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GRAS Notification for the Use of Lactic Acid Bacteria to Control Pathogenic Bacteria in Meat and Poultry Products

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Division of Biotechnology and GRAS Notice Review

Volume 1 of 1

Submitted by:

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July 30, 2012

000001.001

Cover Letter

NPC

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July 27, 2012

Antonia Mattia, Ph.D., Director
Division of Biotech and GRAS Notice Review, (HFS 255)
OFAS/CFSAN/FDA
5100 Paint Branch Parkway
College Park, MD 20740

VIA: Federal Express

Re: GRAS Notification for Lactic Acid Bacteria (LAB) Mixture

Dear Dr. Mattia:

Pursuant to the proposed 21 CFR § 170.36 (c) Nutrition Physiology, on behalf of its affiliate Guardian Food Technologies, LLC, claims that the use of Lactiguard™ is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act because we have determined by scientific procedures that such use is Generally Recognized as Safe (GRAS) as a processing aid, acting as a competitive inhibitor to pathogenic organisms in fresh meat and poultry products and ready-to-eat products.

In accordance with proposed regulation, the following information is provided:

Proposed 21 CFR § 170.36 (c)(1)(i) The name and address of the notifier:

Guardian Food Technologies, LLC
4550 West 109th Street, Suite 110
Overland Park, KS 66211

Proposed 21 CFR § 170.36 (c)(1)(ii) The common or usual name of the notified substance:

Lactic Acid Bacteria (LAB) Mixture
Trade Name: Lactiguard™

Proposed 21 CFR § 170.36 (c)(iii) The applicable conditions of use of the notified substance:

For control of *E. coli, Salmonella., Campylobacter, Listeria* and other pathogenic bacteria in fresh chopped/ground, whole muscle cuts, and carcasses of meat and poultry, and RTE meat products.

Proposed 21 CFR § 170.36 (c)(1)(iv) The basis for the GRAS determination:

This GRAS determination is based on scientific procedures.

Proposed 21 CFR § 170.36 (c)(1)(v) Availability of information:

The data and information that are the basis for NPC's GRAS notification are available for FDA review by request at Phoenix Regulatory Associates, Ltd. Copies of references and any additional information may be obtained by contacting:

Dr. Clyde A. Takeguchi, Ph.D.
Phoenix Regulatory Associates, Ltd.,
21525 Ridgetop Circle, Suite 240
Sterling, VA 20166
Phone: (703)-406-0906
Email: ctakeguchi@phoenixrising.com.

Sincerely,

(b) (6)

Tony Arnold, President
Guardian Food Technologies, LLC

Attachment: Original and one CD copy

cc: Cdr. C. Rockwell, DVM, FSIS: letter and one CD copy
Phoenix Regulatory Associates, Ltd.

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GRAS Notification

GRAS Notification for the Use of Lactic Acid Bacteria to Control Pathogenic Bacteria in Meat and Poultry Products

1. Introduction

On June 6, 2005, Nutrition Physiology Corporation, LLC predecessor to Nutrition Physiology Company, LLC (NPC) submitted a GRAS notification that was designated as GRN No. 0171. In a letter dated December 7, 2005, FDA informed NPC that the agency had no questions at that time regarding NPC's conclusion that a mixture of lactic acid bacteria (LAB) is GRAS to control pathogenic bacteria in meat and poultry products. The LAB included *Lactobacillus acidophilus* (NP 35, NP 51), *Lactobacillus lactis* (NP 7), *and Pediococcus acidilactici* (NP 3). On July 20, 2006, NPC submitted a supplement to include ready-to-eat (RTE) meat products. FDA informed NPC on September 13, 2006, that the agency had no questions at that time regarding NPC's conclusion that the LAB mixture used to control pathogenic bacteria in RTE meat products is GRAS.

Since that time, NPC through its affiliate Guardian Food Technologies, LLC (Guardian) has characterized other LAB and has decided to replace NP 35 with NP 28 because NP 35 was difficult to produce commercially, and the new mixture with NP 28 provided the best combination to inhibit pathogens in raw meat and poultry products. Guardian is now submitting a new GRAS notification for the new mixture of LAB, *Lactobacillus acidophilus* (NP 28, NP 51), *Lactobacillus lactis* (NP 7), *and Pediococcus acidilactici* (NP 3).

2. Identity

2.1 Common and Usual Name

Lactic Acid Bacteria (LAB) Mixture
Trade Name: Lactiguard™

2.2 Identity of Microorganisms

2.2.1 *Lactobacillus acidophilus* (NP 28, NP 51)

LAB were isolated from cattle that were culture negative for *E. coli*. Isolate NP 28 (C 28) was identified as *L. acidophilus* using API biochemical analysis at Oklahoma State University (OSU) (Reilly and Gilliand, 1996).

NP 51 (LA-51; 381-IL-28) is a commercially available strain originally isolated from a calf and identified as *L. acidophilus* originally at OSU and again subsequently at University of Nebraska by Dr. Brashears (Brashears *et al.* 2005).

2.2.2 *Lactobacillus lactis* (NP 7)

LAB were isolated from alfalfa seeds and sprouts. Isolate NP 7 (L 7) was identified as *L. lactis* subspecies *lactis* by the API system and selected as the best candidate for a competitive exclusion product (Wilderdyke *et al.* 2004). Inhibitory activity was attributed to organic acids and peptides.

2.2.3 *Pediococcus acidilactici* (NP 3)

LAB were isolated from ready-to eat meats (ham and frankfurters). Isolate NP 3 (D 3) was identified as *P. acidilactici* and selected as one of the best candidates for a competitive exclusion product (Amézquita and Brashears 2002). Inhibitory activity was attributed to organic acids and peptides.

3. Manufacturing

Batches of bacteria have been cultured in a pilot plant setting. Commercialization will require scale-up of the culturing process.

3.1 Growing Conditions

Bacteria are cultured in media specifically designed for each organism by using a 1% inoculum at a temperature range between 35° C to 42° C. The base formulation for culturing microorganisms is the NPC-1 media, consisting of tripticase, casamino acids, yeast extract, and safe and suitable media components. Additions are made on a per strain basis. Glucose and lactate are made as additions to the media depending upon organism.

3.2 Production

The culture time for each strain varies but it takes approximately 20 h from inoculation until late stationary phase. The production process is summarized below and in the attached flow diagrams describing the 1) seed strain maintenance, 2) stock culture production, and 3) finished production [See Figures 1-3].

GRAS Notification – Use of Lactic Acid Bacteria (continued)
3. Manufacturing (continued)

Growth of Microorganism

- Obtain vial of seed bacterium.
- Grow in 10 ml tube of proper media at proper temperature for required time. Ensure purity by microscopy.
- Inoculate 0.5 ml into 500 ml bottle and grow. Ensure purity by microscopy.
- Inoculate 500 ml into 50 l fermentation vessel and grow. Ensure purity by microscopy.
- Inoculate 50 l into 5000 l fermentation vessel and grow.
- Yield estimated by in-fermentation vessel optical density monitoring.
  - Ensure purity by microscopy, cell count obtained by microscopy.
  - Ensure purity by dilution plating and and/or cell streak plating.

Concentration of Microorganism

- 5000 L (or desired volume) of product-filtered through 0.2 μmeter filter system. Filtration for 5000 L takes approximately 4 h.

Freeze Drying Product

- From filtered product, bacterial solids are mixed with lyoprotectants and cryopreservative, and bulking agents.
- Product is placed into freeze dry cabinets in shelves at 1 cm depth.
- Product is frozen to an internal temperature of -30° C, vacuum is applied and temperature is progressively increased.
- Freeze-dry time is approximately 72 h.
- Freeze dried product is removed from shelves and ground into a homogenous powder and stored at -80° C.

Viability and Product Purity

- Obtained by plate count, broth tube enumerations, or by live/dead staining.

Packaging

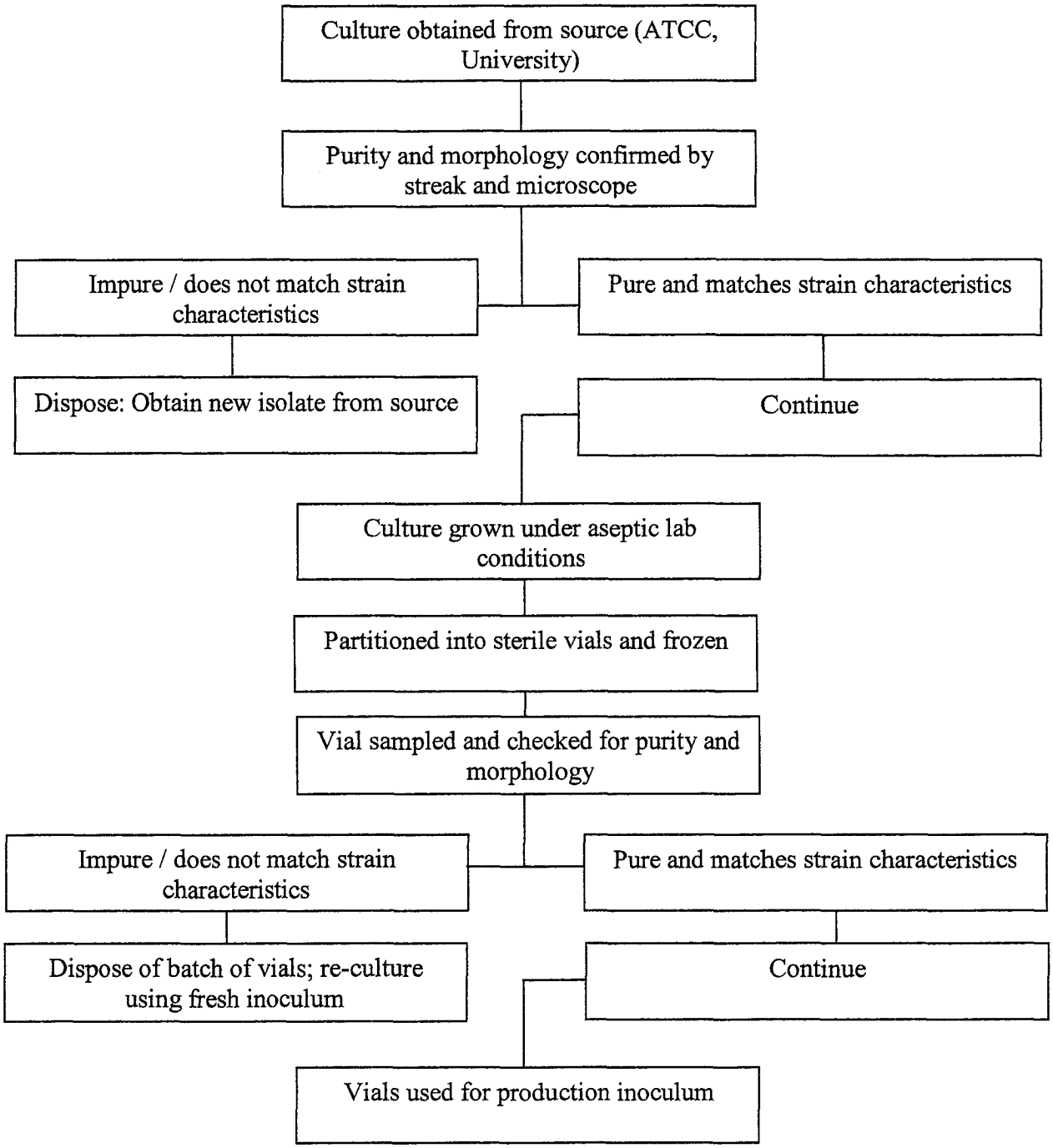
- Product is blended with a USDA approved carrier and packaged.
- Packaging is performed by automatic bagging machine with N_2 blowout.
- Final Product Cell counts are verified by enumeration process.

Mixtures

- Product is mixed and bagged prior to shipping to a customer.

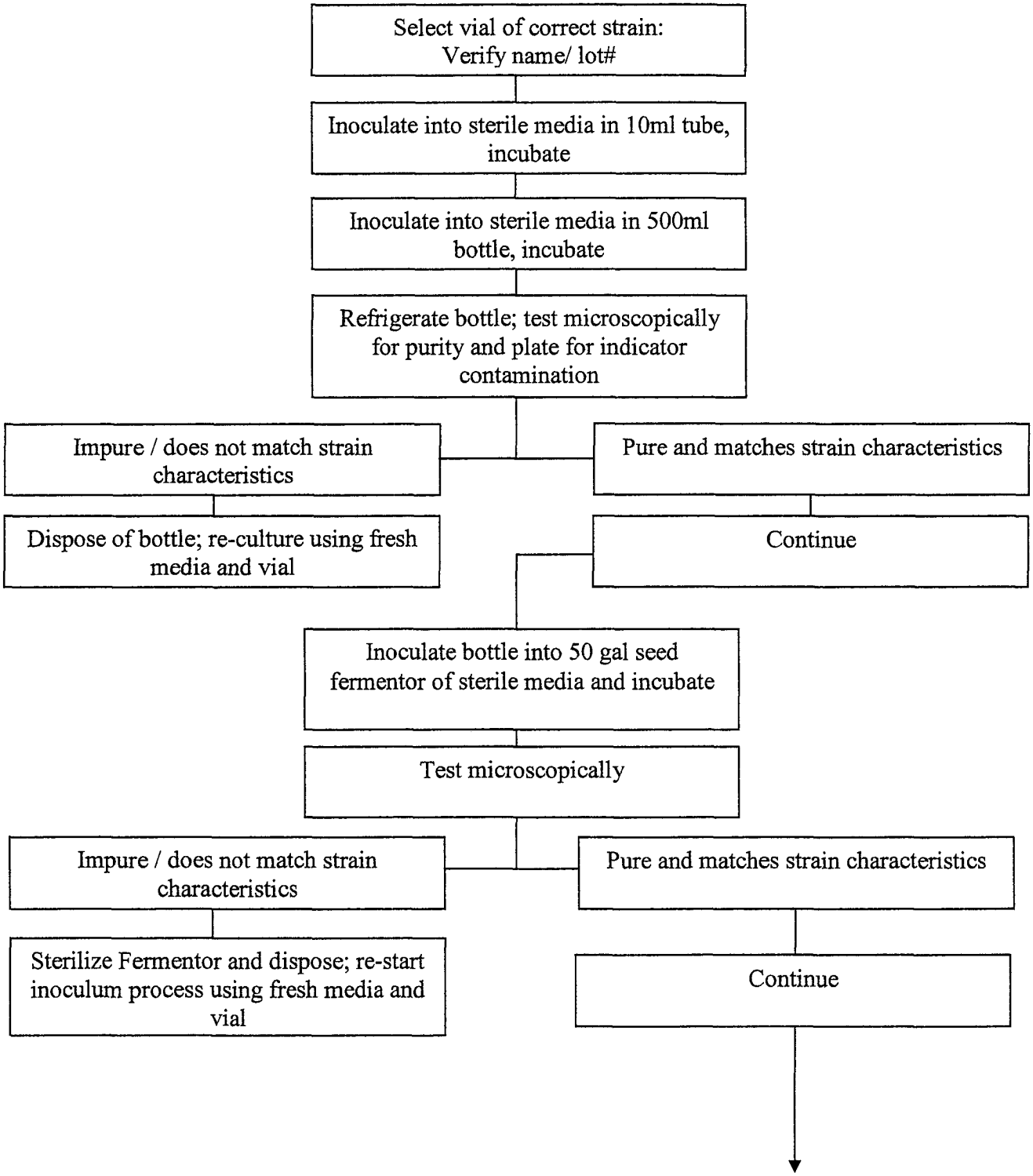
GRAS Notification – Use of Lactic Acid Bacteria (continued)
3. Manufacturing (continued)

Figure 1. Seed Strain Maintenance



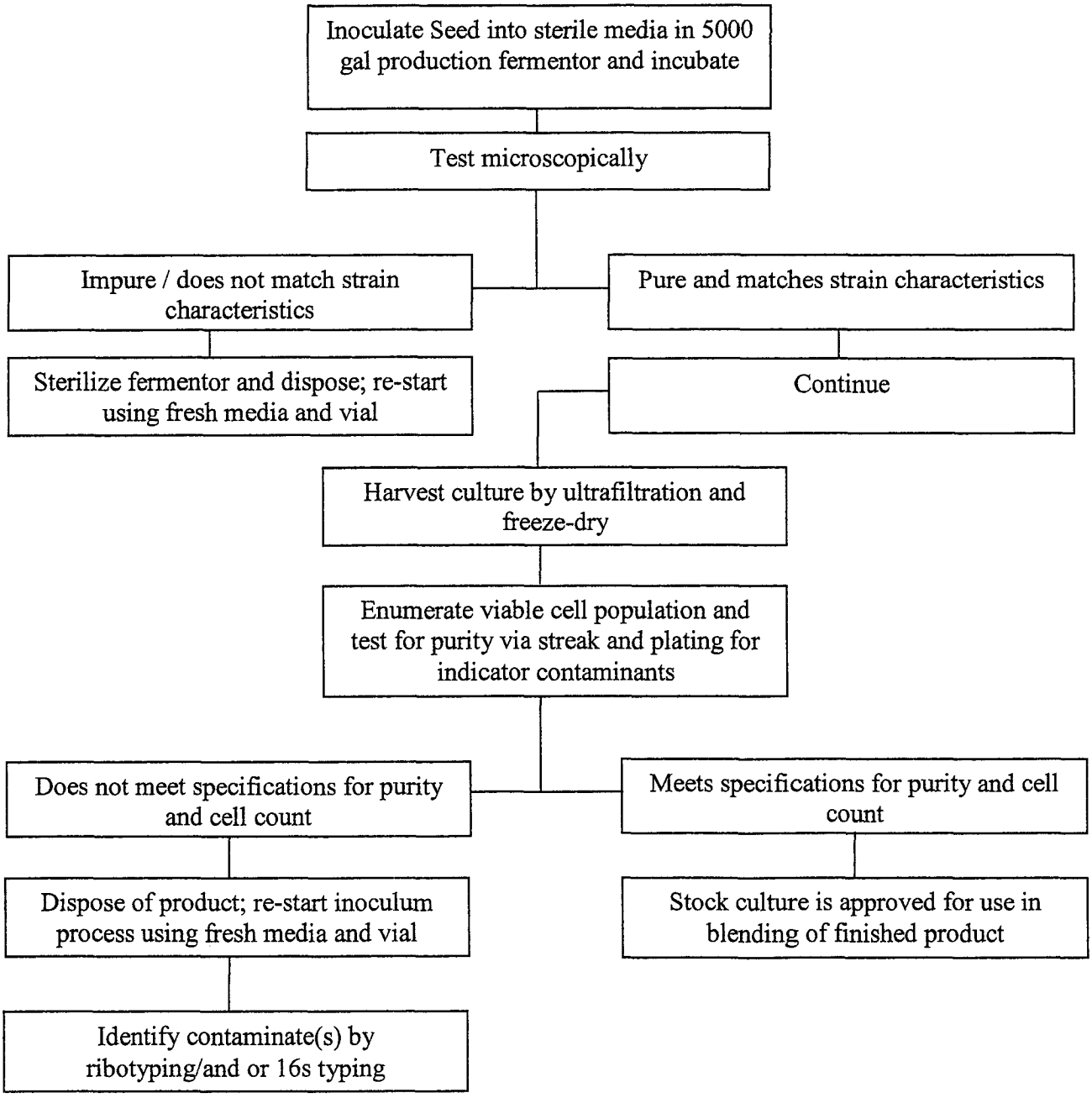
GRAS Notification – Use of Lactic Acid Bacteria (continued)
3. Manufacturing (continued)

Figure 2. Stock Culture Production



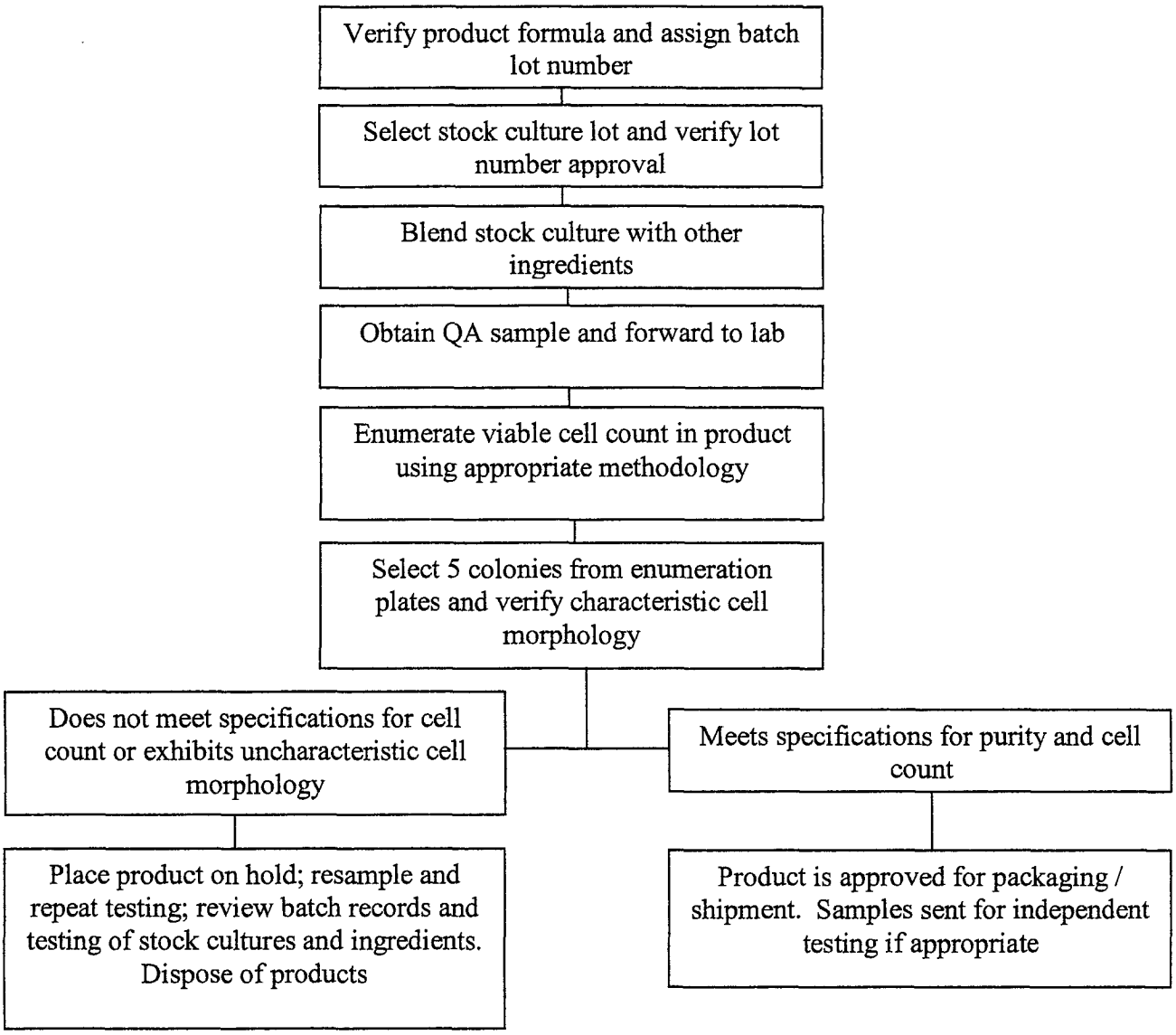
GRAS Notification – Use of Lactic Acid Bacteria (continued)
3. Manufacturing (continued)

Figure 2. Stock Culture Production
(continued)



GRAS Notification – Use of Lactic Acid Bacteria (continued)
3. Manufacturing (continued)

Figure 3. Finished Production



4. Conditions of Use

4.1 Purpose

For control of *E. coli, Salmonella, Campylobacter, Listeria* and other pathogenic bacteria in fresh chopped/ground, whole muscle cuts, carcasses of meat and poultry, and RTE meat products.

4.2 Level of Use

10^6 to 10^8 cfu total lactobacilli/g or cm^2

4.3 Population Expected to Consume the Substance

General population

5. Safety and Effectiveness

5.1 History of Use

LAB species have a long history of safe use in food and food products. Currently, prior-sanctioned uses of LAB starter cultures are allowed for breads (21 CFR Part 136 Bakery products), cultured milk products (21 CFR 131.112 Cultured milk, 131.160 Sour cream, and 21 CFR 131.200 Yogurt), cheese (21 CFR 133.128 Cottage cheese and 21 CFR 133.113 Cheddar cheese), and bacon and sausage products (9 CFR 424.21 Use of food ingredients and sources of radiation).

The use of LAB as competitive inhibitors to undesired or pathogenic microorganisms has been known for several decades. FDA allows LAB and other safe and suitable microorganisms as direct-fed microorganisms in animal feed (36.14 Direct-fed microorganisms; Official Publication, AAFCO, 2011) and as probiotics in dietary supplements. In recent years, the use of LAB as "protective cultures" rather than starter cultures have gained importance. Brashears *et al.* (2005) have reviewed their uses in animal feeding to improve food safety. They state that this concept of microbial antagonism or microbial interference is based on the inhibition of undesired or pathogenic microorganisms by competition for nutrients, by the production of antimicrobial metabolites or by other specific mechanisms. The protective metabolites may include substances such as lactic acid, acetic acid, ethanol, hydrogen peroxide, bacteriocins, and other small molecular weight metabolites.

These metabolites are normally found in traditional foods cultured with LAB and are considered as GRAS. In addition, some of these substances are listed in GRAS and food standard regulations. Lactic acid is affirmed as GRAS (21 CFR 184.1061 Lactic acid) and allowed in standardized foods such as jams and jellies (21 CFR 150.141 Artificially sweetened fruit jelly and 150.161 Artificially sweetened preserves and jams), cheeses (21 CFR Part 133 Cheeses and related cheese products), and in pickles. Acetic acid is a food (vinegar) and GRAS (21 CFR 182.1 Substances that are generally recognized as safe and 184.1005 Acetic acid) and is allowed in cheeses (21 CFR Part 133 Cheeses and related cheese products). Hydrogen peroxide is allowed in cheese making (21 CFR 184.1366 Hydrogen peroxide and 133.113 Cheddar cheese) and in processed foods and ingredients (21 CFR 160.105 Dried eggs, 160.145 Dried egg whites, 160.185 Dried egg yolks, 172.814 Hydroxylated lecithin, 172.892 Food starch-modified). In addition, FDA has allowed the use of nisin, an antimicrobial peptide derived from certain strains of *Streptococcus lactis* (reclassified *Lactococcus lactis* subsp. *lactis)* in processed cheese products (21 CFR 184.1538 Nisin preparation), and from *Lactococcus lactis* subsp. *lactis* as a GRAS substance for ready-to-eat meat and poultry products, and egg products (GRN 000065 Nisin; FSIS Directive 7120.1, 4/6/12).

5.2 Competitive Exclusion

In FCN 0171, NPC reported that a cocktail with four LAB (NP 51, NP 3, NP 7, and NP 35) added to pathogen-inoculated ground beef resulted in a 2.0 log reduction of *E. coli* O157:H7 in 3 days and a 3 log reduction after 5 days of storage. *Salmonella* was reduced to non-detectable levels after 5 days, and sensory studies on uninoculated samples indicated that there were no adverse effects on the sensory properties of the ground beef (Smith et al., 2005).

In this submission, Guardian has compared the old LAB cocktail formulation with several combinations and demonstrated that cocktail number 3 that replaces NP 35 in the old formulation with NP 28 provides the best results for inhibiting pathogens in raw meat and poultry. Zhang et al (in press) has evaluated the inhibition activity of four different LAB cocktails (1) NP 51 and NP 28, (2) NP 51, NP 28 and NP 3, (3) NP 51, NP 28, NP 3, and NP 7, (4) NP 51, NP 3, NP 7 and M 35 against foodborne pathogens commonly associated with ground beef, ground turkey and chicken legs during refrigerated storage.

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

The results indicated that the LAB cocktail number 3 (new formulation) (ca. 10^8 cfu/g) displayed the strongest inhibition on ground beef against *Escherichia coli* O157:H7 (*E. coli* O157:H7), *Salmonella* and non-O157 STECs among four LAB cocktails. The effect of the dose of the new formulation on inhibition activity on ground beef, ground turkey and chicken legs varied. In ground beef, the new formulation gave a 1.5 log reduction of all three pathogens at a dose of 1 x 10^8 cfu/g after 5 days. In ground turkey, the new formulation resulted in a reduction of *Campylobacter* by 1.4 log cycles and a reduction of *Salmonella* by 1.3 log cycles at a dose of 1 x 10^7 and 1 x 10^9 cfu/g, respectively. In chicken legs, the new formulation achieved an almost 1.0 log reduction of both *Salmonella* and *Campylobacter*.

5.3 Spoilage

Packaging studies were conducted by Hoyle and coworkers with the old formulation (Hoyle 2005; Hoyle, Brashears and Brooks 2005) comparing traditional packaging and MAP packaging of beef patties at refrigeration (0° C) and abusive temperatures (10° C). The studies were conducted to determine if LAB masked color and odor changes typically associated with the spoilage of ground beef displayed under refrigerated (0° C) or abusive (10° C) temperatures. Microbial and sensory analyses were conducted to determine spoilage endpoints. Packaging consisted of traditional (foam trays overwrapped with permeable film) and MAP packaging (80% O_2 and 20% CO_2). To mimic industry practice, one-half of the MAP samples contained 1000 ppm added rosemary oleoresin. Packages were stored in display cases with a light intensity of approximately 1900 lux.

Samples displayed at 0° C were collected at 0, 24, 48, 72 and 48 hours. Samples displayed at 10° C were collected at 0, 12, 24 and 36 hours. Color tests were conducted on 48 hr and 36 hr samples. Sensory and odor panels were conducted on all sampling intervals. After panel testing, half of the patties were used for microbial analysis and the other half assayed for thiobarbituric acid (TBA) assay. The researchers used six media to isolate and enumerate microorganisms present in the samples. They were Trypticase Soy Agar (nonfastidious and fastidious microorganisms), Pseudomonas F Agar (*Pseudomonas aeruginosa* and other fluorescin-producing pseudomonads), YM Agar (yeasts, molds and other aciduric microorganisms), Violet Red Bile Agar (coliforms), Lactobacilli MRS Agar (*Lactobacillus* spp.), and STAA Agar with supplement SR151E (*Brochothrix thermosphacta*).

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

At 0° C, traditionally packaged LAB samples had significantly lower yeast and mold (YM) counts than controls throughout display. Among traditional packages, there were no significant differences in coliform, *Brochothrix thermosphatca* (BT), and *Pseudomonas* spp. counts between LAB treatments. At abusive storage temperatures, there were no significant differences in coliform, YM, BT, and *Pseudomonas* spp. counts between traditionally packaged LAB treatments. At 0° C and 10° C, total plate counts and LAB populations in both traditional and MAP packaged inoculated samples were significantly higher than the control. In MAP packaging, no significant differences existed between LAB treatments for YM, coliform, and *Pseudomonas* spp. Samples containing oleoresin had significantly lower coliform and total plate counts at both temperatures. No significant differences in sensory, color, and odor existed between LAB and controls for traditional and MAP, indicating spoilage was not masked. Furthermore, results indicate rosemary oleoresin inhibits spoilage organism growth in modified atmosphere systems.

Addition of LAB to ground beef at refrigeration temperatures did not significantly ($P > 0.05$) affect the ground beef color in patties stored at 0° C when evaluated by trained panelists (Hoyle 2005). However, the trained panelists did detect a significant ($P < 0.05$) difference over time in patty color. For patties stored at 10° C, trained panelists detected a significant ($P < 0.05$) difference in patty color over time, but no significant ($P > 0.05$) were found between treatments. Although there was a significant difference in color when patties were stored over time, both consumer and trained panelists did not see a significant ($P > 0.05$) difference between uninoculated patties and inoculated patties.

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

Odor panels were also conducted at sampling times. The trained panelists were asked to identify how strong the smell was and then characterize the smell. Consumer panelists were asked if the meat smelled fresh and would they consume the meat. The trained odor panel determined there was a significant ($P < 0.05$) difference in odor when the patties were stored at 0° C over the sampling period. However, there was not a significant ($P > 0.05$) difference between the uninoculated and inoculated patties stored at 0° C. At 10° C, trained panelists also determined there was a significant ($P < 0.05$) difference in patty odors over time, but did not find a significant difference ($P > 0.05$) between treatments. Consumer panelists responses were significantly ($P < 0.05$) different for patties stored at 0° C over time, but the responses were not significantly ($P > 0.05$) different between treatments. In addition, consumer panelists responses were significantly ($P < 0.05$) different over time for ground beef patties stored at 10° C, but the responses were not significantly ($P > 0.05$) between treatments. As the storage period progressed, consumer responses showed the odor of the meat to not be fresh and those consumers were less likely to eat the patties they had smelled. In addition, as the storage time progressed, trained panelists determined that the strength of the odor had increased.

A study was conducted to determine the effect of ground beef packaged under modified atmosphere conditions consisting of 80% oxygen and 20% carbon dioxide (Hoyle 2005). The ground beef was divided into four different treatment groups which included an uninoculated control, LAB only, LAB with rosemary oleoresin, and an uninoculated control with rosemary oleoresin. A portion of each treatment group was displayed under refrigeration temperatures (0° C) and the remainder was displayed under abusive temperatures (10° C).

For those samples displayed at refrigeration temperatures, samples with added LAB had a significantly higher ($P < 0.05$) total plate count than those without LAB. This is due to the inoculation of the samples with the bacteria. In addition, total plate counts for samples with rosemary oleoresin were significantly lower ($P < 0.05$) than the controls, 4.37 and 4.67, respectively. The LAB samples containing rosemary oleoresin had a significantly higher population of LAB than the other samples, while the control samples without rosemary oleoresin had a significantly lower population of LAB than the remaining samples. There were no significant differences between all treatments in populations of *Pseudomonas* spp. and YM counts. While there was no significant differences in coliform populations for LAB samples, samples containing rosemary oleoresin had a significantly lower coliform count than those samples without the oleoresin.

GRAS Notification – Use of Lactic Acid Bacteria (continued)
5. Safety and Effectiveness (continued)

Trained sensory panelists for samples stored at refrigeration temperatures did not detect differences between samples containing LAB and those that do not. However, the panelists did detect significant differences between samples with or without rosemary oleoresin over the display period. Trained panelists did not detect differences in odor between LAB and control samples, but did detect significant differences in samples containing rosemary oleoresin. No significant differences were found between controls and samples containing LAB by consumer panelists, but they did detect significant differences between samples with rosemary oleoresin and controls throughout the display period. Significant differences in consumer odor panels were found for samples with rosemary oleoresin and controls, but samples with LAB and controls were not different statistically. Hunter color analysis did not detect significant differences for LAB and control samples. L values were not significantly different between samples with rosemary oleoresin and controls. However, rosemary oleoresin and control sample A values were significantly different. Throughout the storage period, B values for samples containing rosemary oleoresin and controls were significantly different.

For those samples stored at abusive temperatures, LAB inoculated samples with and without rosemary oleoresin had significantly higher total plate counts than control samples with and without the oleoresin. The LAB populations throughout the display period were significantly higher in samples with LAB than those without. In addition, significant differences were found between samples with oleoresin and controls throughout the storage period. No significant differences in all treatments were found in *Pseudomonas* spp. Significant differences were found in coliform and YM counts between combinations of LAB and rosemary oleoresin samples and controls.

Trained panelists detected significant differences in color between samples with oleoresin and controls over time, but did not detect differences between LAB samples and controls. No significant differences were found in odor between LAB samples and controls, but trained panelists did detect differences in samples with rosemary oleoresin and controls throughout the storage period. Consumer panelists also found significant differences in color between samples with oleoresin and controls throughout the storage period, but no significant differences were found between LAB samples and controls. Consumer odor panels found significant differences in rosemary oleoresin samples and controls, but did not find differences between LAB samples and controls.

Initial TBA values were not significantly different between treatments. After 24 and 36 hours of display, the treatment groups without added oleoresin had significantly ($P < 0.05$) higher TBA values than those treatment groups with added resin. These results indicate that the addition of rosemary oleoresin slowed lipid oxidation in the presence or absence of LAB.

5.4. RTE Meat Products

Amezquita and Brashears (2002) isolated 49 strains of LAB from commercially available ready-to-eat (RTE) meat products and were screened for their ability to inhibit the growth of *Listeria monocytogenes* at refrigeration temperatures. The three most inhibitory strains were identified as *Pediococcus acidilacti* (D3), *Lactobacillus casei* (D6), and *Lactobacillus paracasei* (15) and used in the study. Four strains of strep^R *L. monocytogenes* were combined for the pathogen cocktail.

The antilisterial activity quantified in associative cultures of the three inhibitory strains at 5° C for 28 days resulted in a 3.5 $\log_{10}$ cycle reduction compared to its initial level.

A cocktail combining the three strains was used on 1) a commercially available frankfurter, 2) a pork-based laboratory –produced frankfurter, and 3) a commercially-available cooked ham. The products were treated a) with the pathogen, b) with both the pathogen and LAB, c) or with the LAB, and compared to the background control. The treated products were packaged and stored at 5° C for 28 days. The pathogen count was 4.2, 4.7, and 2.6 $\log_{10}$ cycles lower than controls, respectively, with the numbers of LAB increasing by only 1 $\log_{10}$ cycle.

There was no significant difference in the antilisterial activity detected in frankfurters whether the LAB strains were used individually or as combined cultures. The results of the triangle test on frankfurters treated with the D3 (NP 3) strain compared to control showed no significant differences in sensory and quality changes during the 8-week evaluation period. The authors stated that the use of this competitive inhibition method represented a potential antilisterial intervention in RTE meat products, because it did not grow but continued to produce metabolites that inhibited the growth of the pathogen at refrigeration temperatures without causing sensory changes.

6. Exposure

In order to estimate the amount of LAB and metabolites are consumed, we have used the product category and RACC values from 21 CFR 101.12, including 55 g as serving size for RTE meat products (similar to sausage products) and ¼ lb (113 g) as serving size for ground meat. We have assumed that there are 10^8 cfu of LAB/g of food product, an average consumption of products per week, and that all ground meat and poultry products will contain LAB and metabolites. However, because such ground meat and poultry products will be cooked prior to consumption, there is no added exposure to LAB from these products. The LAB population will be about the same when consuming RTE meat products with or without LAB treatment because there will be minimal change in LAB growth at refrigerated temperatures.

Based on these assumptions, we estimate that there may be no increase in exposure due to use of LAB as a competitive inhibitor in fresh meat and poultry products and in RTE meat products.

Table 1. Estimated Consumption Value for LAB

Food	Serving size, g	LAB/g	Frequency/wk, average	LAB/serving x 10^8
Sausage products	55	10^8	2	110
Yogurt, etc.	225	10^8	2	450
Cheese	30	10^8	2	60
Pickles	30	10^8	1	30
Dietary suppl	1 cap/tab	10^8 to $5x10^8$	2-3/da 14/wk	70
Total				**720**
Ground meats*	113	10^8	3	0
RTE meat products	55	10^8	2	110

*cooked prior to consumption

GRAS Notification – Use of Lactic Acid Bacteria (continued)

7. Basis for Safety-Conclusion

There is a long history of safe use of LAB as starter cultures in traditional fermented foods, other food products and dietary supplements. The LAB have also been used as competitive inhibitors in feed for food-producing animals.

Although the technical effects of LAB have been known for a long time, the use as a protective culture in food products has become more common and was the basis for NPC's GRN 0171 that identified four LAB cultures used in the original LAB formulation. This new formulation replaces NP 35, a *Lactobacillus acidophilus* strain, with another safe and suitable strain, NP 28. The new formulation displayed the strongest inhibition on *E. coli* 0157.H7, *Salmonella* and non 0157 STECs in ground beef, and *Campylobacter* and *Salmonella* in ground turkey and on chicken legs. These LAB cultures will be produced using safe and suitable ingredients and standard bacteria culturing processes.

Packaging and spoilage studies have confirmed that the use of LAB mixtures does not mask spoilage in traditional and MAP packaged beef patties at refrigerated and abusive temperatures. Because meat and poultry products will be cooked prior to consumption, there is no added exposure to LAB from these products.

Studies on RTE meat products demonstrated that the use of LAB as a competitive inhibitor to control pathogenic microorganisms in RTE meat products is safe and effective, and will not increase consumer exposure to LAB.

Therefore, based on the history of use of LAB as starter cultures, identity of the individual strains, studies using LAB as protective cultures in fresh meat and poultry products and RTE meat products, and the estimated consumption of these products, Guardian Food Technologies, LLC concludes that the use of LAB mixtures as a competitive inhibitor for pathogenic microorganisms is considered generally recognized as safe.

8. References

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Zhang, Q., J.C. Brooks, D.C. Campos, and M.M. Brashears, in press. Inhibition of food-borne pathogens in ground beef, ground turkey and chicken legs using lactic acid bacteria cocktails. *Meat Science.*

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Inhibition of food-borne pathogens in ground beef, ground turkey and chicken legs using lactic acid bacteria cocktails

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Abstract

The microbial inhibition of Lactic Acid Bacteria (LAB) cocktails on ground beef, ground turkey and chicken legs against foodborne pathogens was determined. A variety of combinations of NP51, NP28, NP7, NP3 and NP35 were examined to formulate the best combination to inhibit pathogens in raw meat and poultry. The results indicated that the LAB cocktail of NP28+NP51+NP3+NP7 displayed the strongest inhibition on ground beef against *Escherichia coli* O157:H7 (*E. coli* O157:H7), *Salmonella* and non-O157 STECs among four LAB cocktails. In ground beef, the 51, 3, 7, 28 combination gave a 1.5 log reduction of all three pathogens at a dose of 1 x 10^8cfu/g after 5 days. In ground turkey, the same combination resulted in a reduction of *Campylobacter* by 1.4 log cycles and a reduction of *Salmonella* by 1.3 log cycles at a dose of 1 x 10^7 and 1 x 10^9cfu/g, respectively. In chicken legs, the combination achieved an almost 1.0 log reduction of both *Salmonella* and *Campylobacter*.

Keywords: Lactic acid bacteria; Antimicrobial activity; Raw meat; *Salmonella*; *E. coli* O157:H7; Non O157 STECs; *Campylobacter*

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52 **1. Introduction**

53 *Escherichia coli* O157:H7 (*E. coli* O157:H7), *Salmonella*, and *Campylobacter* are
54 the most commonly reported enteric bacterial pathogens causing illness in humans
55 (Samadpour et al., 2006; Cook et al., 2009). *E. coli* O157:H7 is a significant human
56 pathogen and has been linked to several foodborne-disease outbreaks involving
57 ground beef. It is estimated that approximately 73,000 cases of illness are caused by
58 this pathogen annually in the United States and that approximately 85% of these cases
59 result from foodborne transmission (Niebuhr and Dickson, 2003). *Salmonella* is
60 responsible for the majority of cases of foodborne bacterial gastroenteritis and has
61 been implicated in several outbreaks (Min et al., 2006). Lactic acid bacteria (LAB) are
62 a group of Gram-positive bacteria united by a constellation of morphological,
63 metabolic, and physiological characteristics (Mohankumar and Murugalatha, 2011).
64 LAB play a key role in food fermentations where they provide taste and texture, and
65 increase the nutritional value of fermented foods such as yoghurt, ripened cheese and
66 meat products, as well as some vegetables (Concha-Meyer et al., 2011; Narbutaite et
67 al., 2008). A large amount of research has focused on LAB for food biopreservation
68 and some has shown that LAB and their metabolites (which are generally regarded as
69 safe by the FDA and European Union) could inhibit and reduce food-contaminating
70 microorganism growth on meat, meat products and seafood (Silva et al., 2002). It has
71 been shown that antimicrobial substances produced by LAB include organic acids,
72 hydrogen peroxide, diacetyl and bacteriocins (Abrams et al., 2011). Brown et al.
73 (2011) found LAB could inhibit *E. coli* O157:H7 and *Clostridium sporogenes* in
74 spinach packaged in modified atmospheres. Maragkoudakis et al. (2009) also found a
75 significantly reduced growth of *Listeria monocytogenes* and *Salmonella enteritidis* in
76 raw chicken meat with the combination of *Enterococcus faecium* PCD71 and

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77 *Lactobacillus fermentum* ACA-DC179. Although there have been many reports on the
78 use of LAB combinations to inhibit pathogens in product in recent years (Echeverry et
79 al., 2010; Smith et al., 2005; Concha-Meyer et al., 2011), to date there has not been a
80 comprehensive study comparing the efficacy of a variety of combinations of LAB to
81 inhibit a wide range of pathogens in raw meat and poultry. Therefore, the objective of
82 this study was to evaluate the inhibition activity of LAB cocktails against foodborne
83 pathogens commonly associated with ground beef, ground turkey and chicken legs
84 during refrigerated storage.

85 **2. Materials and Methods**

86 *2.1 Microbiological cultures and growth conditions*

87 Experiments involving the four different pathogens were performed. Two strains of
88 *Salmonella typhimurium* (ATCC 14028 and Heioel Sheloon 3347-1,originally isolated
89 from cattle), two strains of *E. coli* O157:H7 (A4 966 and A5 944,originally isolated
90 from cattle), two strains of *Campylobacter* (*jejuni* ATCC 43470 and *coli* ATCC 43485,
91 originally isolated from turkey) and two strains of non-O157 STECs (EC 026 and EC
92 0111, originally isolated from cattle) were used for this study. These cultures were
93 obtained from the stock culture collection at Texas Tech University. For the
94 *Salmonella*, *E. coli* O157:H7 and non-O157 STECs studies, frozen stock cultures
95 were grown individually in 10 ml of Trypticase soy broth (TSB) at 37 °C for 24 h and
96 passed explain? three times prior to experimental use. For the *Campylobacter* study,
97 two frozen stock cultures were grown in Bolton broth base (Neogen Corp., Lansing,
98 Mich.) containing 5% horse blood at 37 °C for 4 h and at 42 °C up to 24 h. The
99 cocktail combinations of the pathogens were prepared by mixing equal volumetric
100 parts of each freshly cultured strain into a sterile container.

101 *2.2. Preparation of LAB cocktails*

102 Five commercially prepared LAB strains with antimicrobial activity from Culture
103 System Inc. (Mishawaka, Ind., U.S.A.) included NP28, NP51, NP3, NP7 and NP35.
104 Strain NP 51 is *Lactobacillus acidophilus* originally isolated from cattle, strain NP35
105 is *Lactobacillus crispatus* also isolated from cattle, strain NP3 is *Pediococcus*
106 *acidilactici* isolated from commercial hot dogs, strain NP7 is *Lactococcus lactis subsp*
107 *lactis* isolated from alfalfa sprouts and strain NP28 is *Lactobacillus acidophilus*
108 originally isolated from cattle. These cultures were prepared and packaged
109 commercially in freeze-dried 10-g portions in maltodextrin and, upon arrival at Texas
110 Tech University laboratory, maintained at –20 °C until use. To prepare the LAB
111 cocktails, five LAB strains (10 g) were individually suspended in 90 ml of buffered
112 peptone water (BPW, Oxoid) and they were mixed with equal volumetric parts of each
113 BPW suspension to prepare LAB cocktail. Four different combinations of LAB
114 cocktails of (1) NP51 and NP28, (2) NP51, NP28 and NP3, (3) NP51, NP28, NP3 and
115 NP7, (4) NP51, NP3, NP7 and M35 were held at ambient temperature for 1 h to allow
116 for metabolic activity of the cultures prior to use (Brown et al., 2011).

117 *2.3 Antimicrobial treatment application on ground beef: Four LAB cocktails*

118 Antimicrobial treatments included the four LAB cocktails described above. Freshly
119 prepared ground beef was obtained from Texas Tech Meat Laboratory which had not
120 been previously subjected to any antimicrobial treatments during the slaughter or
121 preparation process.

122 During each of three replications for each pathogen, 500 g of ground beef was
123 processed. Ground beef (500 g) was inoculated individually with *E. coli* O157:H7,
124 *Salmonella* and non-O157 STEC cocktails, with a target populations of approximately
125 3.0×10^3 cfu/g for each pathogen. After inoculation, the ground beef was then
126 individually inoculated with four different combinations of the LAB cocktails. The
127 desired concentration of 1.0×10^8 cfu/g was verified by plating serial dilutions onto
128 De Man, Regosa, and Sharpe agar (MRS agar, EMD). Control samples of ground beef
129 were taken before intervention application. After attachment, ground beef for each set

130 of treatments was divided into equal portions (50 g) and stored at 4 °C.

131 2.4 *Antimicrobial treatment application on ground beef: dose-response of the LAB*
132 *cocktail*

133 This study followed the same protocol as the previous study except that each
134 portion ofground beef was individually inoculated with the same LAB cocktail at
135 three different concentrations (1.0×10^7, 1.0×10^8 and 1.0×10^9 cfu/g).

136 *2.5 Antimicrobial treatment application on ground turkey: dose-response of the LAB*
137 *cocktail*

138 Freshly prepared ground turkey was obtained from Texas Tech Meat Laboratory
139 and had not been previously subjected to any antimicrobial treatments. Ground turkey
140 (500 g) was inoculated individually with cocktail mixtures of *Salmonella* and
141 *Campylobacter*, with target populations of pathogen ataround 3.0×10^3 cfu/g. After
142 inoculation, the ground turkey was individually inoculated with the same four LAB
143 cocktail combinations used in previous ground beef study. The desired concentrations
144 of LAB were 1.0×10^7, 1.0×10^8 and 1.0×10^9 cfu/g and this wasverified by plating
145 serial dilutions onto MRS agar. Control samples of ground turkey were taken before
146 intervention application. After attachment, ground turkey for each treatment was
147 divided into equal portions (50 g) and stored at 4 °C.

148 *2.6 Antimicrobial treatment application on chicken legs: dose-response of the LAB*
149 *cocktail*

150 One hundred fresh chicken legs (120 ± 10 g) with skin were purchased from a local
151 supermarket on day one of study initiation. Fifty chicken legs were inoculatedwith
152 *Salmonella* and 50 with *Campylobacter* with a target population of approximatelyt 3.0
153 $\times 10^3$ cfu/g. After inoculation, the chicken legs were individually inoculated with the
154 same LAB cocktail used in previous study. The desired concentrations of LAB at 1.0

155 $\times 10^7$, 1.0×10^8 and 1.0×10^9 cfu/g were verified by plating serial dilutions onto MRS
156 agar. Control samples of chicken legs were taken before intervention application.
157 After attachment, three chicken legs from each treatment and all samples were stored
158 at 4 °C.

159 *2.7. Microbiological analysis*

160 Samples were evaluated on days 0, 1, 2, 3 and 5. One package from each study and
161 treatment combination (ground beef and turkey) was sampled at random and 25 g
162 samples were weighed into filtered homogenizer bags (Model 400 Bags 6041,
163 Stomacher Lab System Seward Limited, London, UK). Buffered Peptone Water
164 (BPW, 225 ml) was added to each bag. Samples were stomached (Model 400, Seward
165 Medical, London, UK) at 230 rpm for 2 min and serially diluted for enumeration. Two
166 chicken legs from each treatment at each time point were randomly sampled into
167 filtered homogenizer bags and BPW (250 ml) was added to each bag. Samples were
168 hand stomached for 5 min and serially diluted for enumeration. *E. coli* O157:H7 and
169 non O157 STECs dilutions were respectively plated onto MacConkey agar with
170 tryptic soy agar (TSA) layer and incubated at 37 °C for 24 h. Similarly, *Salmonella*
171 dilutions were plated onto xylose lysine decarboxylase (XLD) agar with TSA layer
172 and incubated at 37 °C for 24 h. TSA overlays were used to facilitate recovery of
173 injured cells (Echeverry et al., 2010). Selective plating for *Campylobacter* was on
174 modified CCDA agar (Campylobacter Blood-Free Selective Agar, Neogen) and these
175 plates were incubated at 42 °C up to 48 h under microaerobic conditions (Bosilevac,
176 J.M., 2007). All the bacterial counts were recorded as colony forming units per gram
177 (cfu/g).

178 *2.8. Statistical analysis*

179 Analyses of variance and significant difference were conducted to identify

180 differences among means by one-way ANOVA using SPSS software (version 13.0 for
181 Windows, SPSS Inc., Chicago, IL, USA). Student-Newman-Keuls test was used for
182 comparison of mean values among treatments, and to identify significant differences
183 ($p < 0.05$) among treatment.

184 **3. Results and Discussion**

185 *3.1 Inhibition activity of LAB cocktails in ground beef*

186 Pathogens (E. *coli* O157:H7, *Salmonella* and non O157 STECs) were not detected
187 (detection limit, 10 cfu/g) from ground beef before treatment with the LAB cocktails.
188 The inhibition effect of four LAB cocktails (NP28+NP51, NP28+NP51+NP3,
189 NP35+NP51+NP3+NP7 and NP28+NP51+NP3+NP7) against pathogens is illustrated
190 in Figure 1A to Figure 1C. *E. coli* O157:H7, *Salmonella* and non O157 STECs
191 increased from approximately 3.5 log cfu/g to 5.0 log cfu/g after 5 days of refrigerator
192 storage without LAB cocktails. Compared with control samples, samples treated with
193 the four LAB cocktails showed a reduction in pathogens of approximately 0.5 to 1.5
194 log cfu/g from days 1 to 5. In previous studies, some LAB strains displayed their
195 inhibition activity against *E. coli* O157:H7 in live cattle (Brashears et al., 2003), *E.*
196 *coli* O157:H7 and *Salmonella* in cattle carcasses (Smith et al., 2005), *Listeria*
197 *monocytogenes* in hot dogs (Amezquita et al., 2000), and various foodborne
198 pathogens in alfalfa sprouts (Harris et al., 2002). *E. coli* O157:H7 decreased to 3.1 log
199 cfu/g on day 2 but increased to approximately 4.0 log cfu/g on days 3 and 5 with the
200 LAB cocktail of NP28+NP51 (Fig. 1A). *Salmonella* remained almost the same until
201 day 3 and grew to 3.7 log cfu/g with NP28+51 (Fig. 1B). Non O157 STECs remained
202 almost the same until day 2 and grew to 3.9 log cfu/g with the combination of
203 NP51+NP3+NP7+NP35 (Fig. 1C). Generally, all the inhibition activity of LAB
204 cocktails against these three pathogens was weaker on day 1 than on other days.

205 During the whole storage period, the LAB cocktail of NP28+NP51+NP3+NP7
206 effectively inhibited the growth of *E. coli* O157:H7, *Salmonella* and Non O157
207 STECs. In addition, the most significant reductions on *E. coli* O157:H7 (1.5 log cfu/g),
208 *Salmonella* (1.4 log cfu/g) and non O157 STECs (1.7 log cfu/g) were obtained after
209 the treatment of NP28+NP51+NP3+NP7 on day 5 in comparison with the control
210 samples. Therefore, the LAB cocktail of NP28+NP51+NP3+NP7 was used in
211 subsequent experiments.

212 *3.2 Inhibition activity of LAB cocktails in ground beef*

213 As shown in Fig. 2A, there were significant ($p < 0.05$) reductions observed in *E.*
214 *coli* O157:H7 on ground beef with three dose treatments of NP28+NP51+NP3+NP7
215 compared to the control sample. On days 1, 2 and 3, there was no significant ($p < 0.05$)
216 difference in this pathogen on ground beef with treatments. The counts of *E. coli*
217 O157:H7 on the samples treated with 1×10^8 and 1×10^9 of NP28+NP51+NP3+NP7
218 were significantly lower than those with 1×10^7 on day 5. During the storage period,
219 there was no significant ($p > 0.05$) difference in the *E. coli* O157:H7 reductions
220 between treatments of 1×10^8 and 1×10^9. All the *Salmonella* counts on ground beef
221 with treatments were reduced steadily from day 1 to 5 (Fig. 2B). Compared with the
222 control sample, *Salmonella* decreased significantly on the samples with three
223 treatments and there was no significant difference ($p > 0.05$) in the effect of treatment
224 dose on *Salmonella* reduction. Subsequently, 1×10^7, 1×10^8 and 1×10^9 of
225 NP28+NP51+NP3+NP7 individually showed from 0.5 to 1.6 log cfu/g reductions of
226 non-O157 STECs compared with the control during 5 days of refrigerator storage (Fig.
227 2C). There was no significant ($p > 0.05$) difference in non O157 STECs reduction
228 with three treatments on days 1 and 2 (during the first two days). In contrast,
229 treatments of 1×10^8 and 1×10^9 of the LAB cocktail displayed significant ($p < 0.05$)

230 inhibition in 1×10^7 on days 3 and 5. Castellano et al. (2011) found that the LAB
231 combination of *Lactobacillus curvatus* CRL705 and *Lactococcus lactis* CRL1109
232 with Na_2EDTA effectively inhibited the growth of *E. coli* O157:H7 and indigenous
233 coliforms in ground-beef patties.

234 *3.3 Inhibition activity of LAB cocktail in ground turkey*

235 The *Salmonella* and *Campylobacter* reductions on ground turkey with the LAB
236 cocktail of NP28+NP51+NP3+NP7 were shown in Fig. 3A and 3B, respectively.
237 There were significant ($p < 0.05$) reductions in both pathogens observed with all the
238 treatments by the LAB cocktail. *Salmonella* on the control sample increased
239 approximately 0.7 log cfu/g after 5 days of storage (Fig. 3A). *Salmonella* on the
240 samples treated with 1×10^8 and 1×10^9 of NP28+NP51+NP3+NP7 kept decreasing
241 and there was no significant ($p > 0.05$) difference between these two treatments
242 during 5 days of storage. *Salmonella* on the sample treated with 1×10^7 of the LAB
243 cocktail decreased on days 1 and 2 but increased on days 3 and 5. *Campylobacter* on
244 the control sample got to the maximum count (4.0 cfu/g) on day 3 (Fig. 3B).
245 *Campylobacter* was reduced within the first 3 days and remained the same until day 5
246 in all treatments. On day 1, there was no significant effect of LAB cocktail dose on
247 the reduction of *Campylobacter*. The 1×10^7 cocktail treatment displayed
248 significantly ($p < 0.05$) stronger inhibition activity than the other two treatments since?
249 day 2. The lowestt numbers of *Salmonella* (2.4 cfu/g) and *Campylobacter* (2.4 cfu/g)
250 were obtained with 1×10^9 and 1×10^7 of NP28+NP51+NP3+NP7 on day 5 and day 3,
251 respectively. Burfoot and Mulvey (2011) applied a 4% concentration lactic acid to
252 significantly ($p < 0.001$) reduce *Campylobacter* on turkey carcasses on the day after
253 slaughter.

254 *3.4 Inhibition activity of LAB cocktail in chicken legs*

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255 As shown in Fig. 4A and 4B, *Salmonella* and *Campylobacter* on chicken legs with
256 no treatment increased 0.4 log cfu/g after 3 days of refrigerator storage and then
257 decreased. Compared to the control sample, there were significant ($p < 0.05$)
258 reductions in these two pathogens with three treatments from day 1 to day 5. The
259 treatment of 1×10^8 of NP28+NP51+NP3+NP7 displayed the strongest inactivation
260 against *Salmonella* and resulted in the lowest *Salmonella* counts (3.0 log cfu/g) on day
261 5 (Fig. 4A). There was no significant ($p > 0.05$) difference observed in inhibition
262 effect on *Campylobacter* by three treatments (Fig. 4B). It has been found that two
263 LAB strains of food origin (*Enterococcus faecium* PCD71 and *Lactobacillus*
264 *fermentum* ACA-DC179) could be respectively applied as protective cultures in
265 chicken meat against *Listeria* and *Salmonella* without any detrimental effect on
266 biochemical parameters related to spoilage of the meat (Maragkoudakis, et al., 2009).
267 Bucher et al. (2012) found that lactic acid dip treatments could significantly decrease
268 *Salmonella* contamination on broiler chicken carcasses during primary processing.
269 Heres et al. (2004) studied the effect of acidification of food on reducing the
270 *Campylobacter* and *Salmonella* carriage in broiler chickens and found the number of
271 *Campylobacter* was reduced 2-3 log in an in vitro experiment. In addition, carvacrol
272 vapour could effectively inhibit and eliminate *Salmonella* on the surface of raw
273 chicken (Burt et al., 2007).

274 **4. Conclusion**

275 The antimicrobial ability of many LAB against pathogens is primarily attributed to
276 the production of organic acids and/or bacteriocins. In our study, different LAB
277 cocktails displayed varied inactivation against *E. coli* O157:H7, *Salmonella*, and non
278 O157 STECs on ground beef. The LAB cocktail of NP28+NP51+NP3+NP7 could
279 effectively inhibit and reduce food-borne pathogens on ground beef, turkey and

280 chicken legs. The food and feed applications of all LAB strains used have been
281 approved by USDA. In addition, LAB cocktails can be provided to processors in
282 various forms (frozen, liquid or freeze-dried) and application can be easily
283 implemented into current operations by adding the cocktails into ground beef products
284 during processing. Therefore, LAB cocktails are excellent antimicrobial treatments
285 against pathogens on raw meat. On the basis of the results obtained from this work,
286 more studies need to be done on the physical, chemical, and sensory properties of
287 meat after these interventions are applied to determine if meat quality stays intact
288 without being adversely affected.

289 **Acknowledgement**

290 Funding was provided by the International Association for Food Industry Excellence
291 foundation.

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380 **Figure captions:**

381 **Fig. 1** Inhibition of *E. coli* O157:H7 (A), *Salmonella* (B) and non O157 STECs (C) by
382 different LAB cocktails (1 x 10^8) in ground beef stored at 4 °C for 5 days. Data are
383 expressed as mean value ± SD of three independent experiments.

384 **Fig. 2** Inhibition of *E. coli* O157:H7 (A), *Salmonella* (B) and non O157 STECs (C) by
385 the LAB cocktail of 28, 51, 3 and 7 at different doses in ground beef stored at 4 ±°C
386 for 5 days. Data are expressed as mean value ± SD of three independent experiments.

387 **Fig. 3** Inhibition of *Campylobacter* (A) and *Salmonella* (B) by the LAB cocktail of 28,
388 51, 3 and 7 at different doses in ground turkey stored at 4 °C for 5 days. Data are
389 expressed as mean value ± SD of three independent experiments.

390 **Fig. 4** Inhibition of *Campylobacter* (A) and *Salmonella* (B) by the LAB cocktail of 28,
391 51, 3 and 7 at different doses in chicken legs stored at 4 °C for 5 days. Data are
392 expressed as mean value ± SD of three independent experiments.

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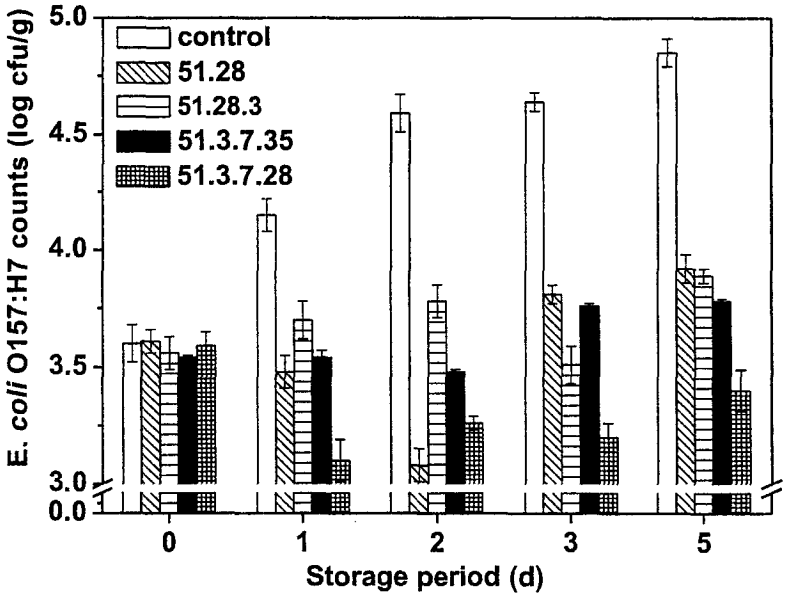
E. coli O157:H7 counts (log cfu/g)
5.0
4.5
4.0
3.5
3.0
0.0
control
51.28
51.28.3
51.3.7.35
51.3.7.28
0
1
2
3
5
Storage period (d)

Fig. 1A

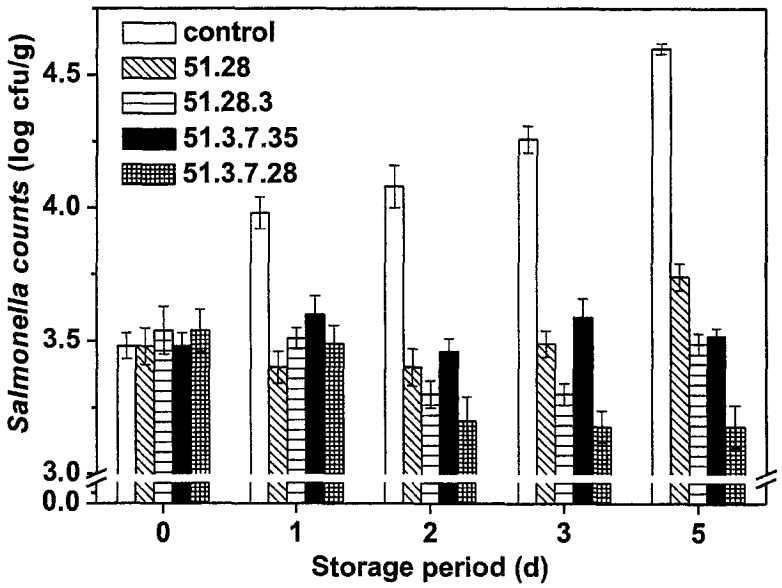
control
51.28
51.28.3
51.3.7.35
51.3.7.28
Salmonella counts (log cfu/g)
4.5
4.0
3.5
3.0
0.0
0
1
2
3
5
Storage period (d)

Fig. 1B

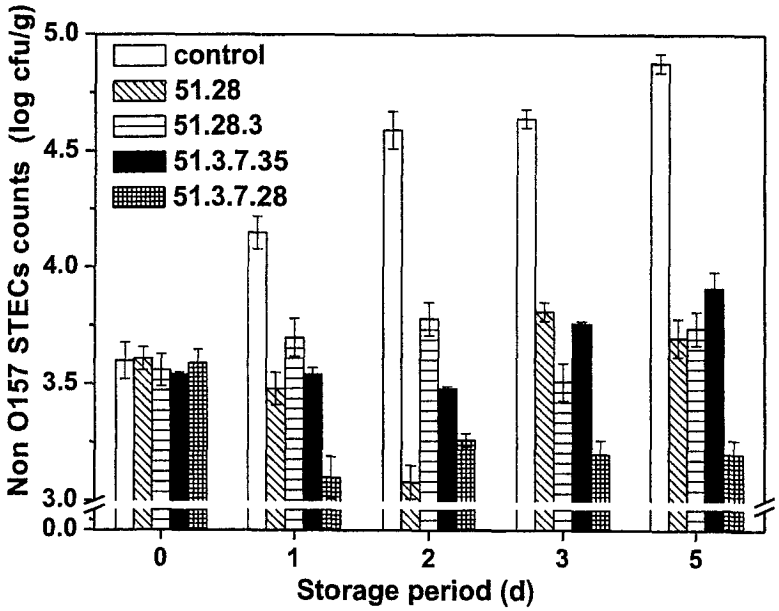
Non O157 STECs counts (log cfu/g)
5.0
4.5
4.0
3.5
3.0
0.0
control
51.28
51.28.3
51.3.7.35
51.3.7.28
0
1
2
3
5
Storage period (d)

Fig. 1C

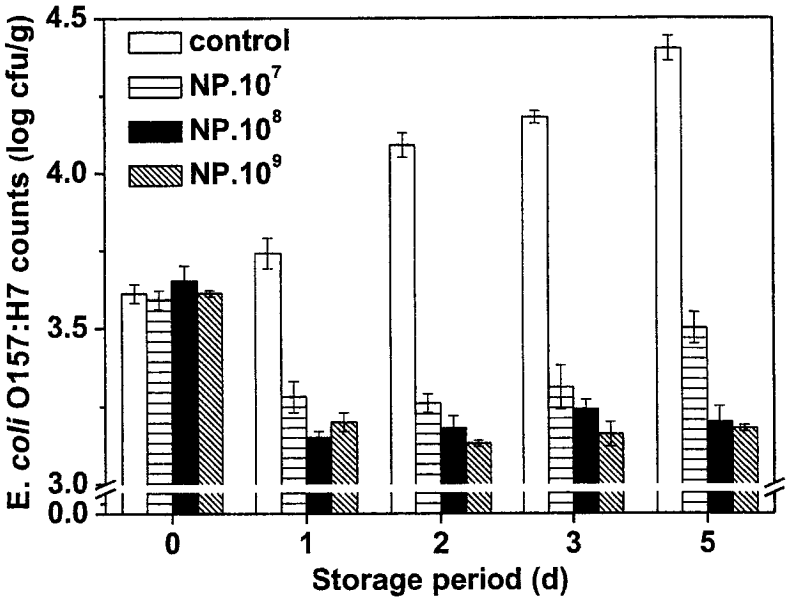

Fig. 2A

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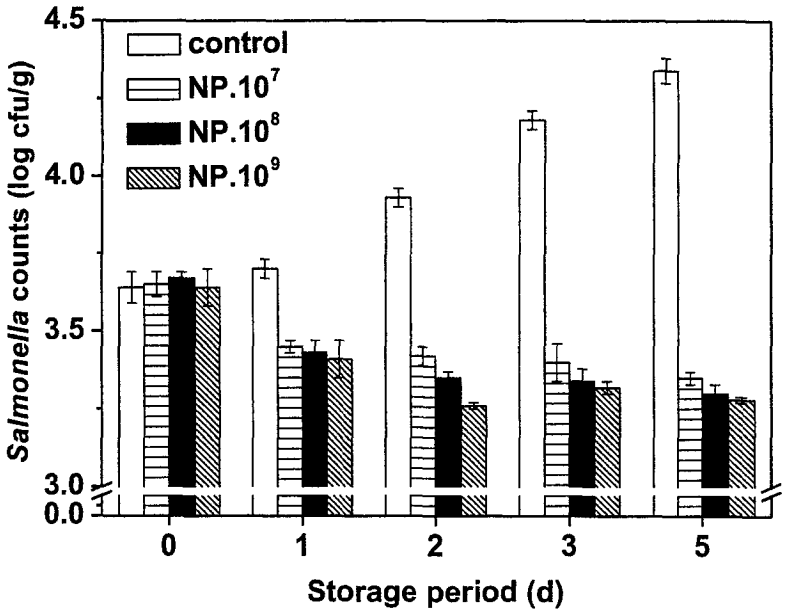

4.5
4.0
3.5
3.0
0.0
control
NP.10^7
NP.10^8
NP.10^9
Salmonella counts (log cfu/g)
0
1
2
3
5
Storage period (d)

Fig. 2B

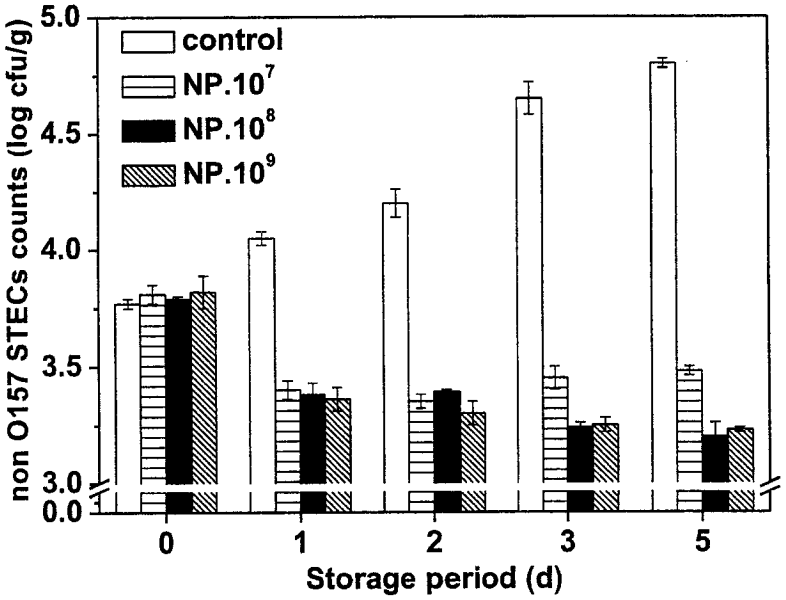

control
NP.10^7
NP.10^8
NP.10^9
non O157 STECs counts (log cfu/g)
5.0
4.5
4.0
3.5
3.0
0.0
0
1
2
3
5
Storage period (d)

Fig. 2C

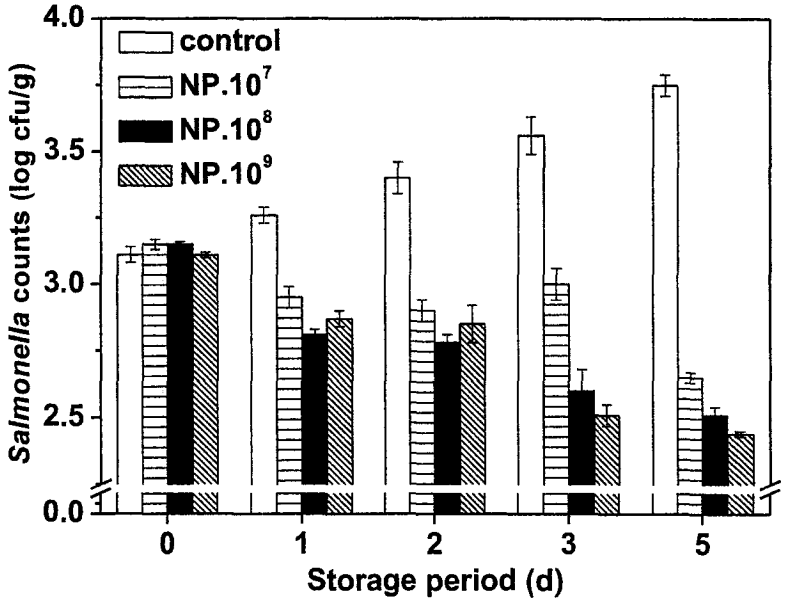

4.0
3.5
3.0
2.5
0.0
control
NP.10^7
NP.10^8
NP.10^9
Salmonella counts (log cfu/g)
0
1
2
3
5
Storage period (d)

Fig. 3A

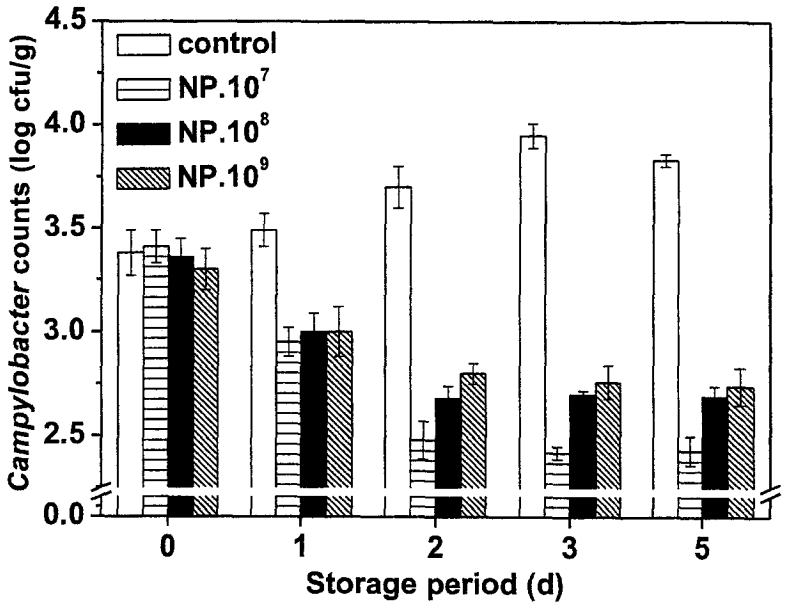

4.5
4.0
3.5
3.0
2.5
0.0
control
NP.10^7
NP.10^8
NP.10^9
Campylobacter counts (log cfu/g)
0
1
2
3
5
Storage period (d)

Fig. 3B

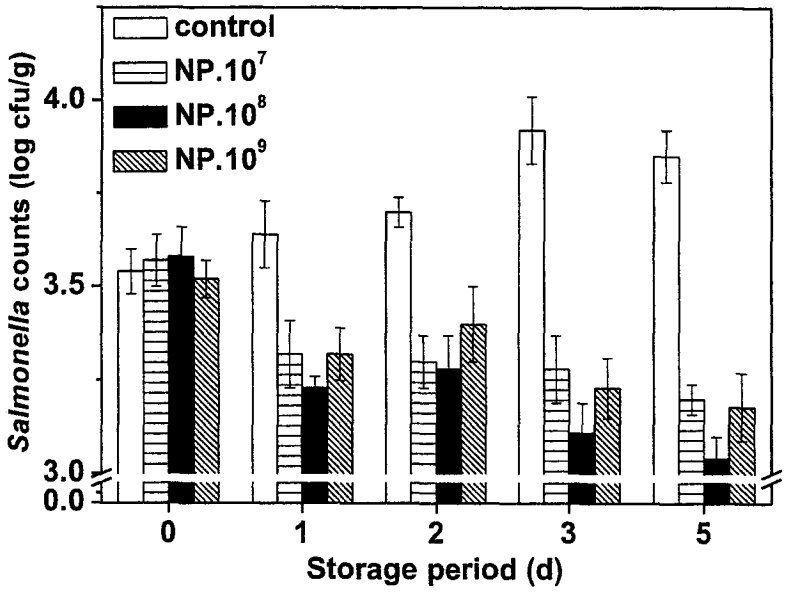
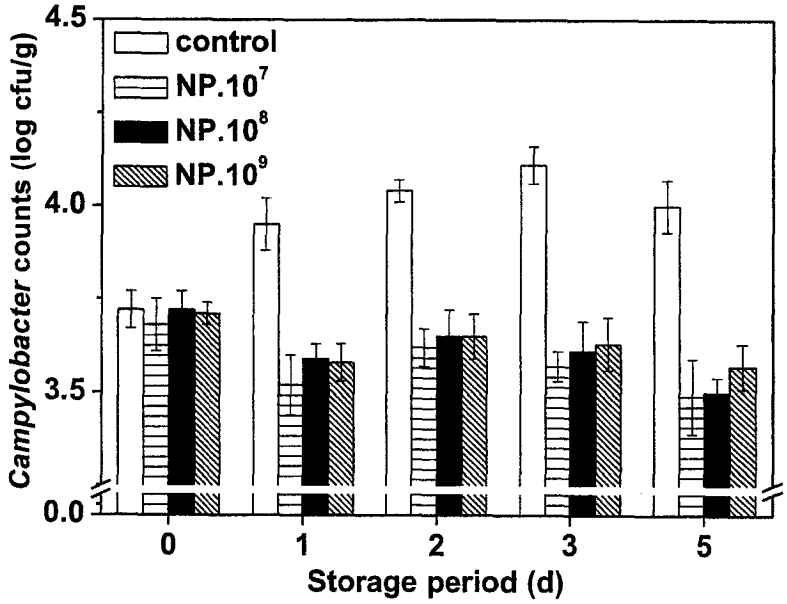

control
NP.10^7
NP.10^8
NP.10^9
Salmonella counts (log cfu/g)
4.0
3.5
3.0
0.0
0
1
2
3
5
Storage period (d)

Fig. 4A



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540 Fig. 4B

SUBMISSION END