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Fate of Spoilage Microorganisms Associated with the Production of Pickled Sausage Using a Cold Fill Process

ABSTRACT

Pickling is a means of preserving a variety of foods, including meat products. Cooked sausages have traditionally been pickled using a heated solution of vinegar, salt, and spices in a process known as hot filling. However, hot fill pickling can result in quality defects. Alternatively, room temperature brine does not cause the various quality defects resulting from heated brine. To date, no study has determined the efficacy of a cold fill pickling process to inhibit the growth of lactic acid bacteria (LAB), yeasts, and molds associated with acidified meat products. Therefore, the efficacy of cold fill pickling of cooked sausages, using a brine solution (5% acetic acid and 5% salt at 22–23ºC and pH ~2.70), was validated for the reduction of spoilage microorganisms by inoculating sausages with select LAB (6.98 $\log_{10}$ CFU/g), yeasts (4.49 $\log_{10}$ CFU/g), and molds (4.29 $\log_{10}$ CFU/g) over 28 days of pickling. The results revealed reductions of 5.51 $\log_{10}$ CFU/g in 24 h for LAB counts, 3.89 $\log_{10}$ CFU/g in 48 h for yeast counts, and 4.09 $\log_{10}$ CFU/g in 24 h for mold counts. To our knowledge, this experiment is the first to demonstrate that cold fill pickling effectively reduces and inhibits spoilage microorganisms.

INTRODUCTION

Pickling is a means of preservation that has been used for many years to extend and maintain the shelf life of various types of food products, such as fruits, vegetables, eggs, and meat. In particular, pickled sausage is a common acidified meat product that is generally prepared by curing and cooking sausages, which are then covered with a heated brine solution containing an organic acid (typically acetic or citric acid), salt, spices, food coloring, and sometimes preservatives. The sausages are acidified or pickled in containers which are sealed, letting the brine solution penetrate into the sausages, allowing the acidification of the meat product. The pickling process utilizes a combination of microbiological inhibitory effects, which together prevent the growth and survival of microorganisms. Meat products may be preserved by several

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methods, including placing product in an acidic environment (reducing the pH), using salt to decrease the water activity (a_w), and sealing the storage container to decrease the oxygen level. Leistner and Gorris referred to the use of multiple processing factors to inhibit microorganisms in food as "hurdle technology" *(15)*. The use of multiple, sub-lethal factors to kill and inhibit unwanted microorganisms works by disturbing the homeostasis of microorganisms on multiple fronts simultaneously, resulting in the destruction of microorganisms and allowing for the preservation of the food product *(13)*. Preventing the spoilage of such products is critical to ensure pickled sausages remain of good microbiological quality and are safe for consumption.

The preparation of pickled sausage has traditionally used either a cold fill or hot fill process, in which cooked sausages are covered with either a cold or hot brine solution to begin the pickling process. However, hot filling has generally resulted in significant quality defects, including cloudiness, turbidity, or discoloration of the brine, which make the product less appealing to consumers. To avoid these quality defects, many commercial pickled sausage processors may utilize a cold fill process, in which the brine solution is kept at room temperature prior to pickling.

The pickling of sausages has been regarded as a safe method of meat preservation by federal regulatory authorities. However, recent incidences of spoilage contamination involving pickled meat products have prompted the United States Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) to reconsider the microbiological stability of pickled sausages subjected to a cold fill pickling process *(17)*. Current U.S. regulations addressing acidified foods (21 CFR parts 114 and 120) *(32, 33)* are mainly focused on canned food products intended to ensure processors properly address the potential growth of *Clostridium botulinum*. Title 21 CFR part 114 *(32)* states that "…the pH must be maintained at or below 4.6…" with no specification to the type or concentration of the acid, while title 21 CFR part 120 *(33)* states that "…manufacturers of acidified fruit and vegetable products must demonstrate that their process achieves a 5-log reduction in appropriate acid-tolerant pathogenic bacteria, as validation of product safety." It does not appear that either of these policies has been applied to pickled meat products or used to address issues with spoilage. Current USDA-FSIS policies require processors of pickled meat products to demonstrate stability and safety by monitoring and recording several quantitative parameters, including pH, a_w, salt concentration, temperature and overall formulations. To date, no specific scientific validation has been performed to demonstrate which intrinsic and extrinsic factors are important to ensure the stability and safety of pickled meat products. The lack of scientific validation has generated additional concerns from Federal regulators about post-processing contamination, since it is unknown if a cold fill process might allow for the survival and growth of spoilage microorganisms.

Research on the microflora categorized as spoilage microorganisms associated with acidified meat products is limited. To our knowledge, no scientific literature exists that addresses either: (1) the microbial stability of pickled meat products or (2) the growth or survival of spoilage microorganisms associated with acidified meat and sausage products using a cold fill pickling process. Recent studies have demonstrated the effectiveness of pickling processes using various organic acids to reduce the growth and survival of pathogenic bacteria in fruits and vegetables *(1, 13)*, cucumber purees, vegetable brines and pickles *(5, 6, 7)* and hard cooked, pickled eggs *(22, 26, 29)*. Research on pickled eggs may be most relevant to pickled meat products, because of their similar protein content and susceptibility to microbial contamination, although these studies did not address spoilage microorganisms such as LAB, yeasts, and molds. Therefore, the purpose of this study was to validate the efficacy of a cold fill pickling process of cooked sausages to prevent the growth and survival of LAB, yeasts, and molds associated with ready-to-eat (RTE) sausage products.

MATERIALS AND METHODS

In this study, a cooked, smoked, and cured sausage product was placed into an acidified brine (5% acetic acid (wt/vol); 5% salt (NaCl, wt/wt); 22–23°C; pH ~2.70) solution and held at room temperature for 28 days. *Table 1* highlights the spoilage microorganisms chosen for the challenge study, based on their prominence in fresh and RTE meat products, relevant isolation sources, and availability.

Antagonism of select lactic acid bacteria and fungi

Preliminary studies (data not shown) indicated that the lactic acid bacteria (LAB) cultures *(Table 1)* identified for use in this challenge study inhibited selected mold cultures. All three LAB cultures inhibited both *Penicillium nalgiovense* strains; *Lactobacillus sakei* ATCC 15521 inhibited both *Cladosporium cladosporioides* strains; and *L. curvatus* 51436 inhibited *C. cladosporioides* 11275. No inhibition occurred between the selected yeast and mold cultures. Therefore, the inoculation and spoilage challenge study of sausages by fungi and LAB cultures were performed separately.

Preparation of selected lactic acid bacteria cultures and challenges study inoculum

LAB cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Lyophilized cultures were transferred into fresh de Man, Rogosa and Sharpe (MRS) broth (Difco, Sparks, MD). Both *L. curvatus* cultures were incubated aerobically at 30°C for 48 h, while *L. sakei* was incubated at 30°C in a microaerophilic environment (5.0% O_2, 10% CO_2, 85% N_2) in a CO_2 incubator (VWR International, West Chester, PA), following ATCC recommendations. Colonies were then re-suspended in fresh MRS broth containing 10% glycerol (VWR, Radnor, PA) and stored at

Table 1. Spoilage microorganisms inoculated onto sausages prior to cold-fill pickling

Spoilage Microorganism	Genus/Species	ATCC[1]/ID	Isolation Source
Lactic Acid Bacteria (LAB)	*Lactobacillus curvatus*	51436	fermented sausage
	Lactobacillus curvatus	PTA5150	Kimchi (fermented Korean dish)
	Lactobacillus sakei	15521	moto (sake starter culture)
Yeast	*Debaryomyces hansenii*	90624	salty pickle
	Debaryomyces hansenii	10619	horse meat sausage
	Candida zeylanoides	26318	Spanish sausage
	Candida zeylanoides	24745	grape must
Mold	*Cladosporium cladosporioides*	60549	frozen lamb carcass
	Cladosporium cladosporioides	11275	wheat bread
	Penicillium nalgiovense	96457	mold-ripened sausage
	Penicillium nalgiovense	96460	mold-ripened salami

[1]American Type Culture Collection (ATCC); Manassas, VA.

-80°C until future use. Working stocks were maintained on MRS agar (Difco) and stored at 4°C.

To prepare the separate LAB inoculum, frozen stocks were transferred twice into fresh MRS broth and streaked onto MRS agar, under the same incubation conditions already described. Single colonies of each LAB were isolated and transferred into 10 ml of fresh MRS broth. Following incubation, 10 ml of each culture were transferred to 990 ml of fresh MRS broth and incubated for an additional 24 h at the same temperature and atmospheric conditions as previously described to obtain a cell concentration of ~8 $\log_{10}$ CFU/ml. Each single LAB inoculum was prepared in duplicate, providing a total inoculum volume of 6 liters. Two liters of each LAB culture were mixed in a sterilized metal container under a biological safety hood to produce a homogenous LAB cocktail (6 liters total) for the sausage inoculation immersion bath as described previously *(22, 26)*. This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Yeast cultures

Yeast cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Upon receipt, yeast cultures were transferred into fresh yeast mold (YM) broth (Difco, Sparks, MD) and incubated aerobically at 25°C for 72 h, following ATCC recommendations. Yeast cultures were then re-suspended in YM broth containing 10% glycerol (VWR, Radnor, PA), and stored at -80°C. To create working stocks, the yeast freezer stock cultures were streaked onto Malt Extract Agar (MEA; Difco, Santa Maria, CA) slants in sterile, screw top test tubes, incubated at 25°C for 72 h, and stored at 4°C until use.

To prepare the yeast inoculum, single colonies of each yeast strain were individually transferred with a sterile 1 µl loop from MEA slants to 10 ml of fresh YM broth and allowed to grow aerobically at 25°C for 24 h. Incubated yeast cultures (10 ml) were transferred to 490 ml of fresh YM broth and incubated aerobically at 25°C for an additional 72 h to obtain a cell concentration of ~6 $\log_{10}$ CFU/ml for each of the yeast cultures. Five hundred ml of each of the four yeast cultures were mixed in a sterilized metal container under a biological safety hood to produce a homogenous yeast cocktail (2 liters total) for the sausage inoculation immersion bath. This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Mold cultures

Mold cultures were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Each mold culture was transferred to fresh YM broth and incubated aerobically at 25°C for 96 h, following ATCC recommendations. Molds were then re-suspended in YM broth containing 10% glycerol (VWR, Radnor, PA), and stored at -80°C for future use. Cultures were transferred from freezer stocks, using a sterile 10 µl loop, to fresh MEA slants prepared in 125-ml glass bottles, by spreading the inoculated loop across the agar slant surface. Agar slants were then incubated aerobically at 25°C for 96 h and stored at 4°C until use.

To prepare the mold inoculum, 50 ml of sterile 2% (wt/vol) buffered peptone water (BPW; HiMedia, Mumbai, India) with Tween 80 (Acros Organics, Geel, Belgium) at 0.03% (wt/vol) was poured into each MEA bottle slant, and a sterile 10 µl loop was used to scrape off and collect the mold spores for transfer into the BPW. Tween 80 was added to the

BPW to prevent mold spores from clumping while keeping spores in suspension. The BPW/Tween 80 spore solution was filtered through sterile cheesecloth to separate mold spores from other fungal debris and transferred to a sterile 500-ml glass bottle. This process was repeated five times for each individual mold culture to ensure complete removal of spores and produce a spore solution of 500 ml. Through this technique, spore concentrations of ~6 $\log_{10}$ spores/ml were produced for each mold culture, based on plate counts performed using a pour plating technique. Briefly, 1 ml of BPW spore solution was added to molten 1% (wt/vol) MEA in a 15 ml Falcon test tube (VWR, Radnor, PA) and held in a 50°C water bath (Precision, Winchester, VA). The 15-ml Falcon tubes were then vortexed, pour-plated, and incubated at 25°C for 72 to 96 h before enumeration.

To prepare the final inoculum, 500 ml of each of the four mold cultures were mixed in a sterilized metal container under a biological safety hood to produce a homogenous mold cocktail (2 liters total) for the sausage inoculation immersion bath. The 2-liter yeast cocktail was then mixed with the 2-liter mold spore cocktail to produce homogeneous yeast and mold cocktail inoculum (4 liters total) for the sausage inoculation immersion bath. This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Preparation of sausages

Sausages used in this study were manufactured at The Pennsylvania State University Meat Laboratory and formulated as a percent of the ingredient weight of the total batch weight (wt/wt) with the following ingredients: beef trim (80% lean; 22% wt/wt), pork trim (80% lean; 44% wt/wt and 50% lean; 22% wt/wt), water (7% wt/wt), salt (2.2% wt/wt), dextrose (0.44% wt/wt), ground black pepper (0.28% wt/wt), sodium tripolyphosphate (2500 ppm), ground mustard (0.55% wt/wt), granulated garlic (0.55% wt/wt), ground ginger (0.013% wt/wt), sodium erythorbate (540 ppm) and curing salt (6.25% $NaNO_2$; 156 ppm). Sausages were stuffed to a weight of 130.4 g into a 35-mm curved, edible collagen casing (Devro Ltd., Moodiesburn, Scotland, UK), using a vacuum stuffer with a twist linker attachment (Handtmann VF608 Plus Albert Handtmann Maschinenfabrik GmbH & Co., Biberach, DE). A large diameter (> 30 mm), collagen type casing was chosen, because it demonstrated the most resistance to brine penetration and acidification, compared with skinless and natural type casings, in preliminary experiments (data not shown). The sausages were smoked and cooked in a Kerres Smoke Air® JS 1950 (Kerres Anlagensysteme GmbH, Backnang, DE) to a core temperature of 70°C to comply with the time-temperature requirements in Appendix A for meeting lethality performance standards *(30)*. After cooking, the sausages were transferred to a walk-in cooler (1–2°C) and chilled to 4°C in accordance with Appendix B *(31)*. *Table 2* illustrates the thermal processing schedule used to smoke and cook the sausages. The average measurements of the cooked and chilled sausages were: length 144 mm ±

Table 2. Thermal processing schedule of sausages used in the spoilage challenge study

Function	House Temperature (°F)	% Relative Humidity	Core Temperature (°F)	Time (minutes)
Steam Cook	120	90	-	00:15
Dry Cook	135	0	-	00:15
Steam Cook	163	90	-	00:10
Dry Cook	135	0	-	00:15
Heissrauch	140	0	-	00:15
Dry Cook	140	0	-	00:20
Heissrauch	140	0	-	00:15
Dry Cook	140	0	-	00:20
Heissrauch	140	0	-	00:10
Dry Cook	140	0	-	00:10
Steam Cook	154	90	-	00:10
Dry Cook	140	0	-	00:10
Steam Cook	165	90	158	00:00
Dry Cook	140	0	-	00:10
Shower	0	0	-	00:15

Note: "Heissrauch" — hot smoking step

2, width 31 ± 2 mm, wt 127 ± 2 g. Sausages were placed in food grade vacuum pouches measuring 5.5 cm × 9.5 cm (3 mil standard barrier, nylon-polyethylene pouches with an oxygen transmission rate (OTR) of 52 cc/m^2/24 h at 23°C; JVR Industries, Lancaster, NY). The pouches were vacuum sealed (Smith SuperVac Digimat, Clifton, NJ) and stored in a walk-in cooler at 2°C (< 21 days) until they could be transported to The Pennsylvania State University Muscle Foods Microbiology Laboratory for the microbiological challenge study. Three separate batches of sausages were made for each of the 3 inoculation and sampling replications. Fat analysis was conducted in duplicate for each of the 3 batches of sausages, following a modified Babcock Fat Analysis procedure *(11)*.

Preparation of brine solution

Food-grade distilled white vinegar (5% acidity (50 grain acetic acid); H. J. Heinz Company, Pittsburgh, PA) and non-iodized salt (NaCl; Morton, Chicago, IL) were purchased from local supermarkets. Pickling brine (5% salt (wt/vol) and 5% acetic acid (vol/vol)) was mixed using a magnetic stir bar (VWR, Radnor, PA) and stirrer (VWR, Henry Troemmer LLC., Thorofare, NJ) 24 h prior to the inoculation and subsequent pickling of sausages, covered with aluminum foil and allowed to fully equilibrate to room temperature (~22–23°C). This procedure was repeated in the same manner for each of the 3 replications of the challenge study.

Inoculation of sausages with selected LAB or selected yeast and mold

Precooked and chilled (~2°C) sausages (n = 144 total for 3 replications for each treatment group) were aseptically removed from the vacuum packaged bags and transferred to sterilized metal bins under a biological safety hood. The LAB cocktail (6 liters total) was poured over the sausages (n = 48 per replication) and allowed to sit for 30 minutes with gentle periodic mixing, following the methods described previously *(22, 26)*. The LAB and yeast/mold inoculations were repeated in the same manner for each of the 3 replications of the challenge study.

Negative controls

In addition to the inoculated sausages, negative control sausages also were utilized. Precooked and chilled (~2°C) sausages (n = 36 total for 3 replications) were aseptically removed from the vacuum packaged bags, transferred to sterilized metal bins under a biological safety hood and covered with 3 liters of sterile BPW. The sausages (n = 12 per replication) were allowed to sit for 30 minutes in the sterile BPW with gentle, periodic mixing as already described. Three replications of the negative control sausage group were performed for the challenge study.

Pickling of sausages

Following the inoculation of sausages through the immersion bath technique, sausages were removed aseptically from the inoculum and placed into sanitized 1 gal (3.8 L) plastic (oriented polyethylene terephthalate (PET) with an OTR of 4.5 cm^3-mil/(24 h) (100 in^2) (atm) at 25°C) food grade screw-top containers (Pretium Packaging, Hazleton, PA). All sausages inoculated with LAB, separate from the fungal inoculation, were evenly distributed into 4 separate containers (n = 11 per container), with the exception of 4 sausages that were withdrawn from the inoculation bath for initial sampling. Yeast and mold-inoculated sausages and the negative control sausages also were aseptically transferred to containers as previously described. Room temperature (~22–23°C) pickling brine was poured into each container, allowing for a headspace of 0.5 inches (0.19 cm), and the containers were capped and sealed, simulating a cold fill commercial pickling and packaging process. The pickled sausages were stored at room temperature (~22–23°C) for the remainder of the challenge study (28 days) for each of the 3 replications. A total of 24 containers were utilized for each of the LAB and fungal treatment groups for the duration of the study, while 3 containers were used for the negative control group.

Sampling and microbial analysis

Sausages were sampled and analyzed to determine initial inoculation concentrations, pH, a_w, and salt concentrations of both the individual sausages and pickling brine. Three sausages were removed at the initial sampling (time 0) for each of the LAB- and fungal-inoculated samples, while one sausage from the negative control was sampled and evaluated for microbial analyses. Additional sausages (n = 1 per treatment per time point) were used to measure pH, a_w, and % salt for treatment and control groups. This sampling protocol was repeated for all treatment and control groups at various sampling times (2, 4, 6, 12, 24, 48, 72, 168, 336, 504, and 672 h) for each of the three replications.

To enumerate surviving LAB, yeast, and mold cultures on sausages at each sampling time, pickled sausages were aseptically removed from their respective containers, individually placed into 3500 ml filtered stomacher bags (Interscience, Rockland, MA), weighed, diluted with five times the volume of the sausage with sterile BPW, and stomached for 1.5 minutes at 360 rpm (Interscience Jumbo Mix Stomacher, Rockland, MA). The stomachate (~35 ml) of each pickled sausage was serially diluted in 9 ml of BPW and aliquots of 0.1 ml were spread plated in duplicate onto selective agars to enumerate both LAB and fungal cultures. Aliquots of 1 ml of the stomachate were spread plated in duplicate once viable counts reached the detection limit. MRS agar (Difco) was used for the enumeration of LAB cultures, while Dichloran-Rose Bengal Chloramphenicol agar (DRBC; Difco) was used for enumeration of yeast

and mold cultures. DRBC spread plates were incubated (upright) aerobically at 25°C for 3 to 5 days, while MRS spread plates were incubated aerobically (inverted) at 30°C for 48 h. For the negative control sausages, any naturally occurring microflora was monitored using Aerobic Count Plate (AC) and Yeast Mold (YM) Petrifilm™ (3M, St. Paul, MN). AC Petrifilm™ was incubated both aerobically and in microaerophilic conditions (5.0% O_2, 10% CO_2, 85% N_2) at 30°C for 48 to 72 h while YM Petrifilm™ was incubated aerobically at 25°C for 96 h.

Enrichment procedures

During each sampling of inoculated pickled sausage, 1 ml aliquots of stomachate and associated brine solution were transferred to 9 ml of YM broth and MRS broth for enrichments of fungi and LAB, respectively. Yeast and mold enrichments were incubated aerobically at 25°C for 3 days before plating onto DRBC plates, which were incubated at 25°C for 3 to 5 days. LAB enrichments were incubated aerobically at 30°C for 48 h before plating onto MRS agar plates, which were incubated at 30°C for 48 to 72 h.

Sausage and brine chemical analysis

Brine pH was measured using a Thermo Scientific Orion 4 Star pH ISE Benchtop meter with a connected Orion 9103BNWP Semi-Micro Combination pH Probe (Beverly, MA). Sausage pH was measured using a Thermo Scientific Orion 4 Star pH ISE Benchtop meter with an Orion 8163BNWP Ross Combination Spear Tip pH Electrode (Beverly, MA). The sausage designated for pH measurements were first cut in half, using a cross-sectional cut, after which the tip of the pH probe was inserted into the center of the cut half. The other portion of the cut sausage was used for water activity (a_w) and salt concentration analysis. A_w was analyzed using a water activity meter (Aqua Lab 4TE; Decagon Devices, Pullman, WA). Percent salt concentrations for sampled sausages and pickling brine were determined using Chloride Quantabs (Hach, Loveland, CO) according to a method previously described *(28)*.

Statistical analysis

Means of the 3 replications of LAB, yeast, and mold plate counts were calculated and converted to $\log_{10}$ CFU/g for statistical analysis. The limit of detection for the MRS and DRBC spread plates was 0.40 $\log_{10}$ CFU/g, while the limit of detection for the Aerobic Count and Yeast and Mold Petrifilm™ was 0.70 $\log_{10}$ CFU/g. Comparison of spoilage microorganism populations was determined at each time point, within and between groups, using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test at $\alpha = 0.05$. pH, a_w, and salt concentrations of sausages and brine were also determined, using a one-way analysis of variance (ANOVA) with Tukey's HSD test at $\alpha = 0.05$. Analysis of Variance (ANOVA) tests were conducted using SAS (SAS software version 9.3, SAS Institute Inc., Cary, NC).

RESULTS

Inoculated pickled sausages, with use of a cold-fill process and storage at room temperature (~22–23°C), experienced rapid and significant decreases ($P \leq 0.05$) in microbial populations measured in this study *(Table 3)*. Within the first 24 h of pickling, LAB populations were reduced ~5.51 $\log_{10}$ CFU/g, with a total reduction of 6.58 $\log_{10}$ CFU/g at the conclusion of 28 days of storage. After 7 days, LAB populations were reduced to undetectable levels. Enrichments of LAB-inoculated sausages and brine were negative after 21 days *(Table 3)*. Similarly, all mold and yeast populations were reduced to undetectable levels within the first 24 and 48 h of the study, respectively *(Table 3)*. Yeast and mold populations were reduced 3.89 $\log_{10}$ CFU/g and 4.09 $\log_{10}$ CFU/g, respectively, by day 28 of storage. Enrichments of yeast and mold populations were negative after 48 h *(Table 3)*, while brine enrichments were not positive for the organisms at any sampling period. Significant changes ($P \leq 0.05$) in pH of the inoculated sausages and brine were observed throughout storage *(Table 4)*. The pH of inoculated sausages and brine equilibrated to 3.92 ± 0.04 and 3.94 ± 0.06, respectively, in the 28-day study. Similarly, negative control sausages experienced a decline in sausage pH, coupled with an increase in brine pH *(Table 4)*. There was no significant difference observed in a_w throughout the study, with values ranging between 0.95 and 0.97. The salt concentration of cold fill pickled sausages rose slightly from 2.26% to 2.80%, while the salt concentration of the brine decreased from 4.86% to 4.30% over the course of the study. Average fat concentration assessed through the modified Babcock fat analysis procedure for all 3 replicates was 26.4%, with a range of 24.5 to 29.5% fat (data not shown).

DISCUSSION

The idea of combining several antimicrobial processes, often at sub-lethal levels, in an effort to increase the microbial safety of food products, is known as hurdle technology *(13)*. It is theorized that the combination of antimicrobial processes works better than any single intervention to inhibit the growth of potential spoilage or pathogenic microorganisms in foods. Typically, the process of pickling sausage utilizes heat, acetic acid, and salt, which in combination can increase the shelf life and microbial stability of the acidified meat product *(23)*.

Weak acids, like acetic acid ($pK_a = 4.76$), exert antimicrobial effects through the action of their undissociated form, which can diffuse through bacterial and fungi cell membranes, altering the homeostasis of the cell. In addition, the dissociated form of weak acids can concentrate within the cell, cause the buildup of protons (H^+), resulting in the acidification of the cytoplasm and cell interior *(12, 13)*. The accumulation of protons and subsequent decline in pH can

Table 3. Average counts (log_{10} CFU/g) of lactic acid bacteria (LAB), yeast (*Debaryomyces hansenii* and *Candida zeylanoides*) and mold (*Penicillium nalgiovense* and *Cladosporium cladosporioides*) inoculated onto sausages and their respective results for enrichment. Aerobic and yeast and mold counts of the negative control are included

Storage Time (hours)	LAB	LAB Enrichment	Yeast	Yeast Enrichment	Mold	Mold Enrichment	Aerobic Count (negative control)	Yeast & Mold (negative control)
0	6.98^{a}	+	4.29^{a}	+	4.49^{a}	+	1.36^{a}	≤ 0.70^{a}
2	3.76^{b}	+	0.85^{b}	+	2.24^{b}	+	1.36^{a}	≤ 0.70^{a}
4	3.46^{b}	+	0.61^{b}	+	1.45^{bc}	+	1.36^{a}	≤ 0.70^{a}
6	3.16^{bc}	+	≤ 0.40^{b}	+	1.10^{cd}	+	1.36^{a}	≤ 0.70^{a}
12	2.30^{cd}	+	≤ 0.40^{b}	+	0.53^{d}	-	0.80^{a}	≤ 0.70^{a}
24	1.63^{de}	+	0.62^{b}	-	≤ 0.40^{d}	-	0.86^{a}	≤ 0.70^{a}
48	0.43^{e}	+	≤ 0.40^{b}	-	≤ 0.40^{d}	-	0.86^{a}	≤ 0.70^{a}
72	1.15^{e}	+	≤ 0.40^{b}	-	≤ 0.40^{d}	-	0.86^{a}	≤ 0.70^{a}
168	≤ 0.40^{e}	+	≤ 0.40^{b}	-	≤ 0.40^{d}	-	≤ 0.70^{a}	≤ 0.70^{a}
336	0.45^{e}	+	≤ 0.40^{b}	-	≤ 0.40^{d}	-	≤ 0.70^{a}	≤ 0.70^{a}
504	0.55^{e}	+	≤0.40^{b}	-	≤ 0.40^{d}	-	≤ 0.70^{a}	≤ 0.70^{a}
672	≤ 0.40^{e}	-	≤ 0.40^{b}	-	≤ 0.40^{d}	-	≤ 0.70^{a}	≤ 0.70^{a}

$^{a\text{-}e}$ Different letters represent significant difference among values within each column ($P = 0.3690$). Log_{10} CFU/g values at each time point are the mean of (n = 9) 3 sausages/replication. Enrichment results are the sum of 3 sausage and 4 brine samples/replication.

"+" = positive enrichment result and "-" = negative enrichment result.

cause damage to cell membranes, denaturation of proteins, enzymes and DNA, and metabolic exhaustion *(14, 31)*. The accumulation of anions also is thought to contribute significantly to the inhibition of microorganisms, causing high turgor pressure, potential leakage from the cell, production of free radicals, and oxidative stresses *(8, 23, 24)*. It is also important to note that weak acids exhibit greater antimicrobial effects in lower pH environments *(2, 12)*. Therefore, the low pH of the brine used in this study (pH 2.72) likely aided in the reduction of LAB and fungal populations. In addition, since the pH of the brine solution used in this study was below the pK_a value of acetic acid, this step ensured that the majority of the weak acid was in the undissociated form and able to penetrate cell membranes.

The addition of salt in acidified food products is thought to increase the antimicrobial activity of acetic acid, by lowering the a_w and increasing osmotic pressure *(13)*. Although the addition of salt has inhibitory effects on bacteria and fungi, this process also can result in an environment which favors LAB *(4, 25)*. Although salt was a component of both the sausages and brine examined in this study, significant changes in a_w were not observed throughout the 28 days of storage. Therefore, any observed antimicrobial effects were not due to decreasing a_w. These results are similar to those observed by Richard and Cutter *(22)* and Scheinberg et al. *(26)*, who also noted no significant changes in a_w while investigating pickled eggs.

The decrease in spoilage microorganism populations coincided with the decrease in pH of the inoculated sausages within the first 24 h of storage in pickling brine. This result suggests that the total reduction and inhibition of these microorganisms was driven mainly by the decline in pH. This result is in agreement with Richard and Cutter *(22)*, who demonstrated a > 4.85 log_{10} CFU/ml reduction in hard cooked eggs inoculated with a cocktail of *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in 24 h at 22°C, with a similar associated drop in pH. The authors also noted that the reduction of pathogenic microorganisms was significantly higher ($P < 0.05$) in the hard cooked eggs stored at room temperature (22°C) than in eggs stored at refrigeration temperatures (4°C). Similar results were observed by Scheinberg et al. *(26)*. Results of the pickled egg research suggest that temperature is also an important factor in the acidification process. Studies by Leistner and Gorris *(15)* and Leistner *(14)* have also reported evidence supporting the

Table 4. pH of inoculated sausage and brine, and pH, a_w, and salt concentration of negative control sausages throughout 28 days of storage

	LAB		Yeast + Mold				Negative Control		
Storage Time (hours)	pH Sausage	pH Brine	pH Sausage	pH Brine	pH Sausage	pH Brine	a_w	Salt Concentration – Sausage	Salt Concentration – Brine
0	6.54^{ab}	2.72^{d}	6.52^{a}	2.72^{e}	6.44^{a}	2.61^{c}	0.97^{a}	2.26^{a}	4.86^{a}
2	6.80^{a}	3.40^{abcd}	6.62^{a}	3.03^{de}	-	-	-	-	-
4	6.54^{ab}	3.26^{bcd}	6.54^{a}	3.12^{cde}	-	-	-	-	-
6	6.32^{ab}	2.98^{cd}	6.55^{a}	3.23^{cde}	-	-	-	-	-
12	6.23^{ab}	3.12^{bcd}	6.33^{a}	3.03^{de}	6.26^{a}	3.08^{bc}	0.96^{a}	2.19^{a}	4.64^{a}
24	5.81^{b}	3.26^{bcd}	6.08^{a}	3.19^{cde}	6.09^{a}	3.17^{abc}	0.97^{a}	2.29^{a}	4.71^{a}
48	4.59^{c}	3.33^{bcd}	4.67^{b}	3.30^{bcde}	-	-	-	-	-
72	4.26^{c}	3.61^{abc}	4.21^{b}	3.39^{abcd}	-	-	-	-	-
168	4.13^{c}	3.93^{ab}	4.05^{b}	3.76^{abc}	3.97^{b}	3.64^{ab}	0.96^{a}	2.69^{a}	4.73^{a}
336	4.26^{c}	4.20^{a}	4.11^{b}	3.93^{ab}	-	-	-	-	-
504	3.89^{c}	3.95^{ab}	3.92^{b}	3.91^{ab}	3.88^{b}	3.82^{ab}	0.95^{a}	2.53^{a}	4.44^{a}
672	3.88^{c}	3.89^{ab}	3.96^{b}	4.00^{a}	3.96^{b}	3.94^{a}	0.96^{a}	2.80^{a}	4.30^{a}

$^{a\text{-}e}$ Different letters represent significant difference in values within each column ($P = 0.2062$). All numerical values are the mean of (n = 3) 1 sausage/replication).

hypothesis that temperature impacts the ability of acids and preservatives to produce antimicrobial effects in food systems.

Among the three groups of spoilage microorganisms utilized in the current study, select LAB cultures demonstrated the greatest acid tolerance, exhibiting higher populations throughout the study, when compared to select yeast or mold cultures *(Table 3)*. The addition of salt, decrease in pH, the reduction of aw, and brine injection have caused shifts in the microflora of meat products to favor LAB *(4, 10)*. The pickling process in this study may have created an environment which initially allowed LAB populations to persist longer than both the yeast or mold cultures. Noonpakdee et al. demonstrated that LAB have the potential to become more acid-tolerant if viable cells are exposed to sub-lethal acidic conditions for an extended period of time *(19)*.

In other studies, synergistic effects between decreased pH and increased sodium chloride concentrations also resulted in decreased yeast populations *(3, 34)*. The 5% salt and 5% acetic acid brine solution utilized in this study achieved significant reductions in yeast populations that suggest a synergistic inhibitory effect of the brine. Other studies have found that yeast populations can be decreased by the addition of as little as 1% acetic acid, and yeast growth can be entirely inhibited at concentrations of 3.5 to 4% *(35)*. Although molds can survive at various pH levels and salt concentrations (2–3% wt/wt; *16, 21*), these factors did not appear to aid in the survival or growth of select molds (*P. nalgiovense, C. cladosporioides*) used in this study. While previous studies have noted the abilities of LAB, yeasts, and molds to adapt, resist, and break down food preservatives, the acidity of the brine solution used in this study was likely too high for the microorganisms to adapt to or counteract its lethality *(9, 18, 19, 20, 27)*. To our knowledge, this is the first study that has measured the survival of spoilage microorganisms in a pickled meat product.

Furthermore, this study demonstrated the ability of a cold fill pickling process to inhibit the growth and survival of LAB, yeast, and mold on a pre-cooked RTE pickled sausage product. The use of industry standard sausage production parameters (casing type, diameter, formulation) and spoilage microorganisms associated with meat products, also greatly enhance the applicability of this study. The findings of this study will be most useful as Hazard Analysis Critical Control Point (HACCP) validation for manufacturers of pickled sausage that utilize a cold fill process. Future work by these authors will examine the efficacy of the cold fill pickling process on sausages inoculated with relevant strains of *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus*.

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