4% purasal, 2.5% ε-polylysine, and 1.2% nisin were effective in inhibiting the growth of *S. aureus*. For the BCS only purasal and nisin were incorporated. They were added in the same concentrations as in the IPS, but were incorporated only in the bread. Results were similar; however, there was less *S. aureus* growth in the BCS control than in the IPS control. Though all three ingredients inhibited the growth of *S. aureus*, nisin appeared to be the most effective based on its ability not only to inhibit growth, but also to reduce the original bacterial population. It also had less effect on the sensory properties.

15. SUBJECT TERMS

MEALS	RATIONS	SHELF LIFE	FOOD SAFETY	FOOD PACKAGING
YEASTS	BACTERIA	PH FACTOR	FOOD STORAGE	WATER ACTIVITY
OXYGEN	MOISTURE	SANDWICHES	MICROBIOLOGY	MICROORGANISMS
MOLDS(ORGANISMS)		FOOD PRESERVATION		STAPHYLOCOCCUS AUREUS

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PREFACE

The U. S. Army Natick Research, Development and Engineering Center (NSRDEC) performed a series of challenge studies, during the period October 2003 to September 2007, under the AH99 Combat Feeding Directorate (CFD) program, program element number 662786. This program evaluated three bioactive ingredients (BIs) for potential incorporation into the combat ration intermediate moisture (IM) products already in the military system and those being developed by CFD. The purpose of the study was to improve the sensory quality and safety of IM shelf stable sandwiches by increasing their water activity (a_w) and pH levels while maintaining the microbial stability of the products. They can be susceptible to growth of *Staphylococcus aureus* (pathogenic bacteria that threaten foods) at the a_w and pH required to improve sensory quality. The BIs were added to maintain stability. Two sandwiches (the Italian pocket and the bacon cheddar pocket) were reformulated at the higher a_w and pH levels with BIs added. They were evaluated to determine the efficacy of the BIs on *Staphylococcus aureus* inhibition.

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THE EVALUATION OF NOVEL BIOACTIVE INGREDIENTS FOR COMBAT RATION INTERMEDIATE MOISTURE (IM) PRODUCTS TO ASSURE THE MICROBIOLOGICAL SAFETY

1. INTRODUCTION

This report presents the results and findings of research performed at the U.S. Army Natick Research, Development and Engineering Center (NSRDEC) to improve the safety and sensory quality of combat ration intermediate moisture (IM) products while maintaining microbial stability of the products. Several challenge studies were conducted on the effects of adding specific bioactive ingredients (BIs) to IM sandwiches to accomplish this goal.

Military IM products, specifically shelf stable sandwiches, are required to maintain microbial stability for two years at 80^{o} F. The current IM products have good microbial stability and have been well received in the field by the warfighter. However, there is a need to develop new classes of IM products and to improve the safety, stability, and sensory characteristics of the existing products. IM sandwiches are developed using hurdle technology, which uses a series of barriers to inhibit the growth of various organisms (e.g., bacteria, yeast, and mold). The current IM components rely on controlling the water activity (a_w), pH, and oxygen content of the products. The IM sandwiches were developed to have a low pH (4.8 to 5.4) and an intermediate a_w (0.84-0.89) to ensure microbial stability. See Table 1 for current a_w and pH ranges for the different shelf stable sandwiches.

Table 1. Shelf stable IM sandwiches and their required a_w and pH levels

IM Sandwich	a_w	pH
Barbecue Chicken Barbecue Beef	0.84-0.89	4.8
Nacho Cheese Pepperoni	0.84-0.89	4.8
Italian Pocket	0.86-0.89	5.2
Bacon Cheddar	0.84-0.88	5.4

The a_w is controlled by incorporating various humectants and by varying the baking times and temperatures. The pH is controlled by choosing naturally acidic ingredients or by incorporating food grade acids. The sandwiches are packaged in foil, tri-laminated pouches, which prevent the transmission of both oxygen and moisture. Oxygen absorbing sachets are incorporated into the package to absorb residual oxygen.

Military IM sandwiches appear to be susceptible to microbial compromise especially when considering the final a_w and pH. The targeted a_w and pH (0.88 to 0.92 and 6.0, respectively) suggest that the product is potentially hazardous. That is, the product will support the growth of

pathogenic bacteria specifically *S. aureus*. Because these products were validated by conducting microbial challenge studies with *S. aureus*, at the required lower a_w and ph, they are not considered to be potentially hazardous (Powers et. al., 1999). In this current study BIs are added to determine if microbial stability can be maintained at higher a_w and pH levels. By increasing the a_w and pH a potentially better tasting product can be produced, and new products can be developed.

The objective of the study described in this report was to maintain the microbial stability of the IM product while relaxing the strict conditions of a_w and pH. BIs were added to determine, if microbial stability can be maintained at higher pH levels. By increasing the a_w and pH a potentially better tasting product can be produced, and new classes of IM products can be developed. Challenge studies with five strains of *S. aureus*, the main bacterial concern with IM sandwiches, were conducted on two of the IM products, the Italian pocket sandwich (IPS) and the bacon cheddar sandwich (BCS), to determine the effectiveness of the BIs to inhibit growth of *S. aureus* at the higher a_w and pH.

2. MATERIALS AND METHODS

A model system was used to determine the effectiveness of various BIs. The model system indicated that the commercial BIs nisin (Nisaplin®Danisco USA Inc., New Century, KS), ϵ-polylysine (Save-ory™ PL50, Chisso America Inc., Rye, NY), and Purasal®P (PURAC America Inc., Lincolnshire, IL) would be effective at relatively low concentrations against *S. aureus*. Nisin, a polypeptide, inhibits growth of Gram positive bacteria by binding to the cytoplasmic membrane, forming pores and causing the leakage of low molecular weight compounds (Ray, 1992). ε-Polylysine is a straight-chain homopolymer of 25 to 35 L-lysine units with a broad antimicrobial spectrum (Geornaras and Sofos, 2005). It exhibits its antibacterial effects through growth suppression or to a certain extent bacterial inactivation (Hiraki, 2000 and Hiraki et al., 2003). The exact mechanism of action is not well understood. Purasal®P is a combination of potassium lactate and sodium diacetate. The mode of action of these organic acids on bacteria is by starvation or metabolic inhibition by the undissociated acid molecules (Jay, 1992).

In performing challenge studies it was necessary to provide a more uniform product to eliminate inconsistencies in the results. Thus, the test sandwiches were ground in a sterile blender prior to inoculation. The IPS and the BCS were reformulated with the BIs incorporated. To increase the sensory acceptability, the targeted a_w range and pH for the IM products were 0.88 to 0.92 and 6.0, respectively. The IPS included all three candidate BIs. Because of the high tomato content of the IPS, the pH was low. To increase the pH of the sandwich, sodium bicarbonate was added to the formulation. This change was also required in a previous study using nisin as a preservative in a burrito IM product (Muller et al., 2003, 2005). The BCS included only two of the BIs: purasal and nisin. Like the IPS, sodium bicarbonate was also included in the BCS to increase the pH.

Five strains of *S. aureus* bacteria, obtained from the American Type Culture Collection (ATCC), were used to inoculate ground IPS and BCS. The strains were ATCC 6538, ATCC 8095, ATCC 13567, ATCC 27154, and ATCC A100. The particular BIs and their concentrations chosen for each challenge were not determined in advance. They were determined as the testing progressed, based on the results of the previous test(s). The challenges performed are listed in Chapter 3.

2.1 Inoculum Preparation and Method of Inoculation

The reactivation procedure was similar to the method described by ATCC for the recovery of bacteria from freeze-dried cultures (ATCC, 1985). Activated cultures of the strains were maintained on slants of tryptic soy agar supplemented with 0.5 % yeast extract (TSB-YE), with weekly transfers. The cultures of each organism were prepared separately by transferring a loopful of the stock cultures into tryptic soy broth TSB (Difco) and incubating at 35° C for 18 to 24 hr. Subsequently, 0.1 mL of the 18-to-24 hr growth of each strain was placed in 9.9 mL of sterile TSB, incubated at 35° C overnight, harvested by centrifugation (3000 x g for 10 min at 10° C), and washed (3x) in 0.1 % sterile peptone (Difco) water. Finally, cell densities were adjusted (0.1 % peptone water) to approximately 10^7 CFU/mL, using previously prepared standard growth curves (optical density$_{540nm}$ vs. Log_{10} cell numbers). The five cultures were mixed in equal proportions and diluted so that a volume of 10 µL added to ground IPS or BCS (20 g in a stomacher bag) produced a targeted concentration of approximately $10^3 – 10^4$ CFU/g.

2.2 Sample Storage

After inoculation, the inoculum was distributed in the samples by hand mixing for 15 to 30 sec, Then, the samples were rolled up in a stomacher bag, placed inside tri-laminated Meal-Ready-to-Eat (MRE) pouches with oxygen scavenger (Mitsubishi Technologies, Buffalo, NY., one sachet/pouch), and heat sealed (AIE-610VA/GA,vacuum sealer, Pac-N-Seal, Inc., Newport News, VA). Samples were stored for 180 days at 25^{o} C. Initially, samples (in triplicate) for micro analysis were withdrawn at 0, 3, and 7 days for analysis; subsequently, samples were taken at 7, 14, 21, 33, 49, 62, 125, and 180 days.

2.3 Microbiological Analysis

MRE pouches were removed from a large walk-in environmental room (25^{o} C) and brought to the lab for analysis. The pouches were opened, the stomacher bag in each pouch was removed and unfurled, and an appropriate volume of diluent (Butterfield's Phosphate Buffer, pH 7.2) was added. The stomacher bag was placed in the stomacher (Stomacher 400 Circulator, J.A. Seward, London, England) and blended for 2 min. Samples were serially diluted (10-fold) in Butterfield's Phosphate Buffer (pH 7.2), and then appropriate aliquots were taken, placed on duplicate Staphylococcus 110 agar (S 110; Hardy Diagnostic, Santa Maria, CA) plates, and spread using a sterile bacterial cell spreader (Lazy-L-Spreaders™, LabSource, Willowbrook, IL). Plates were incubated, aerobically, at 35^{o} C for 48 hr before counting.

2.4 Water Activity (a_w) and pH

The a_w measurements were determined in triplicate using the Aqua Lab Model 3TE (Decagon Devices Inc., Pullman, WA) instrument. Plastic disposable sample cups (Decagon Devices, Inc.) were half filled with ground sandwiches and inserted into the instrument for a_w measurements at 25^{o} C.

The pH was measured in triplicate using an Orion 3 Star pH meter (Thermo Electron Corp., Beverly, MA) that had a flat surface electrode (Orion). The pH of the ground sandwich was determined by mixing it in boiled/cooled, distilled deionized water (1:1, w/v) at 25^{o} C.

2.5 Minimum Inhibitory Concentration (MIC) of Antimicrobial Ingredients

Three overnight cultured strains of *S. aureus* (ATCC 8095, ATCC 6538, and ATCC 13567) were used in the model study. These strains were tested against different concentrations of purasal, ε-polylysine, and nisin in tryptic soy broth (TSB). One hundred µL of each overnight culture was used to inoculate 10 mL of double strength (2x) TSB (providing ~10^4 CFU/mL). Stock solutions (25 mg/ml) of Purasal and 50 % ε-polylysine were prepared and filter sterilized (0.45µm; Nalge Company, Rochester, NY). Stock solutions of nisin ranged from 500 IU/mL to 5000 IU/mL.

Using microtiter plates (200 µL wells), growth of *S. aureus* strains in the presence of the bioactive ingredients were monitored in TSB broth. The microtiter plate setup was as follows:

1. Three empty wells were setup as blanks.
2. Four wells were set up as negative controls (200 µL of TSB, no cells).

3. Positive control wells contained 100 µL of inoculated 2x TBS + 100 µL of sterile water.
4. One hundred µL of the 2x TSB/organism were added to 20 wells. Then 5 to 100 µL (5 µL interval increase to each well, i.e., 5, 10, 15, 20…100) of the stock solution of antimicrobial was added to each of the wells containing the 2x TSB/organism. The volume in each well was brought to 200 µL with sterile water.
5. Using the same plate, step four was repeated for each antimicrobial ingredient.
6. Microtiter plates were sealed with gas permeable sealing membranes (Breathe-easy™, Diversified Biotech, Boston, MA), placed in a Thermo Max Microplate Reader (Molecular Devices Corp., Sunnyvale, CA), and incubated at 37° C for 30 hr.
7. Microtiter plates were read (OD_{650nm}) at 0 hr, 8 hr, 24 hr, and 30 hr.

2.6 IPS Preparation

The BIs were incorporated into both the 48 g of IPS filling and the bread. The filling was manually enrobed in the divided bread dough and sealed. The ingredients are listed by percentage of each sample in Table 2. The formed sandwiches were placed in a proof box (Hobart, Troy, OH) at 90º F and 86 % RH for 1 hr. They were then baked in a convection oven (Hobart, Troy, OH) at 425º F for 11 min. After baking the sandwiches were bulk packed (10 IP/pouch) in tri-laminated pouches between 80º F to130º F and refrigerated until inoculation.

Table 2. IPS ingredients by percentage of each sample

Ingredients	Control (%)	Purasal Treated (%)	ε-Polylysine Treated (%)	Nisin Treated (%)
Flour, bread	28.25	28.00	27.64	27.92
Italian Tomato Sauce	20.23	19.42	19.86	19.99
Water	17.15	15.49	16.77	16.94
Marinated/Cooked Sausage	12.19	11.70	11.96	12.04
Pepperoni	9.57	9.19	9.40	9.46
Shortening	4.75	4.68	4.65	4.70
Glycerol	2.83	2.78	2.77	2.79
Yeast (Instant Dry)	1.24	1.22	1.21	1.22
Mozzarella Cheese Powder	1.20	1.15	1.18	1.18
Salt	0.66	0.65	0.65	0.65
Sucrose Ester	0.55	0.54	0.54	0.55
Sodium Bicarbonate	0.50	0.30	0.00	0.48
Control S (ADM)	0.28	0.27	0.27	0.27
Gum Arabic	0.28	0.27	0.27	0.27
Calcium Sulfate	0.14	0.14	0.13	0.14
Xanthan Gum	0.14	0.14	0.14	0.14
Sorbic Acid (Encapsulated)	0.06	0.05	0.05	0.05
Bioactive Ingredient	0.00	4.01	2.51	1.21

2.6.1 IPS Filling. The IPS filling was prepared using a rotating vacuum infuser (Dayton). The uncooked ground Italian sausage was infused with water, rice syrup, glycerol, salt, and pepper for 25 min at 25 rpms and 27 mmHg at 50º F, as outlined in Figure 1. The sausage was then cooked in a steam jacketed kettle on medium until all visible moisture was gone. The cooked sausage was mixed with a BI (either purasal, ε –polylysine, or nisin), tomato sauce, pepperoni, cheese powder, and sodium bicarbonate.

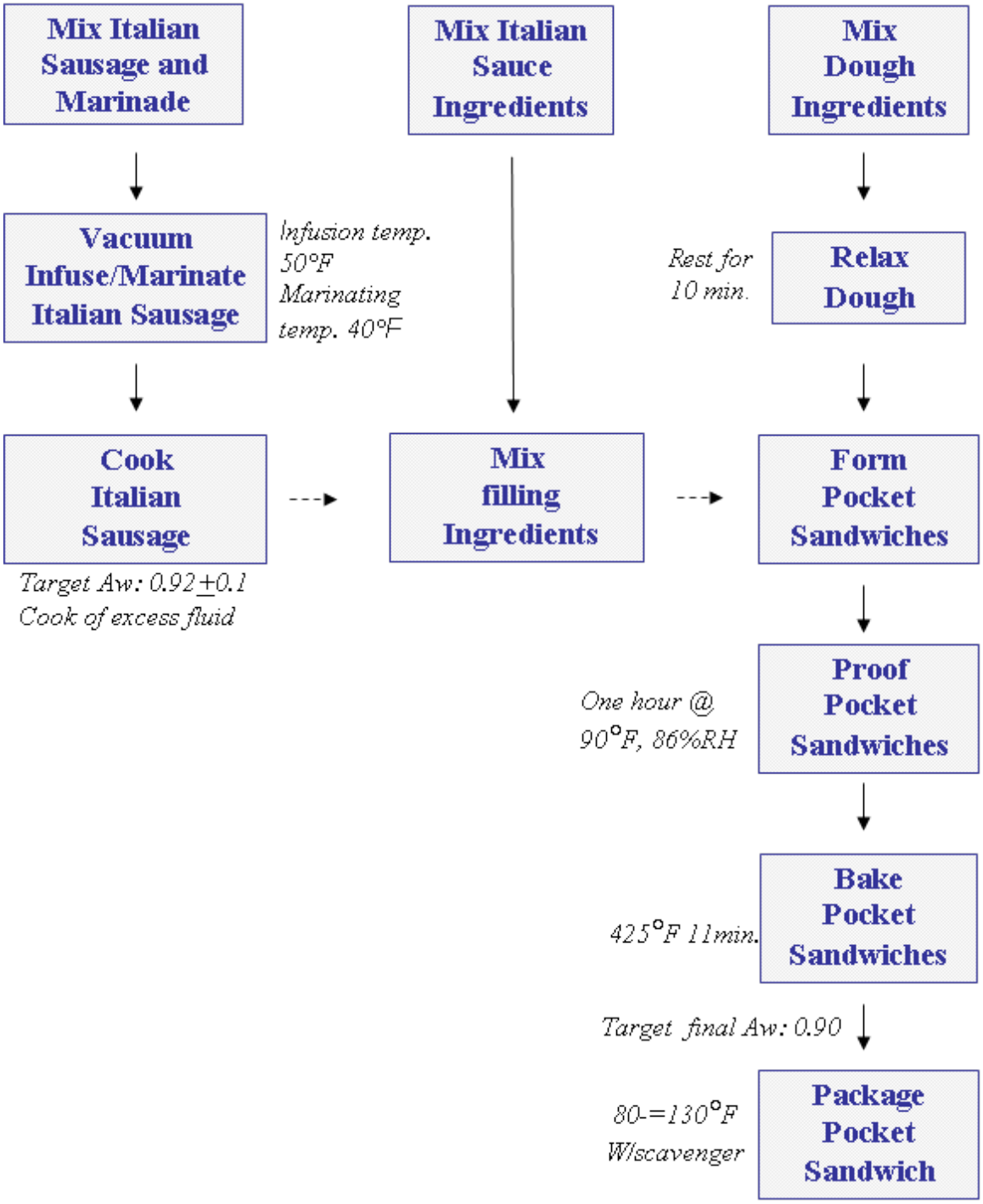

Figure 1. Flow diagram of IPS processing

2.6.2 IPS Bread. The ε-polylysine and nisin were added with the dry ingredients, and the purasal was mixed with the water. The IPS bread dough was made by mixing the dry ingredient for 2 min on low speed. The shortening was added and mixed for 2 min on low speed. Water and glycerol were added and mixed for an additional 2 min on low speed and 8 min on medium speed. The total mixing time was 12 min. The bread dough was divided into 63 g pieces using a dough divider (Fortuna Automat, Adamatic, Eaton, NJ) and allowed to rest for 10 min.

2.7 BCS Preparation

The BIs (purasal and nisin) were incorporated into the bread only. Seventeen grams of commercial pre-cooked bacon was manually enrobed in 75 g of bread dough, sealed, and docked. The ingredients are listed by percentage of each sample in Table 3. The formed sandwiches were placed in a proof box at 90º F and 86 % RH for 1 hr. The sandwiches were baked in a convection oven at 425º F for 11 min. After baking the sandwiches were bulk packed (10 BCS/pouch) in tri-laminated pouches between 80º F to130º F and refrigerated until inoculation.

2.7.1 BCS Bread. The nisin was added to the dry ingredients, and the purasal was mixed with the water. Dehydrated egg white and sodium bicarbonate were added to give a pH of 6.0. The BCS bread dough was made by mixing the dry ingredient for 2 min on low speed. The shortening was added and mixed for 2 min on low speed. Water and glycerol were added and mixed for an additional 2 min on low speed and 6 min on medium speed. Cheddar chips were added during the last 2 min of mixing. The total mixing time was 12 min. The bread dough was divided into 75 g pieces using a dough divider and allowed to rest for 10 min.

Table 3. BCS ingredients by percentage of each sample

Ingredients	Control (%)	Purasal Treated (%)	Nisin Treated (%)
Flour, Bread	48.43	47.14	47.14
Water	24.80	22.00	24.80
Cheddar Betreflakes	6.99	6.99	6.99
Egg Whites, Dehydrated	6.30	6.30	6.30
Shortening	5.17	5.17	5.17
Glycerol	2.80	2.80	2.80
Yeast (Instant Dry)	1.86	1.86	1.86
Salt	0.93	0.93	0.93
Sucrose Ester	0.93	0.93	0.93
Control S (ADM)	0.47	0.47	0.47
Gum Arabic	0.42	0.42	0.42
Butter Flavor	0.33	0.33	0.33
Calcium Sulfate	0.23	0.23	0.23
Xanthan Gum	0.23	0.23	0.23
Sodium Bicarbonate	0.10	0.10	0.10
Sorbic Acid (Encapsulated)	0.09	0.09	0.09
Bioactive Ingredient	0.00	4.00	1.20

2.8 Sandwich Ingredients

The full names, descriptions, and manufacturers of the ingredients listed in Tables 2 and 3 are: flour, wheat, bleached, bread (Abbys Best, Habathem, SC); hydrogenated shortening (Primex, Ach Food, Memphis, TN); glycerol (KIC Chemicals, Amonk, NY); dried egg white (Deb-El-Foods Corp. Elizabeth, NJ), yeast (instant, Lesaffre Yeast Co., Milwaukee, WI); sucrose ester (S1670, Montello Inc., Tulsa, OK); control S (Archer Daniel Midland (ADM), Caravan Trading Co., CA); xanthan gum (Keltrol F, Kelco, San Diego, CA); gum Arabic (Gum technology, Coyote Brand); calcium sulfate (Spectrum, New Brunswick, NJ); encapsulated sorbic acid 70 % active (Balchem, Slate Hill, NY); rice syrup, clarified (WRSRDCL or PPRSRDCL California Natural Products Larhrop); pre-cooked bacon (Hormel, Fast and Easy ™); cheddar flakes (SensoryEffects Somerset cheddar – 12421 regular flakes, Loders Croklaan, Channahon, IL); butter flavor (Colony Flavor, Elk Village, IL); Pepperoni (Hormel, Austin, MN); Italian sausage (Sysco, Houston, TX); and mozzarella cheese powder (Kerry, Beloit, WI).

3. RESULTS AND DISCUSSION

Table 4 shows the results obtained from the model system with three candidate BIs tested against three strains of *S. aureus*. These three candidates inhibited *S. aureus* at relatively low concentrations in ideal growth conditions for the bacteria. The criteria to determine effectiveness of the BI was the concentration needed to inhibit the growth of *S. aureus* for 24 hr at 37˚ C in liquid bacterial medium (tryptic soy broth). The concentration at which the microtiter plate reader, set at 650 nm, indicated no growth after 24 hr was the determined MIC concentration for tested BI against the selected bacterium.

Table 4. Minimum inhibitory concentration (MIC[1]) of ε-polylysine, purasal, and nisin required to prevent growth of three strains of *S. aureus* in the model system

	Staphylococcus aureus		
Bioactive Ingredient	**ATCC 8095**	**ATCC 6538**	**ATCC 13567**
ε-Polylysine	5.63 mg/ml	1.88 mg/ml	3.13 mg/ml
Purasal	5.5 %	5 %	5.5 %
Nisin	75 IU/ml	4.5 IU/ml	5 IU/ml

[1] Concentration at which there is no growth after incubation for 24 hrs at 37° C

The challenge studies performed using all five strains of *S. aureus*. The studies included:

1. An IPS with a pH range of 5.2 to 5.4 with only one BI added, 2 % purasal, and a commercially prepared IPS as a negative control.
2. IPS with a pH range of 5.8 to 6.2 with all three BIs added and a commercially prepared BCS (with a_w of 0.86 and pH of 5.1) as a negative control. The concentrations of the BIs were 2 % purasal, 2 %ε-polylysine, and 0.6 % nisin. The study was conducted over 180 days at 25º C.
3. IPS with a pH range of 6 to 6.5 with BI concentrations of 4 % purasal, 2.5 % ε-polylysine, and 1.2 % nisin.
4. BCS with a pH range of 5.8 to 6.1 with only two BIs added, 4 % purasal and 1.2 % nisin.

Figure 2 shows the inhibition of the five strains of *S. aureus* in the IPS control, the purasal-treated IPS, and the IPS field item, found in the first challenge. This result indicates that the IM product is microbiologically stable at the low pH; however, the effectiveness of the purasal could not be determined at the low pH.. The final a_w and pH of the samples were: control 0.91, 5.2; purasal 0.91, 5.4; and field item 0.89, 5.2. Neither the control nor the purasal-treated product supported the growth of *S. aureus*. It is evident that a higher pH is required to obtain growth of *S. aureus* bacteria in the sandwich.

In the second challenge study, reported in Figure 3, the pH range of the IPS products was significantly higher than in the first challenge. The starting inoculum of the five strains of *S. aureus* was 10^4 to 10^5 bacteria/ml, which was higher than the desired range of 10^3 to10^4 bacteria/ml. The final a_w and pH of the samples were: control 0.90, 6.1; purasal 0.91, 5.9; ε-polylysine 0.9, 06.2; nisin 0.91, 5.8; and the BCS field item 0.86, 5.1. The growth of *S. aureus* in the control IPS sample increased with time, indicating the higher pH was necessary to obtain

growth. The growth in other samples containing BIs also increased. For the purasal sample, at certain sampling times (42 and 63 days), the growth of *S. aureus* was greater than the control. The other two BIs showed some inhibitory effect on the *S. aureus*. It was evident that a higher concentration of the BIs may be required to sufficiently inhibit the strains of *S. aureus*. The negative control BCS did not promote growth of *S. aureus* throughout the sampling period, which was expected, since it had a lower a_w and pH.

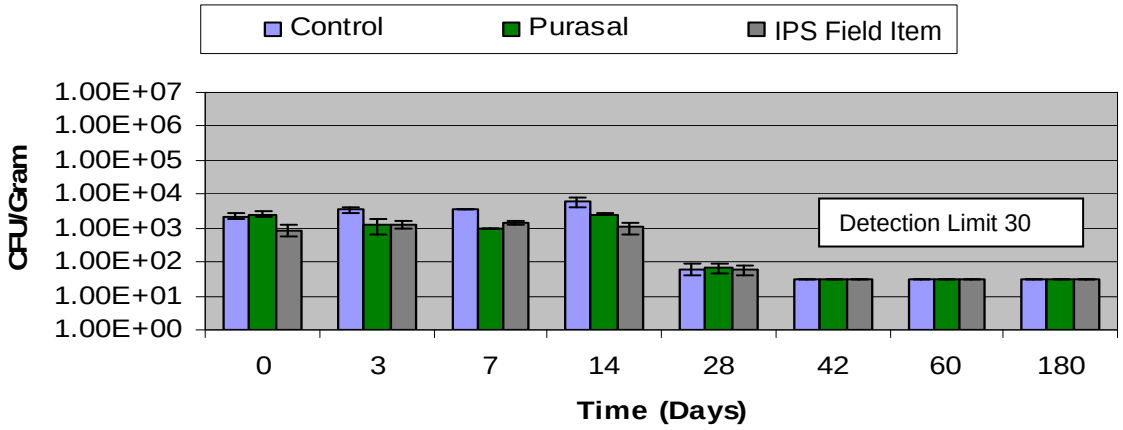

Figure 2. Growth of *S. aureus* in IPS at pH 5.2 to 5.4 with addition of 2 % purasal and growth in IPS negative control field item

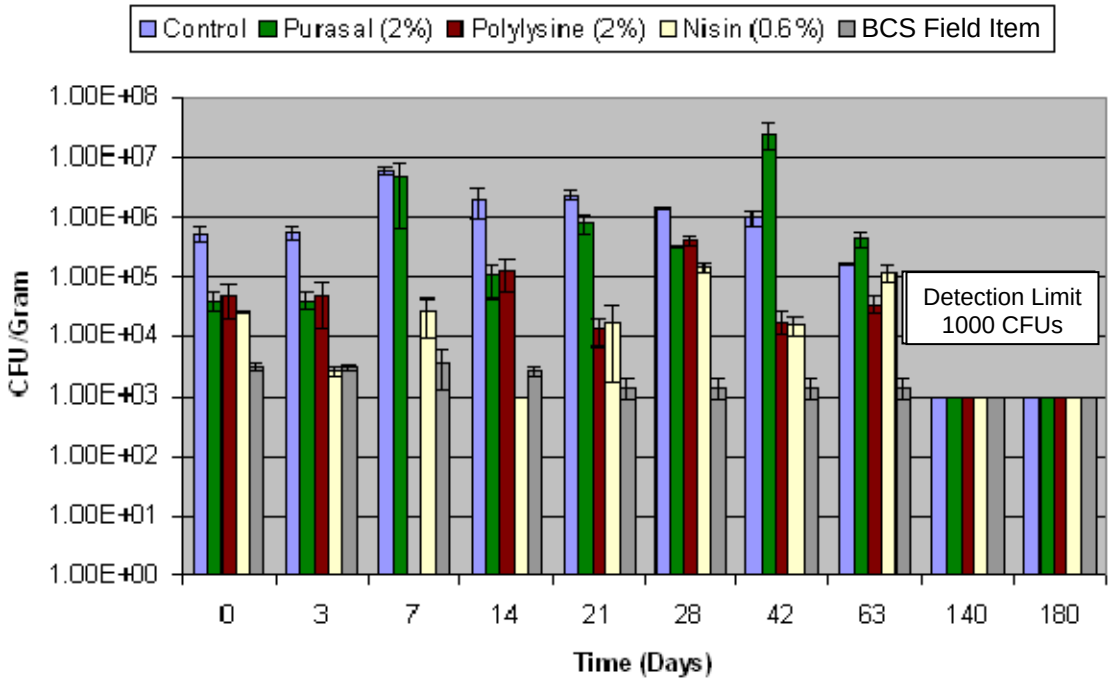

Figure 3. Growth of *S. aureus* in IPS at pH 5.8 to 6.2 with addition of 2 % purasal, 2 % ε-polylysine, and 0.6 % nisin and growth in BCS negative control field item

The level of each BI added to the IPS was increased in the third challenge study, reported in Figure 4. In the control sample the *S. aureus* grew well for the first 50 days. The three BIs at the

higher concentration were all effective in inhibiting the five strains of *S. aureus*. The inoculum was at the desired level, $10^1 - 10^3$ bacteria/ml. The samples were kept at 25º C over the duration of the study. The final a_w and pH of the samples were: control 0.90, 6.0; purasal 0.89, 6.1; ε-polylysine 0.90, 6.3; and nisin 0.90, 6.5. It is evident that at the higher a_w and pH, the IPS would be microbiologically compromised without the addition of the BIs. All three of the BIs appeared to be effective in inhibiting the growth of the five strains of *S. aureus* at the concentrations employed in the study.

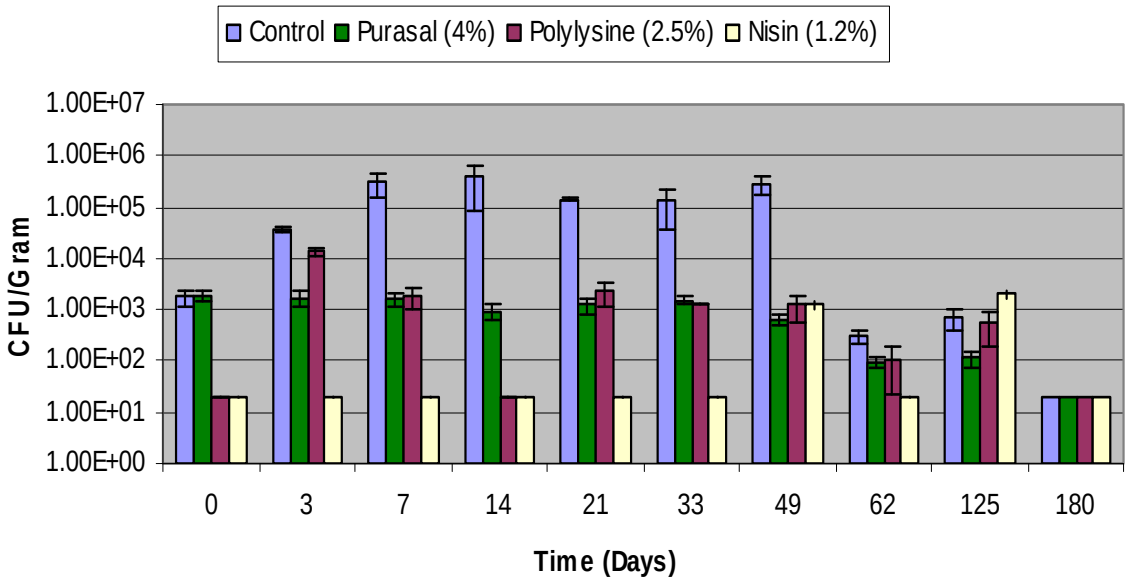

Figure 4. Growth of *S. aureus* in IPS at pH 6.0 to 6.5 with addition of 4 % purasal, 2.5 % ε-polylysine, and 1.2 % nisin.

Figure 5 shows the results of the fourth challenge study, conducted on the BCS. The initial *S. aureus* inoculum was 10^1–10^3 bacteria/ml. The final a_w and pH of the samples were: control 0.89, 5.8; purasal 0.87, 6.1; and nisin 0.88, 6.1. ε-Polylysine was not used in this study because preliminary sensory analysis showed that at the higher concentration it can cause some sensory issues in a BCS. A soapy flavor could be detected at levels above 2 %.

It appears that at this elevated pH, the BCS control sample was fairly stable, with growth above the original inoculum only occurring at day three. The purasal and nisin seemed to maintain the microbial stability of product, but this was difficult to evaluate because of the lack of significant growth in the control sample. It may require a higher pH, for the product to obtain growth of *S. aureus* in the control, to adequately assess the effectiveness of the BIs.

Table 5 lists each sandwich tested and its BI concentration, a_w, pH, and presence or absence of *S. aureus* growth.

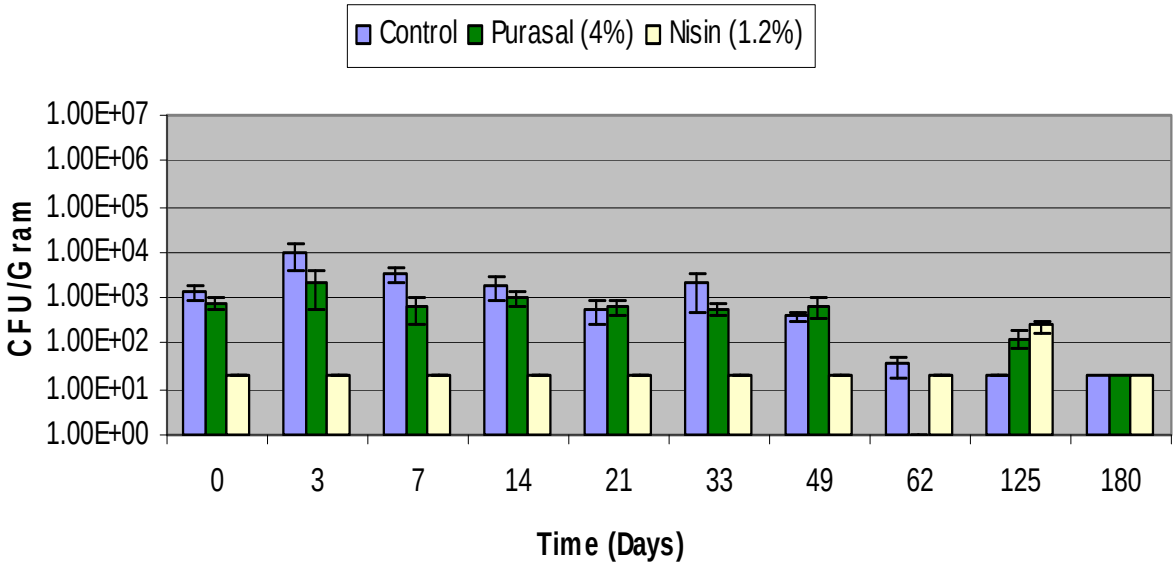

Figure 5. Growth of *S. aureus* in BCS at pH 5.8 to 6.1 with addition of 4 % purasal and 1.2 % nisin

Table 5. BI concentration, a_w, pH, and presence of *S. aureus* growth in the reformulated IM sandwiches

BI Treatment	a_w	pH	*S. aureus*
IPS			
None	0.91	5.2	(-)
None	0.90	6.1	(+)
None	0.90	6.1	(+)
2.0 % Purasal	0.91	5.4	(-)
2.0 % Purasal	0.91	5.9	(+)
4.0 % Purasal	0.89	6.1	(-)
0.6 % Nisin	0.91	5.8	(+)
1.2 % Nisin	0.90	6.5	(-)
2.0 % Polylysine	0.90	6.2	(+)
2.5 % Polylysine	0.90	6.3	(-)
BCS			
None	0.89	5.8	(-)
4.0 % Purasal	0.87	6.1	(-)
1.2 % Nisin	0.88	6.1	(-)

(+)Growth
(-)No Growth

4. CONCLUSIONS

It was apparent, in doing the challenge studies, that the IM sandwiches must be at a higher pH (6) and/or a_w, to support the growth of *S. aureus* in the positive control, to evaluate the BIs. This indicated that the current IM products are microbiologically safe at their current a_w and pH levels. The easiest way to increase the pH of these sandwiches was by incorporating sodium bicarbonate into the formulation. The three BIs evaluated in this study were nisin, ε-polylysine, and purasal. All three showed effectiveness in inhibiting the five strains of *S. aureus* tested in the challenge studies with the IPS at the targeted higher a_w and pH. Concentration was critical in obtaining the necessary inhibitory level in preventing the growth of *S. aureus* above the initial inoculum level. For the IPS 4 % purasal, 2.5 % ε-polylysine, and 1.2 % nisin were required to prevent any significant growth of the *S. aureus*. It is believed that, because of the variety of ingredients that make up the various IM products, the BI concentrations will have to be adjusted and validated via microbial challenge studies for each IM product. The ε-polylysine at concentrations above 2 % can cause sensory issues. However, this BI becomes more effective at higher pH. Thus, it may have special application potential with products having a pH closer to neutral. The ingredients evaluated in this study will transition into the "Next Generation Combat Breakfast Ration Technologies" and the "Next Generation Hurdle Technologies" (NGHT) programs slated to start in fiscal year 2009.

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- 09 / 003 in a series of reports approved for publication.

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