GR

ORIGINAL SUBMISSION

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[redacted]

JHeimbach LLC

March 2, 2005

RECEIVED
MAR 4 2005
OFFICE OF
FOOD ADDITIVE SAFETY

Robert L. Martin, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Bob:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), Laboratorios Miret, S.A. (LAMIRSA), through me as its agent, hereby provides notice of a claim that the food ingredient described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS), based on scientific procedures, for addition to foods as described to inhibit microbial growth.

As required, three copies of the notification are provided, plus a fourth copy for the USDA's Food Safety and Inspection Service since the intended use of the substance includes direct addition to meat and poultry products.

As I mentioned on the telephone, a separate document is in preparation describing the mode of action of the substance in accomplishing its technical effect, minimum inhibitory concentrations, the results of extensive testing with a variety of meat products and other foodstuffs, and studies of its effect on appearance and organoleptic characteristics of meat and poultry products. This second document, which will be submitted shortly, contains no information bearing in any way on the safety of the substance for its intended use.

If you have any questions regarding this notification, please feel free to contact me at 202-237-8406 or jim@jheimbach.com.

Sincerely,

(b) (6)

James T. Heimbach, Ph.D., F.A.C.N.
President

4530 Broad Branch Road, N.W., Washington, DC 20008 USA
tel. (+1) 202-237-8406 fax (+1) 202-478-0986 JHeimbach@aol.com

I. GRAS EXEMPTION CLAIM

Laboratorios Miret, S.A. (LAMIRSA), through its agent JHEIMBACH LLC, hereby notifies the Food and Drug Administration that the use of LAE described below is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because LAMIRSA has determined that such use is generally recognized as safe (GRAS) through scientific procedures.

(b) (6)

3/2/05

James T. Heimbach, Ph.D., F.A.C.N. Date
President, JHEIMBACH LLC
U.S. Agent for LAMIRSA, VEDEQSA (Grupo LAMIRSA)

A. Name and Address of Notifier

Laboratorios Miret, S.A. (LAMIRSA)
Polig. Industrial Can Parellada
Géminis, 4
08228 Terrassa
Barcelona
Spain

Contact: Xavier Rocabayera
Telephone: 34 93 731 50 94
Facsimile: 34 93 783 58 87
E-mail: xrocabayera@vedeqsa.com

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B. Name of GRAS Substance

The common name of the substance that is the subject of this Generally Recognized as Safe (GRAS) determination is lauramide arginine ethyl ester, or LAE; for ingredient labeling purposes it is listed as "lauric arginate." Dehydrated LAE contains 85–95% of its active ingredient, ethyl-N^{α}-lauroyl-L-arginate hydrochloride, while non-dehydrated LAE contains 71–81% active ingredient. (Unless otherwise specified, all references to LAE or lauric arginate refer to the dehydrated form, which differs from the non-dehydrated form only in that most of the water has been removed by drying.) The Chemical Abstracts Service registry number for ethyl-N^{α}-lauroyl-L-arginate hydrochloride is 60372-77-2. LAE is normally sold dissolved in food-grade propylene glycol under the trade name Mirenat-N. Specifications have been established for LAE to assure that all product sold is food grade.

C. Intended Use and Consumer Exposure

LAE is intended to be directly added to food to inhibit microbial growth. It is intended for use in the food categories listed in Table 1, below.

Table 1. Intended Food Uses of LAE

Up to 200 ppm ethyl-N$^{\alpha}$-lauroyl-L-arginate hydrochloride in the following food categories in 21 CFR 170.3(n)
(3) Beverages and beverage bases, not including dairy products, soft drinks, or alcoholic beverages.
(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.
(7) Tea, including regular, decaffeinated, and instant types.
(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.
(11) Egg dishes including egg roll, egg foo young, egg salad, and frozen multicourse egg meals, but not fresh eggs.
(12) Margarine and margarine-like table spreads, mayonnaise, and spoonable and pourable dressings for salads.
(13) Fish products, including all prepared main dishes, salads, appetizers, frozen multicourse meals, and spreads containing fish, shellfish, and other aquatic animals, but not fresh fish.
(15) Fresh fish, including only fresh and frozen fish, shellfish, and other aquatic animals.
(17) Fresh meats, including only fresh or home-frozen beef or veal, pork, lamb or mutton and home-prepared fresh meat-containing dishes, salads, appetizers, or sandwich spreads made therefrom.
(18) Fresh poultry, including only fresh or home-frozen poultry and game birds and home-prepared fresh poultry-containing dishes, salads, appetizers, or sandwich spreads made therefrom.
(22) Pie fillings.
(24) Gravies and sauces, including all meat sauces and gravies, and tomato, milk, buttery, and specialty sauces.
(29) Meat products, including all meats and meat-containing dishes, salads, appetizers, frozen multicourse meat meals, and sandwich ingredients prepared by commercial processing or using commercially processed meats with home preparation.
(34) Poultry products, including all poultry and poultry-containing dishes, salads, appetizers, frozen multicourse poultry meals, and sandwich ingredients prepared by commercial processing or using commercially processed poultry with home preparation.
(35) Processed fruits and fruit juices excluding apple juice, including all commercially processed juices and juice punches, concentrates, dilutions, ades, and drink substitutes made therefrom, and dried fruits; also including strained fruits and fruit juices excluding apple juice as baby or toddler foods.
(36) Processed vegetables and vegetable juices, including potato salads, raw vegetables, vegetable juices and blends, and tomato sauces
(40) Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable, and combination soups and soup mixes.

Under contract with LAMIRSA, Exponent Inc. estimated 2-day average intakes of LAE on a body-weight basis, based on data from the 1994–96, 1998 Continuing Survey of Food Intakes by Individuals conducted by the U.S. Department of Agriculture. About 99% of the population ages 2 and older are consumers of foods in one or more of the intended LAE use categories. Assuming that LAE is present at a concentration of 225 parts per million (ppm, equivalent to mg/kg) in all foods within all permitted food categories in order to provide the listed concentration of 200 ppm ethyl-N^{α}-lauroyl-L-arginate hydrochloride, the estimated mean 2-day average LAE intake for the population 2 years of age and older in the U.S. is 2.5 mg/kg bw/day; the estimated 90th percentile intake is 4.7 mg/kg bw/day.

D. Basis for GRAS Determination

LAMIRSA's GRAS determination for the intended uses of LAE is based on scientific procedures as described under 21 CFR §170.30(b).

The estimated daily intake (EDI) of LAE has been determined to be safe, and has also been determined to be GRAS by demonstrating that the safety of this level of intake is based on publicly available and accepted information and is generally recognized by experts qualified by scientific training and experience to evaluate the safety of substances added to food.

The 90th percentile intake of LAE is estimated to be 4.7 mg/kg bw/day. The safety of this level of intake is based on both *in vitro* and *in vivo* studies of LAE metabolism, toxicokinetics, and toxicity. *In vitro* and *in vivo* and data indicate that ethyl-N^{α}-lauroyl-L-arginate hydrochloride, the active ingredient in LAE, is rapidly metabolized by the hydrolysis of the ethyl ester and lauroyl amide to arginine, lauric acid, and ethanol (Ruckman et al. 2004). Arginine undergoes natural amino acid catabolism to ornithine. The eventual fate of arginine and ornithine is incorporation into proteins or degradation to CO_2 and urea. Lauric acid enters normal fatty acid metabolism. *In vitro* data indicate that the hydrolysis that takes place in the intestinal tract is complete. Animal toxicity studies and genetic toxicity assays show that, under the conditions of these tests, LAE has a low order of oral toxicity, is not mutagenic or clastogenic, and does not produce embryo-fetal toxicity (Ruckman et al. 2004).

A no observed adverse effect level (NOAEL) for subchronic administration of LAE was determined from two 13-week studies, one conducted with Mirenat-N, a 20–25% solution of LAE in propylene glycol, and the other with crystalline LAE (Ruckman et al. 2004). The study employing Mirenat-N administered Mirenat-N at LAE-equivalent dietary concentrations of 691, 2,765, and 10,800 ppm. The NOAEL was the highest dietary level tested, 10,800 ppm, corresponding to LAE doses of approximately 718 and 848 mg/kg bw/day for males and females, respectively. The study conducted with crystalline LAE employed dietary concentrations of 5,000, 15,000, and 50,000 ppm. In this study, indications of local irritation were seen at the highest dietary concentration, including microscopic changes in the stomach as well as unpalatibility reflected in reduced feed consumption and corresponding body-weight reduction, but no indications of systemic toxicity were observed. Thus, the NOAEL for systemic toxicity was identified at the highest level tested, 50,000 ppm, corresponding to LAE doses of approximately 3,714 and 3,915 mg/kg bw/day for males and females, respectively. Because the metabolic data on LAE demonstrate that it is rapidly converted to the natural dietary components

arginine, lauric acid, and ethanol, and does not accumulate in the body, a safety factor of 100 was used to set the acceptable daily intake (ADI) based on the NOAEL for systemic toxicity. Therefore, the ADI for LAE based on the 90-day published study is 37 mg/kg bw/day. The 90th percentile EDI of LAE from the intended uses is 4.7 mg/kg bw/day, only about one-eighth of this figure. Therefore, the intended uses of LAE are determined to be safe and GRAS.

Determination of the safety and GRAS status of LAE for direct addition to food under its intended conditions of use was made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Robert Nicolosi, Ph.D., Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D., who reviewed a monograph prepared by ENVIRON and a revised monograph prepared by JHEIMBACH LLC, as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They critically reviewed and evaluated the publicly available information and the potential human exposure to LAE resulting from its intended uses, and individually and collectively concluded that no evidence exists in the available information on LAE that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public health when LAE is used under its intended conditions of use.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, LAE is GRAS by scientific procedures under the conditions of use described.

Subsequent to the initial GRAS determination, additional metabolic and toxicological studies of LAE were completed. When the results became available, the Expert Panel reviewed this additional information and concluded that it supports their earlier conclusion that LAE is GRAS for its intended uses.

E. Availability of Information

The data and information that serve as the basis for the GRAS determination, as well as the information that has become available since the GRAS determination, will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of James T. Heimbach, Ph.D., President, JHEIMBACH LLC, 4530 Broad Branch Road NW, Washington, DC, 20008, telephone 202-237-8406 and e-mail jim@jheimbach.com.

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II. IDENTITY OF THE SUBSTANCE

A. Chemical Name

The article of commerce that is the subject of this GRAS determination is N^{α}-lauroyl-L-arginine ethyl ester monohydrochloride (LAE), a product manufactured by LAMIRSA. LAE may be dried and used in dehydrated form or used in its original non-dehydrated form, both of which are food grade; the only difference between the two forms is the water content. When LAE is referred to without further qualification, it refers to the dehydrated form. The chemical name for the active ingredient in LAE is ethyl-N^{α}-lauroyl-L-arginate hydrochloride, also known generically as monohydrochloride of L-arginine, N^{α}-lauroyl-ethyl ester.

B. Trade or Common Name

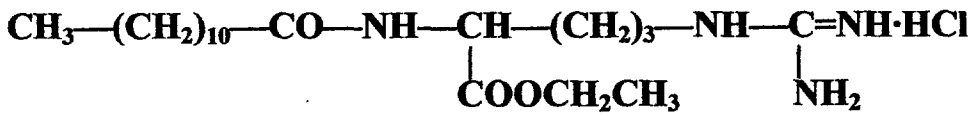
The trade or common names of the article of commerce are lauramide arginine ethyl ester, LAE, and lauric arginate. This last name, lauric arginate, was approved as suitable for use in food labeling in an e-mail to JHEIMBACH from Geraldine June, Food Labeling and Standards Staff, Office of Nutritional Products, Labeling, and Dietary Supplements, dated December 10, 2004 (FDA 2004). The active ingredient of LAE is ethyl-N^{α}-lauroyl-L-arginate hydrochloride, which constitutes 71–81% of food-grade non-dehydrated LAE and 85–95% of food-grade dehydrated LAE. Mirenat-N is the trade or common name of a formulation consisting of LAE dissolved in various concentrations in food-grade propylene glycol.

C. CAS Registry Number

The Chemical Abstracts Services (CAS) registry number for ethyl-N^{α}-lauroyl-L-arginate hydrochloride, the active ingredient in LAE, is 60372-77-2.

D. Molecular and Structural Formula

The molecular formula for ethyl-N^{α}-lauroyl-L-arginate hydrochloride is $C_{20}H_{41}N_4O_3Cl$; the structural formula is shown below:



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E. Production Process

1. Raw Materials

The raw materials used in the manufacture of LAE are:

- Ethyl alcohol (CAS 64-17-5)
- Sodium hydroxide (CAS 1310-73-2)
- Deionized water (CAS 7732-18-5)
- L-arginine monohydrochloride (CAS 1119-34-2)
- Thionyl chloride (CAS 7719-09-7)
- Lauroyl chloride (CAS 112-16-3)

Food-grade ethanol (C_2H_6O) is used as a reactant and a diluent in the first production step. Food-grade sodium hydroxide (NaOH) is used as a production aid to maintain the pH of the reaction mixture during the second production step. Deionized water is used as a diluent during the first and second production steps. These substances meet the specifications provided in *Food Chemicals Codex, 5th Edition* (IOM 2003b).

L-arginine monohydrochloride ($C_6H_{15}N_4O_2Cl$) is an odorless white crystalline powder. Specifications for this compound are listed in Table 2.

Table 2. Specifications for L-Arginine Monohydrochloride

Parameter	Value	Method
Purity	>98% (w/w)[1]	Titration (perchloric or silver nitrate)
Specific Rotation	$[\alpha]^{20}_D$: 21.5–23.0	Polarimetry (8% in 6N HCl)
pH (@20°C)	4.7–6.2	Voltammetry (10g/100mL dH_2O)
pH (@25°C)	4.5–6.5	Voltammetry (2.5% solution)
Heavy metals	<10 ppm	EU-Ph[2]
Iron	<10 ppm	Atomic Absorption

[1]w/w = weight/weight

[2]EU-Ph: European Pharmacopoeia

Thionyl chloride ($SOCl_2$) is a clear colorless to light-yellow low-viscosity liquid with an extremely pungent odor. It is miscible with most organic solvents and is itself a good solvent for many organic compounds. Thionyl chloride is not flammable and is used in the substitution of hydroxyl (OH) groups with chlorine in alcohols, carboxylic acids, and sulphonic acids, as well as as an esterification agent. Specifications are shown in Table 3.

Table 3. Specifications for Thionyl Chloride

Parameter	Value	Method
Purity	≥99.4% (w/w)	Titration
Iron	≤5 ppm	Atomic Absorption

Distilled lauroyl chloride ($C_{12}H_{23}OCl$) is a clear liquid with the specifications listed in Table 4:

Table 4. Specifications for Lauroyl Chloride

Parameter	Value	Method
Purity	≥97.0% (w/w)	HPLC
Free acid (as lauric acid)	≤1.0% (w/w)	Titration
Color	≤200 APHA	ID-11-239

2. Production Process Description

The active ingredient in LAE, ethyl-N^{α}-lauroyl-L-arginate hydrochloride, is a cationic surfactant that is produced from L-arginine monohydrochloride, ethyl alcohol, and lauroyl chloride, with thionyl chloride as an esterification agent.

LAMIRSA's patented production process (Infante et al. 1998) includes two steps followed by a filtration process, as shown in the flow diagram displayed in Figure 1. The two production steps are as follows:

1. Esterification of the carboxyl group of the L-arginine monohydrochloride with ethyl alcohol, using thionyl chloride as an esterification agent and exploiting the heat of reaction to carry out the reaction.
2. Condensation of lauroyl chloride with the α-amino group of ethyl arginine dihydrochloride in an aqueous medium.

In the esterification step, L-arginine monohydrochloride is dissolved in ethanol, and heated to 44–46°C. As soon as this temperature is reached, thionyl chloride is added, causing an exothermic reaction to take place. Consequently, the temperature increases to 74–78°C, and there is a reflux. The temperature shortly reaches a steady state of approximately 62–66°C. During the reaction, a flow of nitrogen (N_2) is added to remove any acid fumes. Once addition of thionyl chloride is completed, the reflux is maintained for an additional 3 hours prior to distillation; this process results in the formation of ethyl arginine dihydrochloride in the form of a viscous brown oil. Deionized water is added, followed by an aqueous solution of sodium hydroxide until a pH of 4–5 is reached.

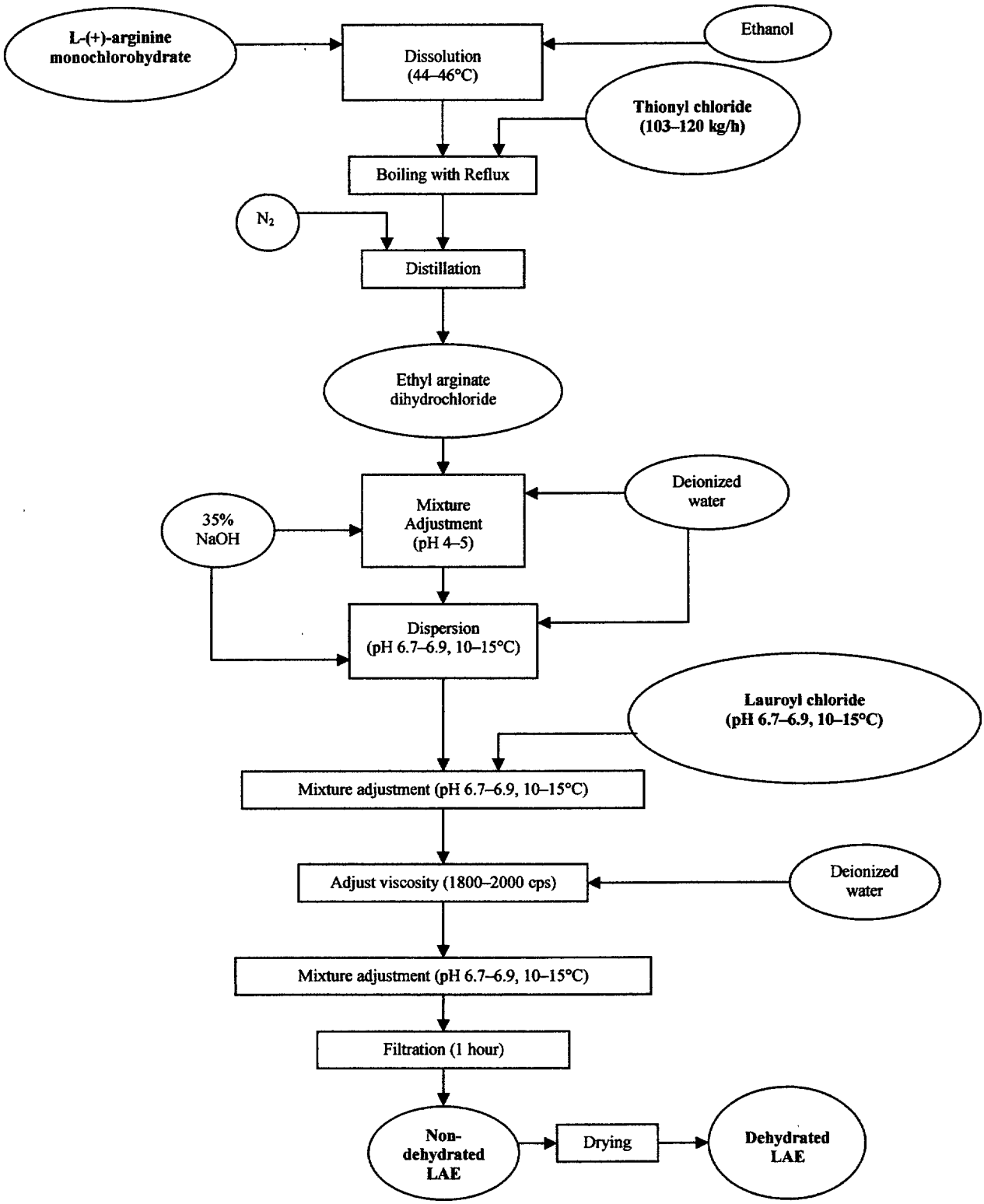

Figure 1. *LAE Production Process*

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In the second step of production, the product obtained in the esterification step is dispersed with deionized water and the pH is adjusted to 6.7–6.9 with aqueous sodium hydroxide. Next, lauroyl chloride is gradually added. The mixture is stirred continuously; the temperature is maintained between 10 and 15°C and the pH between and 6.7 and 6.9 until the reaction is complete. Deionized water is again added to adjust the viscosity of the reaction mixture so that it is between 1,800 and 2,000 centistrokes/second.

The last step in the production of LAE is filtration of the reaction mixture through a press filter. Once filtration is completed, the resulting product (food-grade non-dehydrated LAE) is stored at refrigerated temperatures inside a stainless steel container. This product may be dried to produce food-grade dehydrated LAE, which is stored in a similar manner.

3. Additives and Processing Aids

Thionyl chloride ($SOCl_2$) and sodium hydroxide (NaOH) are used in the production of LAE. Thionyl chloride is used as an esterification agent in the first production step; aqueous sodium hydroxide is used to maintain the pH of the reaction mixture.

F. Product Characteristics

1. Physical and Chemical Properties

The physical and chemical properties of dehydrated LAE are listed in Table 5.

Table 5. Physical and Chemical Properties of Dehydrated LAE

Physical/Chemical Property	Value
Appearance	White hygroscopic powder
Molecular weight	421.02 g/mol
Melting point	50.5–58.0°C
Boiling point	Decomposes from 107°C
Relative density (D^{20}_4)	1.11
Vapor pressure (@ 25°C)	5.45×10^{-4} Pa
Surface tension (@ 19°C, 1 g/L)	25.43 mN/m
Water solubility (@ 20°C)	>247 g/kg
Partition coefficient (K_{ow} @ 20°C)	1.43
Flammability (solids)	Not highly flammable
Explosive properties	Not explosive
Self-ignition temperature (solids)	Does not self-ignite
Oxidizing properties	Non-oxidizing
Particle size distribution	0% <10 μm
Soil adsorption coefficient (K_{oc})	58.0

2. Food-Grade Specifications

The final filtration step employed in the LAE production process described above yields a food-grade product, which is an aqueous paste containing 71–81% of the active ingredient, ethyl-N^{α}-lauroyl-L-arginate hydrochloride. This product may be dried to produce food-grade dehydrated LAE containing 85–95% of the active ingredient. Food-grade specifications for dehydrated LAE are presented in Table 6.

3. Batch Analysis Results

Analysis results for six lots of food-grade dehydrated LAE are shown in Table 6.

These results show that each lot of LAE meets the food-grade specifications and demonstrate that LAMIRSA's production process for LAE is in control and consistently yields a food-grade product.

4. Product Stability

To evaluate product stability, LAMIRSA conducted a series of studies with LAE and Mirenat-N under various conditions. These studies and their findings may be briefly summarized as follows:

1. Stability of LAE in a closed dark container at 25°C for 10 days and 5, 6, 10, and 12 months
   - No significant degradation was found after 12 months.
2. Stability of LAE in Mirenat-N at 30, 50, 75, 100, and 121°C for 1 and 5 hours
   - Stable for 5 hours at ≤100°C and for 1 hour at 121°C.
3. Stability of LAE at 4, 25, and 50°C and pH 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 (using phosphoric, citric, tartaric, malic, and fumaric acids) for 10–52 days
   - At 4°C, no significant degradation if pH >1.5; at 25°C, no significant degradation if pH >2.5; at 50°C, no significant degradation if pH >3.0.
4. Stability of LAE in Mirenat-N for 1–61 days after application to cooked ham
   - No significant degradation was found after 61 days at 4°C.

Overall, the results from these studies show that LAE is stable over a wide variety of conditions. Under severe conditions (e.g., very low pH and high temperature) and over extended periods of time, the product can degrade. However, the resulting degradation products are the same non-toxic compounds as are found as impurities in LAE: N^{α}-lauroyl-L-arginine (LAS), lauric acid, ethyl laurate, arginine, and arginine ethyl ester; no other known products or byproducts are formed during degradation.

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Table 6. Batch Analysis Results for Six Lots of Dehydrated Food-Grade LAE

Formulation: Components, Residuals, Contaminants	Food-Grade Specifications	Batch Number and Results						Mean (n = 6)	Units	Test Method & Document Reference
		3036	5733	7446	8302	8411	10234			
Principal Components										
Ethyl-N$^{\alpha}$lauroyl-L-arginate HCl	85–95	89.4	90.3	88.2	88.1	88.5	88.2	88.8	%	(1) ID-11-1697 (rev. 4)
Other Related Substances										
N$^{\alpha}$-lauroyl-L-arginine (LAS)	≤3	1.5	2.1	1.9	2.3	2.2	1.6	1.9	%	(1) ID-11-1697 (rev. 4)
Ethyl laurate	≤3	1.5	2.0	1.0	1.5	1.1	1.4	1.4	%	(1) ID-11-2004 (rev. 2)
Lauric acid	≤5	2.7	3.0	2.7	2.3	2.6	2.5	2.6	%	(1) ID-11-2004 (rev. 2)
Arginine HCl	≤1	0.3	0.3	0.1	0.4	0.3	0.4	0.3	%	(1) ID-11-2465 (rev. 0)
Ethyl arginine 2HCl	≤1	0.3	0.4	<0.1	<0.01	<0.01	<0.1	0.2	%	(1) ID-11-2465 (rev. 0)
Residuals-Volatiles, Solvents, etc.										
Ethanol	≤0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	%	(2)
Ash	≤2	0.7	0.9	1.5	0.9	0.8	0.8	0.9	%	(3) ID-11-083 (rev. 2)
Water	≤5	4.1	0.9	3.7	2.9	2.6	2.8	2.8	%	(4) ID-11-020 (rev. 4)
SUM of I + II + III		100.5	99.9	99.1	98.4	98.1	97.8	99.0	%	
Heavy Metals, Inorganics										
Heavy metals (as lead)	≤10	<10	<10	<10	<10	<10	<10	<10	ppm	(5)
Arsenic (As)	≤3	<3	<3	<3	<3	<3	<3	<3	ppm	(6)
Lead (Pb)	≤1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	ppm	(6)
Cadmium (Cd)	≤1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	ppm	(6)
Mercury (Hg)	≤1	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	ppm	(6)

Test Method:
(1) HPLC: High Performance Liquid Chromatography
(2) GC: Gas Chromatography
(3) Gravimetry
(4) Karl-Fischer
(5) EU-Ph: European Pharmacopeia
(6) ICP-MS: Inductively Coupled Plasma-Mass Spectrometry

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III. Intended Technical Effect

The intended technical effect of LAE in food is to inhibit microbial growth in the food to which it has been added. A separate document accompanying this GRAS notice describes the mode of action of LAE in accomplishing this effect, minimum inhibitory concentrations, and the results of extensive testing with a variety of meat products and other foodstuffs.

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IV. INTENDED USE AND CONSUMER EXPOSURE

A. Intended Uses and Use Level of LAE

LAMIRSA intends to market LAE dissolved in propylene glycol as Mirenat-N to be directly added to food at a maximum concentration of 200 ppm ethyl-N^{α}-lauroyl-L-arginate hydrochloride (active ingredient) to inhibit microbial growth. (Addition of 200 ppm ethyl-N^{α}-lauroyl-L-arginate hydrochloride is equivalent to addition of approximately 225 ppm dehydrated LAE or 265 ppm non-dehydrated LAE.) Specifically, LAE is proposed for use in the food categories shown below in Table 1, repeated from Chapter I.

Table 1. Intended Food Uses of LAE

Up to 200 ppm ethyl-N^{α}-lauroyl-L-arginate hydrochloride in the following food categories in 21 CFR 170.3(n)
(3) Beverages and beverage bases, not including dairy products, soft drinks, or alcoholic beverages.
(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.
(7) Tea, including regular, decaffeinated, and instant types.
(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.
(11) Egg dishes including egg roll, egg foo young, egg salad, and frozen multicourse egg meals, but not fresh eggs.
(12) Margarine and margarine-like table spreads, mayonnaise, and spoonable and pourable dressings for salads.
(13) Fish products, including all prepared main dishes, salads, appetizers, frozen multicourse meals, and spreads containing fish, shellfish, and other aquatic animals, but not fresh fish.
(15) Fresh fish, including only fresh and frozen fish, shellfish, and other aquatic animals.
(17) Fresh meats, including only fresh or home-frozen beef or veal, pork, lamb or mutton and home-prepared fresh meat-containing dishes, salads, appetizers, or sandwich spreads made therefrom.
(18) Fresh poultry, including only fresh or home-frozen poultry and game birds and home-prepared fresh poultry-containing dishes, salads, appetizers, or sandwich spreads made therefrom.
(22) Pie fillings.
(24) Gravies and sauces, including all meat sauces and gravies, and tomato, milk, buttery, and specialty sauces.
(29) Meat products, including all meats and meat-containing dishes, salads, appetizers, frozen multicourse meat meals, and sandwich ingredients prepared by commercial processing or using commercially processed meats with home preparation.
(34) Poultry products, including all poultry and poultry-containing dishes, salads, appetizers, frozen multicourse poultry meals, and sandwich ingredients prepared by commercial processing or using commercially processed poultry with home preparation.

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Table 1. Intended Food Uses of LAE, continued

Up to 200 mg/kg ethyl-N^{α}-lauroyl-L-arginate hydrochloride in the following food categories in 21 CFR 170.3(n)
(35) Processed fruits and fruit juices excluding apple juice, including all commercially processed juices and juice punches, concentrates, dilutions, ades, and drink substitutes made therefrom, and dried fruits; also including strained fruits and fruit juices excluding apple juice as baby or toddler foods.
(36) Processed vegetables and vegetable juices, including potato salads, raw vegetables, vegetable juices and blends, and tomato sauces
(40) Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable, and combination soups and soup mixes.

B. Estimated Daily Intake of LAE

Under contract with LAMIRSA, Exponent Inc. estimated consumption of foods in which LAE is intended for use, based on data collected in the U.S. Department of Agriculture's 1994–96 Continuing Survey of Food Intakes by Individuals (CSFII) and its Supplemental Children's Survey (CSFII 1998), as provided on CD-ROM (USDA 2000). The CSFII 1994–96 was conducted between January, 1994, and January, 1997, with non-institutionalized individuals in the U.S. Twenty-four-hour diet recall data were collected through two in-person interviews conducted between 3 and 10 days apart from a nationally representative sample of individuals of all ages. The CSFII 1998 was designed as a supplement to CSFII 1994–96 to increase the sample size for children from birth through age 9 years. This survey employed the same methodology as CSFII 1994–96. In the merged surveys (designated CSFII 1994–96, 1998), 21,662 individuals provided dietary intake data on the first survey day, and 20,607 provided data on the second day.

Two-day average intakes of LAE on a body-weight basis were calculated for CSFII 1994–96, 1998 respondents age 2 and older who provided two complete 24-hour diet recalls and who reported consumption of foods in the proposed LAE use categories. All estimates were calculated with USDA sample weights to adjust for variable probabilities of selection, differential non-response rates, and possible deficiencies in the sampling frame.

About 99% of the population age 2 and older reported consumption of foods in one or more of the intended LAE use categories. Based on the assumption that LAE is present at a concentration of 225 ppm, providing 200 ppm ethyl-N^{α}-lauroyl-L-arginate hydrochloride, in all foods within all permitted food categories, the mean 2-day average LAE intake for the population two years of age and older in the U.S. is estimated to be 2.5 mg/kg bw/day, while the estimated 90th percentile intake is 4.7 mg/kg bw/day. The estimated mean and 90th percentile daily intakes of ethyl-N^{α}-lauroyl-L-arginate hydrochloride, the active ingredient in LAE, are estimated to be 2.2 mg/kg bw/day and 4.2 mg/kg bw/day, respectively.

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C. Incremental Daily Exposure to Arginine and Lauric Acid

As is discussed in more detail in the following section, ethyl-N^{α}-lauroyl-L-arginate hydrochloride is rapidly metabolized in the human gut; the primary direct metabolites are L-arginine and lauric acid, both common constituents of the human diet. Arginine is a non-essential amino acid found in meats and other protein-rich foods. Based on data from the Third National Health and Nutrition Examination Survey (NHANES III), the estimated mean daily intake of arginine from food and supplements is 4.2 g/day for the U.S. population (IOM 2003a, Table D-3). Lauric acid (dodecanoic acid) is a 12-carbon-chain saturated fatty acid found in plant oils and other common foods. Allison et al. (1999), using data from the 1989–91 Continuing Survey of Food Intakes by Individuals (CSFII 1989–91), estimated the mean daily intake of lauric acid as 0.7 g/day for the population 3 years and older. Expressed on a body-weight basis for a 70-kg individual, these intakes correspond to intakes of approximately 60 mg/kg bw/day for arginine and 10 mg/kg bw/day for lauric acid.

Each gram of ethyl-N^{α}-lauroyl-L-arginate hydrochloride consumed is metabolized to 0.41 g arginine and 0.47 g lauric acid. Thus, consumption of 4.2 mg/kg bw/day ethyl-N^{α}-lauroyl-L-arginate hydrochloride, the 90th percentile estimated intake, produces 1.72 mg/kg bw/day arginine and 1.97 mg/kg bw/day lauric acid. These amounts represent incremental increases of approximately 3% for arginine and 20% for lauric acid.

V. REVIEW OF SAFETY DATA

A. Introduction

Several *in vitro* and *in vivo* toxicity and metabolism studies have been conducted with LAE, using both crystalline LAE and Mirenat-N, a solution of LAE in propylene glycol. These studies include assessment of the metabolism of LAE in both *in vitro* and *in vivo* models, as well as its genetic, acute, subchronic, and reproductive toxicity. All studies were conducted at Huntingdon Life Sciences (HLS) in the UK under UK, EC, and OECD Good Laboratory Practice (GLP) standards applicable at the time the studies were conducted. All statistical analyses presented in this review were conducted as appropriate by the performing laboratories and included in each study report. A review article describing these studies was published early in 2004 (Ruckman et al. 2004).

B. Toxicokinetics and Metabolism

1. In Vivo Metabolism Study in the Rat after Single Oral Dose (Ruckman et al. 2004)

The objectives of this study were to: a) assess absorption and rates and routes of excretion of radioactivity, and b) characterize the biotransformation of LAE in rats. Four male rats (Sprague-Dawley [Crl:CD®(SD)BR]), 7–9 weeks old, were administered ^{14}C-LAE via a single oral gavage. The LAE was radiolabeled as [arginine-[U-^{14}C]]LAE, the specific activity of the test substance was 0.014 MBq/mg (0.38 μCi/mg), and the radiochemical purity was 99.4%. The test material was prepared as a suspension in 1% methyl cellulose at a nominal concentration of 80 mg/ml. Animals were administered doses of 2.5 ml/kg bw; the actual dose received by each rat was 177–180 mg/kg bw. Rats were housed individually in metabowls, and urine and expired air were collected from each animal at 0–8 and 8–24 hours and at 24-hour intervals for 5 days after dosing. Feces were collected separately at 24-hour intervals for 5 days. After 120 hours, animals were sacrificed, and the following samples prepared for radioassay by liquid scintillation counting (LCS): urine, cage wash, expired air, organs (liver, GI tract), and carcasses. Weights of organs and carcasses were also recorded. High performance liquid chromatography (HPLC), thin layer chromatography (TLC), and chromatographic analysis were conducted on urine samples (0–24 hour pooled).

Results

Mean recovery of radioactivity was high—99.5% of the administered dose. The major route of excretion was as CO_2 in expired air (mean of 23.9% of the dose in the first 24 hours after administration and mean of 36.6% over 5 days), but excretion in urine and feces was low (mean of only 11.8% and 4.3% of the dose over 5 days, respectively). Low excretion in feces indicated the LAE was almost completely absorbed. The largest proportion of radioactivity was retained in the carcass at sacrifice (46.4% of the dose), with 2% and 3.4% measured in the GI tract and liver, respectively. Radioactive components were identified and quantified (0–24 hour pooled urine) by HPLC. This analysis showed a single component with a retention time of 2 minutes, representing 9% of the dose. No unchanged LAE was detected. Since co-eluting metabolites was a possibility (as the material was not well retained by the HPLC column), urine was also analyzed by TLC, using several difference solvent systems. Seven different radioactive

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components were separated, the major component representing 85.6% of the sample radioactivity and 7.7% of the administered dose. The other six components (≤0.2% dose) were more polar than the major component. The major component was shown to co-chromatograph with ^{14}C-urea.

2. Metabolites in Liver and Plasma (Ruckman et al. 2004)

The metabolic profiles in rat plasma and liver *in vitro* and *in vivo* were studied to investigate the biotransformation pathway of ^{14}C-LAE and to assess if the primary metabolite(s) of LAE are natural products, such as arginine and ornithine. The LAE was radiolabeled as [arginine-[U-^{14}C]]LAE, and the specific activity of the test material was 0.015 MBq/mg (0.41 μCi/mg); the radiochemical purity was 99.8%. Male rats (N = 6 weighing 155–165 g) were administered a single gavage dose of ^{14}C-LAE (200 mg/kg bw) in 1% methylcellulose (dose volume of 2.7 ml/kg bw). After treatment, pairs of rats were anesthetized and blood samples taken (cardiac puncture) at 0.5, 1, and 4 hours. Blood was also taken from four untreated control rats and plasma and red blood cells (RBC) separated. The pooled plasma was combined and used for separate *in vitro* experiments. For extraction and chromatography of plasma (1 ml), an aliquot of plasma from each treated rat was added to methanol (5 ml) and the radioactivity measured in the separated precipitate and extract. Metabolites in the extracts were analyzed by HPLC and TLC (including co-chromatography with appropriate reference standards). For *in vitro* experiments, liver S9 (N = 3) was incubated with ^{14}C-LAE (at a nominal treatment rate of 10 μg/ml) at 37°C. At 4, 6, and 24 hours, the incubation was terminated by addition of methanol, followed by immediate separation of the precipitate and recovery of the extract for chromatographic analysis. For the *in vitro* experiments using plasma from control rats, an aliquot of plasma was treated with ^{14}C-LAE at a nominal concentration of 10 μg/ml at 37°C. Samples were taken immediately after treatment and at 1 and 4 hours and prepared for analysis as described above for the *in vivo* samples.

Results

Results from the second experiment with ^{14}C-LAE in rats are divided into the five different aspects of the study:

1. Rat liver S9 *in vitro* experiment, identification of biotransformation products: eight radioactive components were detected (none were detected without S9). The two major radioactive components were unchanged LAE and ornithine (confirmed by HPLC, TLC co-chromatography, and mass spectrometry [MS]). Smaller amounts of N^{α}-lauroyl-L-arginine (LAS) and arginine ethyl ester were also detected in the S9 extracts.

2. Rat liver S9 *in vitro* experiments, quantification of biotransformation products: After 4, 6, and 24 hours incubation of S9 treated with ^{14}C-LAE (10 μg/ml), TLC was used to quantify biotransformation products. Four hours after treatment, >50% of LAE had been metabolized and by 24 hours only 25% LAE was detected. The major metabolite identified was ornithine, accounting for up to 29.3% applied radioactivity (AR) 24 hours after treatment. HPLC showed that this component also contained trace amounts (<2%) of arginine. With time, urea increased from 3.8% AR (at 4 hours) to 7.8% (at 24 hours). LAS and arginine ethyl ester and two unidentified components

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each accounted for <5% AR. Unextractable radioactivity was in the range of 9.2–14.4% AR.

3. Rat plasma experiments, total radioactivity concentrations *in vivo*: Rats administered ^{14}C-LAE at 200 mg/kg bw as a single gavage dose had total radioactivity in plasma increasing from a mean of 14.2 µg equivalents LAE/ml at 0.5 hours post dosing to 118 µg equivalents/ml at 4 hours after dosing.
4. Rat plasma experiments, identification of biotransformation products: After treatment of rat plasma with ^{14}C-LAE at 10 µg/ml *in vitro*, five components were detected. HPLC co-chromatography confirmed the presence of unchanged LAE, N^{α}-lauroyl-L-arginine (LAS), and arginine. HPLC co-chromatography of reference substances with plasma extracts identified arginine, ornithine, LAS and unchanged LAE. Plasma metabolite profiles were also qualitatively similar to those in the *in vitro* liver S9 experiments.
5. Rat plasma experiments, quantification of biotransformation products: After treatment of rat plasma with ^{14}C-LAE (10 µg/ml) *in vitro*, extraction of radioactivity was quantitative at zero-time and declined to 93% and 86.4% AR at 1 and 4 hours after treatment. Analysis of the plasma extract revealed that ^{14}C-LAE was rapidly hydrolyzed to N^{α}-lauroyl-L-arginine. At time zero, only 63.9% AR was recovered as LAE due to rapid hydrolysis (this declined to near the limit of quantification after 1 hour). LAS increased from 32.8% AR at time 0 to 67.2% at 1 hour post treatment and declined to 32.1% at 4 hours. L-arginine and L-ornithine increased to a maximum of 43.9% and 5.1% AR, respectively, after 4 hours.

3. Conclusions

Based on the results of the *in vitro* and *in vivo* metabolism studies, a biotransformation pathway for Ethyl-N^{α}-lauroyl-L-arginate hydrochloride was devised as shown in Figure 2. This pathway has been confirmed in studies in humans (described in Chapter VII) conducted after the original GRAS determination was complete. *In vitro* experiments have shown that ethyl-N^{α}-lauroyl-L-arginate hydrochloride is rapidly metabolized by hydrolysis of the ethyl ester lauroyl amide functions to give the amino acid arginine. Arginine is then further metabolized to ornithine. *In vivo* the radiolabeled ornithine would be incorporated into naturally occurring endogenous compounds via the urea and citric acid cycles. From the *in vivo* metabolism study with ^{14}C-LAE, it was shown that a large proportion of the administered radioactivity was either retained in the carcass 5 days after dosing or eliminated as $^{14}CO_2$. The major metabolite excreted was ^{14}C-urea.

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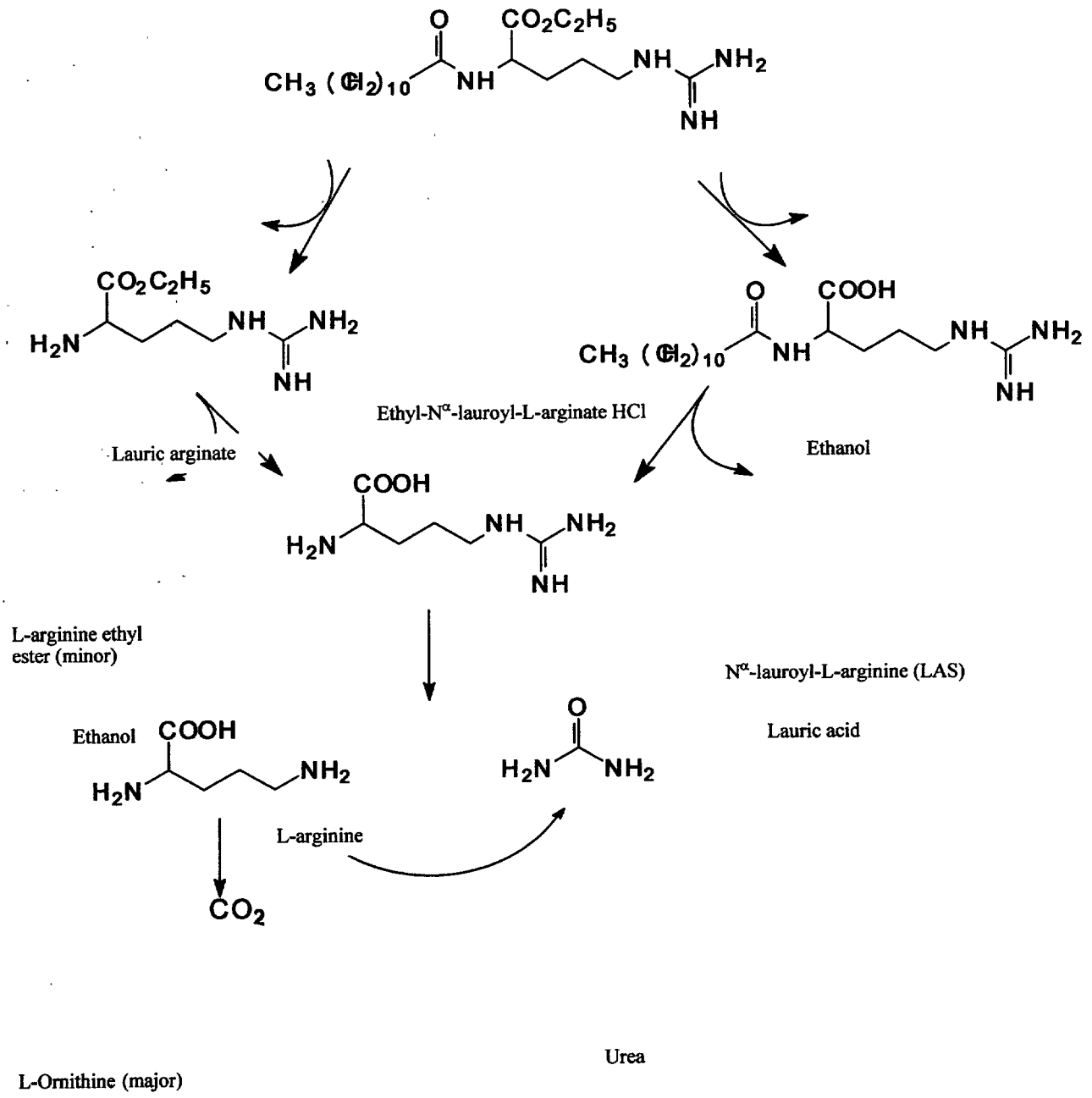

Figure 2. Biotransformation Pathway of Ethyl-N$^{\alpha}$-Lauroyl-L-Arginate Hydrochloride

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C. Genetic Toxicity

A series of studies was conducted on LAE and Mirenat-N to assess the genotoxic potential of these materials.

1. Bacterial Mutation (Ames) Assays with LAE and Mirenat-N (Ruckman et al. 2004)

Bacterial mutation assays were conducted with crystalline LAE or Mirenat-N (a 21.7% solution of LAE in propylene glycol), following OECD Guideline No. 471 (Bacterial Reverse Mutation Test) and U.S. EPA Guideline OPPTS 870.5100 (Bacterial Reverse Mutation Test), which incorporate the *in vitro* techniques described by Ames et al. (1975) and Maron and Ames (1983). Both studies used *S. typhimurium* TA1535, TA100, TA1537, and TA98; the study with crystalline LAE also included *E. coli* CM891. Solubility and range-finding tests determined appropriate solvents (LAE is soluble in DMSO, and Mirenat-N is soluble in water) and concentrations (LAE = 1.6, 5, 16, 54, and 161 µg/plate; Mirenat-N = 5, 15, 50, 150, and 500 µg/plate). Higher concentrations of test materials in their respective solvents were cytotoxic. S9 mix was prepared from homogenized rat livers stimulated by Aroclor 1254. In the main studies, the bacteria were incubated with the test material, ±S9, at 37°C for 30 minutes in glass tubes and then layered over histidine-deficient agar and incubated for an another 72 hours. Appropriate solvent controls (water or DMSO) were included as were positive controls. After the incubation period, appearance of the bacterial lawn was examined and revertant colonies assessed by automatic counters.

Results

In both assays with crystalline LAE or Mirenat-N, there was no evidence of mutagenic activity in any strain of *S. typhimurium* at any non-cytotoxic concentration (up to 161 µg/plate for LAE and 500 µg/plate for Mirenat-N), i.e., the test treatments failed to produce reproducible increases of at least 1.5 times the concurrent solvent/vehicle controls ±S9. Concurrent positive control compounds demonstrated the sensitivity of the assays as well as the metabolizing activity of the S9 liver preparations.

2. In Vitro Human Lymphocyte Chromosomal Aberration Tests with LAE and Mirenat-N (Ruckman et al. 2004)

In vitro mammalian chromosomal aberration tests using human lymphocytes were conducted with LAE or Mirenat-N (a 21.7% solution of LAE in propylene glycol), according to OECD Guideline No. 473 (*In Vitro* Mammalian Chromosome Aberration Test) and U.S. EPA Guideline OPPTS 870.5375 (*In Vitro* Mammalian Chromosome Aberration Test). Human blood, collected from healthy male donors, was pooled and diluted with tissue-culture medium. Aliquots were placed in sterile tissue culture containers and incubated at 37°C for 48 hours. Solubility of LAE (DMSO) and Mirenat-N (water) and top concentration levels were determined. A precipitate was caused by concentrations of LAE above 600 µg/ml; the highest final concentration used for testing was 1,600 µg/ml (so that two insoluble concentration levels were included). For Mirenat-N, the highest concentration was 2,000 µg/ml, as this gave a slight precipitate. After 48 hours, cultures were incubated with LAE (12.5, 25, 50, 100, 200, 400, 800, and 1,600 µg/ml) or Mirenat-N (15.6, 31.3, 62.5, 125, 250, 500, 1,000, or 2,000 µg/ml). Incubations were continued for 3 hours, ±S9 (prepared from Aroclor-induced rat livers). Positive

and solvent controls were included. After 3 hours, cultures were centrifuged, pellets re-suspended in fresh medium, and incubations continued for 15–18 hours. Colchicine (0.1–0.25 μg/ml) was added to stop mitotic activity, and after 2 hours cells were harvested and fixed. Slides were prepared for microscopic examination. Aberrations were scored according to the classification of the ISCN (1985). The number of aberrant metaphase cells in each treatment group was compared with the corresponding solvent control value. In the second phase, cultures were initiated and maintained as previously described. In this continuous treatment regimen, cells were incubated without S9 for 20 hours with 12.5, 25, 50, 75, 100, 150, 200, or 300 μg LAE/ml. In the presence of S9, a 3-hour treatment with LAE was used as in the first phase, but at different concentrations (25, 50, 100, 150, 200, or 300 μg/ml). Solvent and positive controls were included as in the first test with LAE. After 3 hours, cells were centrifuged and re-suspended in fresh medium; incubations continued for another 17 hours (total of 20 hours). Two hours before the end of the 20-hour incubation period, all cultures were treated with the mitotic inhibitor Colcemid (0.1 μg/ml). Cells were harvested and fixed, slides were prepared, and microscopic examination was performed as previously described. For the second phase in the Mirenat-N study similar procedures were followed, with minor variations as noted here: a) without S9, 18-hour harvest at concentrations of 125, 250, 500, 750, 1,000, 1,500, or 2,000 μg/ml; b) with S9, 18-hour harvest at concentrations of 125, 250, 500, 600, 700, 800, or 1,000 μg/ml; c) without S9, 32-hour harvest at concentrations of 125, 250, 500, 1,000, or 2,000 μg/ml; and d) with S9, 32-hour harvest at concentrations of 250, 500, or 1,000 μg/ml. Solvent and positive control cultures were included as before. Three hours after dosing, cultures containing S9 were centrifuged, cells were re-suspended in fresh medium, and incubation continued for another 15 or 29 hours (total incubation time 18 or 32 hours). Cultures treated in the absence of S9 were incubated continuously for 18 or 32 hours. Two hours before the end of incubations, colchicine was added (0.25 μg/ml). Cells were harvested and fixed, slides were prepared, and microscopic examination was performed as previously described.

Results

Neither LAE nor Mirenat-N in the ±S9 caused a statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations when compared to controls. In the study with LAE, statistically significant increases ($p<0.01$; ±S9) were seen at the highest concentration (200 μg/ml) in the proportion of polyploid cells in the first phase of the study. In the second phase with LAE, the same observation was made at 100 and 150 μg/ml but only +S9. These effects were seen at cytotoxic concentrations and, therefore, the biological significance of this observation is questionable. Neither LAE nor Mirenat-N showed evidence of clastogenic activity in the *in vitro* cytogenetics test system. All positive control compounds, however, caused large, statistically significant increases in the proportion of aberrant cells.

3. In Vitro Mouse Lymphoma Cell Mutation Assay with Mirenat-N (Ruckman et al. 2004)

Mirenat-N (a 21.7% solution of LAE in propylene glycol) was tested for mutagenic potential in an *in vitro* mammalian cell mutation assay, performed in compliance with OECD Guideline No. 476 (*In Vitro* Mammalian Cell Gene Mutation Test), U.S. EPA Guideline OPPTS 870.5300 (*In Vitro* Mammalian Cell Gene Mutation Test), and the procedures from Clive and Spector (1975). A maximum concentration of 2,000 μg/ml was selected as the upper limit for the preliminary toxicity test, based on solubility studies. A suspension of L5178Y mouse lymphoma cells was incubated ±S9 (prepared from rat livers stimulated by Aroclor) at Mirenat-N

concentrations of 15–2,000 μg/ml. Cell suspensions (1 x 10^6 cells/ml) were placed on a roller apparatus (3 hours at 37°C), washed, re-suspended, and rolled in bottles for 48 hours. Cell density was adjusted to 2 x 10^5/ml after 24 hours. The growth in suspensions of treated cultures relative to untreated controls was used as a measure of toxicity of Mirenat-N. Results were used to select concentrations for the main test, i.e., maximum concentration for the main study $\leq LC_{80}$. In the main study, cells were prepared as before and cultures incubated with Mirenat-N at four concentrations, ranging between the LC_0 and the LC_{80}, $\pm$S9, for 3 hours. As before, cells were washed, re-suspended, and rolled in bottles for an additional 48 hours. Cell density was adjusted to 2 x 10^5 after 24 hours of the 48-hour incubation. Viability and mutation frequency were assessed after 11–12 days of incubation in bacteriological culture plates in a humidified incubator at 37°C with an atmosphere of 5% CO_2 in air. Colonies of L5178Y cells growing on the plates were counted using an Optomax V image analyzer and results reported based on colonies with a diameter >100 μm. Positive and solvent controls were included.

Results

In the absence of S9, no significant increases in mutant frequency were observed in the cell cultures treated with Mirenat-N at concentrations of 100, 200, 280, and 300 μg/ml in Test 1 and 150, 220, 240, and 280 μg/ml in Test 2. EMS, the positive control, induced highly significant increases in mutant frequency in both tests. In the presence of S9, no significant increases in mutant frequency were observed after treatment with Mirenat-N at concentrations of 300, 400, 425, and 450 μg/ml in Test 1 and 200, 300, 400, and 450 μg/ml in Test 2. 20-Methylchoanthrene, the positive control, induced highly significant increases in mutant frequency in both tests. Cell growth and viabilities were within acceptable ranges for all studies. Thus, based on these results, it is concluded that Mirenat-N did not demonstrate mutagenic potential in this *in vitro* gene mutation assay.

4. Conclusions

Based on *in vitro* results from the Ames assay, the human lymphocyte chromosomal aberration test, and the mouse lymphoma cell mutation assay, LAE and Mirenat-N did not exhibit any evidence of mutagenicity or clastogenicity.

D. Acute Toxicity

1. Single Oral Dose and LD_{50} Studies with LAE and Mirenat-N (Ruckman et al. 2004)

Acute oral toxicity studies of LAE and Mirenat-N were performed in compliance with UK, EC, and OECD GLP regulations. The acute oral toxicity of LAE in male and female rats (HsDSprague-Dawley [CD]) was determined with the stepwise procedure (three animals per step; OECD Guideline No. 423), which is often referred to as the "up-and-down" procedure and has been subjected to international evaluation (Schlede et al. 1994). LAE was formulated at a concentration of 20% w/v in 1% w/v aqueous methylcellulose and administered via oral gavage at a constant dose volume of 10 ml/kg bw in order to achieve the desired dosage (2,000 mg/kg bw). Rats were checked twice daily for 14 days for mortalities; clinical signs and times were recorded at each observation. Body weight of each rat was recorded prior to dosing (Day 1) and on Days 6 and 15. All animals were killed at Day 15 and subjected to macroscopic examination. Another acute oral toxicity study was conducted on Mirenat-N (a 21.7% solution of LAE in

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propylene glycol) using five fasted rats/sex (HsD Sprague-Dawley [CD]) at a single gavage dose of 2,000 mg/kg bw. The material was administered at a volume of 1.9 ml Mirenat-N/kg bw. Clinical signs and body weights were monitored as in the first study above, and all animals were killed at Day 15 and subjected to macroscopic examination.

2. Results

There were no deaths following a single oral gavage dose of LAE or Mirenat-N (2000 mg/kg bw), nor were effects noted on body weight or gross macroscopy at study termination (Day 15). In the LAE study, clinical signs consisted of piloerection and increased salivation, both being evident within 5 minutes of dosing, accompanied in females with unsteady gait and in males by hunched posture. All signs had resolved by Day 3 (males) or Day 4 (females). In the study using Mirenat-N, there were fewer clinical signs, piloerection being the only observation in all rats with complete resolution by Day 2.

3. Conclusions

Based on the absence of adverse effects, the oral LD_{50} of crystalline LAE and of Mirenat-N in male and female rats is >2,000 mg/kg bw.

E. Subchronic Toxicity

1. Three-Month Dietary Studies in Rats with LAE and Mirenat-N (Ruckman et al. 2004)

Two 13-week feeding studies were conducted with LAE under GLP regulations of the UK, EC, and OECD, one with Mirenat-N, a 21.6% solution of LAE in propylene glycol, and one with crystalline LAE in accordance with FDA Redbook guidelines (FDA 1993). Both studies were consistent with OECD Guideline No. 408 (Subchronic Oral Toxicity—Rodent). The main differences relate to the doses of the test materials and the inclusion of a Functional Operational Battery (FOB) in the study with crystalline LAE. For the FOB in the LAE study, an additional 10 animals/sex were included, bringing the total for that study to 20 rats/sex/group, 10 for standard toxicological evaluation and 10 for the FOB. These additional 10 rats/sex/dose included for the FOB study with LAE were also evaluated along with the 10/rats/sex/dose in the standard toxicological evaluation for macroscopic observations, organ weights, and histopathology as described below (thus bringing the total for these parameters in the LAE study to N = 20 animals/sex/dose). Unless otherwise specified, the materials and methods described here are for both studies. After acclimatization, young Crl: CD(SD)BR rats (Mirenat-N) or Han Wistar rats (LAE) were assigned to one control and three treatment groups for each study. The animals were housed five per cage. Doses were selected based on range-finding studies. Rats were fed test material at constant levels in the feed, and the diet was periodically analyzed for homogeneity and concentration. The analytical, feed-intake, and body-weight data allowed conversions from ppm in feed to mg/kg bw/day. Concentrations (in ppm) and the corresponding doses (mg/kg bw/day) are as follows:

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Mirenat-N:	0 ppm = Control		
	3,200 ppm =	220 (males) and	262 mg/kg bw/day (females)
	12,800 ppm =	904 (males) and	1,067 mg/kg bw/day (females)
	50,000 ppm =	3,324 (males) and	3,927 mg/kg bw/day (females)

LAE:	0 ppm =	Control
	5,000 ppm =	384 (males) and 445 mg/kg bw/day (females)
	15,000 ppm =	1,143 (males) and 1,286 mg/kg bw/day (females)
	50,000 ppm =	3,714 (males) and 3,915 mg/kg bw/day (females)

Animals were fed the test materials at the specified levels for 13 weeks. During the in-life phase, the following data were collected: a) clinical signs and mortality were monitored twice daily; b) body weights were collected during acclimatization, on the day treatment commenced, weekly throughout the treatment period, and before necropsy; c) feed consumption was measured weekly; d) ophthalmoscopic examinations were made on all rats before treatment began and again at 13 weeks (control and high-dose groups only); e) blood was taken from fasted rats at 13 weeks for hematological evaluations; f) a complete range of blood chemistries was performed; and g) during week 13, overnight urine samples were collected for evaluation. After 13 weeks, all animals were sacrificed and subjected to detailed necropsy. At that time, organs were dissected free of adjacent fat and weighed; samples of tissues were preserved and selected for histological examination. In both studies, microscopic examinations were done according to the following protocol: a) on the specified tissues from all animals in the control and high-dose groups; b) on any tissue being reported as abnormal at macroscopic examination from any group (in the LAE study this went on to include all animals in the low- and mid-dose groups, as well for that particular abnormal macroscopic tissue); c) on the stomach, which showed a treatment-related change at the high dose for all animals in the low- and mid-dose groups (LAE study only); d) on the lungs, liver, and kidneys from all animals in the low- and mid-dose groups (Mirenat-N study only).

For the FOB in the LAE study, 10 animals/sex/dose were evaluated before treatment, then weekly throughout the treatment period for in-the-hand and standard arena observations. Finally, during Week 12, additional standard FOB measurements were recorded.

2. Results

In the 13-week feeding study with Mirenat-N, no unscheduled deaths were attributed to treatment (one male in the control group died during Week 1 from a ruptured liver). Clinical signs were unremarkable at all doses, except for alopecia in females at termination at dietary concentrations of 12,800 (5 of 10) and 50,000 ppm (8 of 10) when compared to controls (0 of 9). Overall mean body-weight gains for all treated males were similar to controls. In females, body weight was significantly decreased during the 13 weeks at 12,800, and 50,000 ppm (79% [$p<0.01$] and 86% [$p<0.01$] of control, respectively). This finding is not toxicologically significant because it was not dose-dependent and was observed only in females. There were no statistically significant differences between treated groups and controls for feed consumption, feed conversion ratios, water intake, or opthalmoscopic examinations. There were statistically significant decreases in total white blood cells (WBC) in females after 13 weeks at 12,800 ($p<0.05$) and 50,000 ppm ($p<0.01$). However, there was no consistency in the type of WBC contributing to the lower total cell count. Furthermore, these particular parameters often have considerable inter-individual variation, and they were within the range of laboratory controls for this species and strain. Therefore, these findings are not toxicologically significant. Various biochemical parameters were also affected in a statistically significant manner at 50,000 ppm, e.g., in males, increased urea ($p<0.05$) and in females decreased Ca ($p<0.05$), K ($p<0.01$) and P

($p<0.05$). In females Ca was also decreased at 12,800 ppm ($p<0.05$), although there was no apparent dose-response relationship to the effect at 50,000 ppm. Although these differences were statistically significant, the changes were small and inconsistent across the sexes. Thus, no relationship to treatment is suspected. The urinary pH in males was increased ($p<0.01$) at 50,000 ppm. No other effects were noted in the urinalysis. The relevance of this change in urinary pH is unclear, but it is not likely toxicologically relevant in that an opposite change in pH (decrease) was seen in the study with crystalline LAE, it occurred in only one sex, and there were no corroborative pathological findings. At the 13-week terminal macroscopic examination, there were no observations of note except for increased incidence of alopecia in females at the 12,800 and 50,000 ppm concentrations. This change is considered unlikely to be of biological significance. Also in females, the absolute liver weights were increased at 50,000 ppm ($p<0.05$). Since there were no significant differences in relative liver weights, the effect was not seen in males, and there were no macroscopic or microscopic changes in the liver, this finding is not toxicologically significant. There were no other significant changes in organ weights. Finally, no treatment-related microscopic changes were noted. The NOAEL was the highest dose tested in both sexes, 718 mg/kg bw/day of LAE.

In the study with crystalline LAE, there were no unscheduled deaths. Numerous clinical observations were noted at the high (50,000 ppm) dietary concentration, consisting of ungroomed and yellow staining of the coat and brown staining of the muzzle (also observed in males at 15,000 ppm). There were also lower body-weight gains, particularly in the mid- and high-dose groups in the first few weeks of the study. Although body-weight gains were comparable in all groups after Week 2, overall (Weeks 0–13) body weights did not fully recover, with total gains of 79% and 83% of controls for males and females receiving 50,000 ppm, respectively, and 89% of controls for males receiving 15,000 ppm. These effects appear to be related to the initial decreased feed consumption at these two higher doses, which was likely to have been at least partly the result of feed aversion due to poor palatability of the diet (although this diminished after the first week). The FOB evaluation showed no signs of neurotoxicity at any dose. No treatment-related ophthalmic findings, organ-weight changes or macroscopic findings were noted at study termination. There were minor, but statistically significant, increases in MCH ($p<0.01$), MCHC ($p<0.05$) and MCV ($p< 0.05$) and decreased total WBC ($p<0.01$) and lymphocyte counts ($p<0.05$) for males receiving 50,000 ppm. There were, however, no changes in any hematological parameters in females at any dose. Evaluation of blood chemistry at 50,000 ppm revealed minor, but statistically significant, decreased glucose (males, $p<0.01$), decreased total protein (males, $p<0.05$; females, $p<0.01$), decreased cholesterol (females, $p<0.05$), decreased albumin (males, $p<0.01$; females, $p<0.01$) and increased alkaline phosphatase (females, $p<0.05$). Decreased albumin was also observed in females at 15,000 ppm ($p<0.05$). Except for alkaline phosphatase, these clinical chemistry results were within the range of historical controls. This latter finding is not considered toxicologically significant in that it was in only one sex, no other enzymes were elevated, and there were no corroborative histopathological findings. Other intergroup differences in blood chemistry parameters, some of which may have attained statistical significance, were within the range of historical controls or concurrent control values were unusually high. There were no treatment-related changes in blood chemistries in either sex at the low dietary concentration (5,000 ppm). The only significant observation based on urinalysis investigations was low pH in males only at both 15,000 ($p<0.01$) and 50,000 ppm ($p<0.01$). There were no effects in either sex on urinalyses at 5,000 ppm. The

relevance of the changes in urine pH are unclear but are not regarded as toxicologically relevant in that they occurred in only one sex, and there were no corroborative pathological findings. The only histological changes related to treatment with LAE were observed in the area of the forestomach adjacent to the entry of the esophagus (the esophageal groove). The predominant change was parakeratosis, observed in 13 of 20 males ($p<0.01$) and 13 of 20 females ($p<0.01$) at 50,000 ppm and 1 of 20 females at 15,000 ppm. Other non-statistically significant observations in stomach pathology were ulceration (1 of 20 males and 2 of 20 females at 50,000 ppm and 1 of 20 males at 15,000 ppm) as well as erosions (3 of 20) and epithelial hyperplasia (1 of 20) in females only at 50,000 ppm. No histopathological changes were noted in either sex at the low concentration (5,000 ppm). The effects on the forestomach are regarded as local effects, most likely due to physical abrasion by the dry crystalline material, and not evidence of systemic toxicity. The NOAEL for systemic toxicity in this study was the highest achieved dose tested in both sexes, 3714 mg/kg bw/day.

3. Conclusions

In the study with Mirenat-N, there was no indication of toxicity at the highest dietary concentration tested, 50,000 ppm, corresponding to achieved dietary intakes of 3,324 mg/kg bw/day for males and 3,927 mg/kg bw/day for females. Since Mirenat-N contained 21.6% LAE (w/w), these intakes correspond to LAE doses of 718 mg/kg bw/day for males and 848 mg/kg bw/day for females. The NOAEL was the highest dose tested in both sexes, 718 mg/kg bw/day.

In the study with crystalline LAE, localized irritation was observed in the stomachs of rats in the high-dose group. These effects were confined to a specific area of the forestomach, the esophageal groove, and are thought to represent a local response to irritation. There was no correlation between the animals' stomach lesions and weight gain or other indications of biochemical disturbances. Thus they are considered not to be indicative of direct systemic toxicity. No indication of systemic toxicity was observed at the highest dietary concentration tested, 50,000 ppm, corresponding to 3,714 mg/kg bw/day for males and 3,915 mg/kg bw/day for females. The NOAEL for systemic toxicity in this study was the highest achieved dose tested in both sexes, 3,714 mg/kg bw/day.

F. Reproductive and Developmental Toxicity

1. Embryo-Fetal Studies in Rats and Rabbits with LAE (Ruckman et al. 2004)

Two studies, one in rats and one in rabbits and both performed in compliance with UK, EC, and OECD GLP regulations, investigated potential developmental effects of LAE. Many conditions in the two studies were similar; e.g., number of animals and test groups, mating procedures, treatment of pregnant dams, and examination of fetuses. Doses were selected based on range-finding studies. Female CD Rats (Sprague-Dawley origin) were mated with stock males at the laboratory (pregnancy determined by vaginal smears positive for spermatozoa) and New Zealand White rabbits (CD strain) were time-mated by the supplier. At study initiation, pregnant rats were 10–11 weeks old, and pregnant rabbits were 18–26 weeks old. LAE was prepared weekly as a suspension in 1% w/v methylcellulose (homogeneity and stability confirmed by the laboratory). Pregnant animals were gavaged with LAE (10 ml/kg bw/day for rats and 5 ml/kg

bw/day for rabbits) as follows: a) rats (22/dose) on gestation days (GD) 6–19 at doses of 0, 147, 442, or 1,473 mg/kg bw/day; b) rabbits (22/dose) on GD 6–19 at doses of 0, 74, 221, or 736 mg/kg bw/day. All animals were observed twice daily. Moribund animals were sacrificed and, along with any animals that died prematurely, subjected to necropsy (including uterine contents and fetuses, where possible [rabbits only]). Body weights and feed consumption data were collected at varying times for both species from GD 0 through 1 day prior to terminal sacrifice (GD 20 for rats and 29 for rabbits). At study termination, females were killed and subjected to necropsy, followed by examination of the reproductive tract and uterine contents, including weight of the gravid uterus, number of corpora lutea in each ovary, number of implantation sites, number of resorption sites (early and late), and number and distribution of live and dead fetuses in each uterine horn. External examinations at necropsy included fetal weight and sex, notation of external abnormalities, and individual placental weights and abnormalities. All fetuses were killed, then given internal examinations. For rats, this included the neck as well as thoracic and abdominal cavities (50% of each litter); all abnormalities were recorded, and the fetuses were eviscerated prior to fixation, processed, and stained with Alizarin Red for skeletal examinations. In rats, the remaining 50% of fetuses in each litter were placed in Bouin's fixative then examined by the Wilson free-hand serial sectioning technique. For rabbits, the neck and thoracic and abdominal cavities from all fetuses from each litter were examined. This was followed by evisceration; 33% of each litter was decapitated and the heads fixed in Bouin's fluid, then subjected to free-hand serial sectioning and examination for abnormalities. Torsos and remaining intact rabbit fetuses were fixed for subsequent skeletal examination. Eviscerated fetuses were processed using a modification of the Dawson Alizarin Red staining technique, and skeletons were examined for development and abnormalities.

2. Results

In the rat study, several pregnant animals were sacrificed during the dosing phase due to severe clinical signs. At the high dose, three rats were killed *in extremis* on treatment days 2 and 3 (GD 7 or 8). Prior to sacrifice, all three animals showed noisy and gasping respiration, as well as excessive salivation. Two rats had weight loss and one displayed underactive behavior and piloerection. Necropsy revealed various prominent effects in the gastrointestinal tract (GI), consisting of gaseous material in the stomach (and the entire GI tract of one). At the mid dose, two females were similarly affected, although later in gestation (both sacrificed on GD 17). Necropsy revealed results similar to those in the animals sacrificed earlier in the high-dose group. Both animals had grossly normal implantations. The general condition of the surviving animals was satisfactory, and there were sufficient pregnant females (control = 22; low dose = 22; mid dose = 20; high dose = 19) at study termination to permit evaluation of embryo-fetal effects. During ongoing daily gavage treatments, noisy respiration was seen to varying degrees in all groups treated with LAE (0/22 animals in controls; 3/22 in the low dose; 7/22 in the mid dose; 9/22 at the high dose (these numbers include the five animals that were killed prematurely)). The incidence of noisy respiration, however, in the low-dose group was very low, being observed in only three rats on four occasions. The number of animals affected and the number of occasions this was observed was much higher in the top two dose groups. Salivation at dosing was also evident to varying degrees, mainly in the mid dose (14/20 occasionally) and high-dose (all animals on 50% of dosing occasions) groups; only one rat on one occasion in the low-dose group showed salivation. Neither effect was seen in any control animal. Respiratory distress and

associated mortality in several animals in the mid- and high-dose groups were assessed not to be systemic toxic responses to oral ingestion of LAE but more likely due to bronchial irritation if the test material was aspirated (the higher dose suspensions were more viscous; see following discussion of the rabbit study). There were no overall treatment-related effects on body weights or feed consumption. Occasionally, treated animals showed transient effects on both parameters, associated with periods of respiratory distress. Finally, at study termination (GD 20) there were no maternal necropsy findings related to treatment, no effects on fetal survival (as indicated by the extent of pre- and post-implantation loss and the number of live fetuses), and no effects on fetal and placental weights. The incidences of fetal abnormalities and variations were unaffected by treatment at any dose. Thus, fetuses were unaffected even in the presence of maternal respiratory distress at the mid- and high-dose levels.

In the rabbit study, reactions to dosing were largely limited to adverse respiratory reactions, particularly in the mid- and high-dose groups. Five animals in each of these groups displayed noisy respiration, and in the high-dose group, two animals were sacrificed very early in the study and replaced. A total of 76 pregnant rabbits carried live litters to study termination (Gestation day 29). Necropsy of the two rabbits sacrificed early revealed congestion in the lungs and frothy liquid in the trachea. As the study progressed, two additional rabbits were sacrificed for reasons of animal welfare (due to respiratory difficulties)—one each in the mid- and high-dose groups (GD 14 and 9, respectively). Necropsy revealed similar findings as in the rabbits sacrificed earlier. The adverse respiratory reaction was probably related to irritation associated with the higher doses of LAE, and these reactions were greatly reduced when the surface of the gavage catheter was washed clean and left moist rather than just wiped dry. There were no other signs of adverse reactions to treatment. Body weights in the high-dose group were significantly lower when compared to controls throughout most of the treatment period ($p<0.01$ to <0.05, depending on the day of comparison). This is considered treatment-related in that the animals showed some recovery once treatments were completed (GD 19). Similar effects were noted for feed consumption at the high dose. Body weights and feed consumption were unaffected by treatment at 74 and 221 mg/kg bw/day. At study termination, there were no treatment-related necropsy findings, nor were there effects on fetal survival, numbers of corpora lutea, implantations, or live young. Finally, there were no effects of treatments on fetal or placental weights or fetal abnormalities at any dose tested.

3. Conclusions

Respiratory distress was observed in some animals administered LAE by gavage in both of the embryo-fetal toxicity studies described above. However, this effect was not deemed a systemic toxic response to LAE. Rather, it was considered to be due to the potential aspiration, after gavage, of the more concentrated suspensions of LAE, which may have caused bronchial irritation. In the rabbit study, it was found that washing the outside of the catheters and leaving them moist, compared with the previous procedure just wiping them dry, greatly reduced the respiratory effect. It is likely that the same catheter problem existed in the study with rats. Excluding transient changes in body weight and feed consumption, there were no other systemic adverse treatment-related in-life effects of LAE on pregnant females. Furthermore, no adverse treatment-related findings were seen in dams at necropsy. Accordingly, the observed NOAEL of LAE in adult female rats was equal to the highest dose tested, 1,473 mg/kg bw/day.

In adult female rabbits, the NOAEL of LAE was the mid dose, 221 mg/kg bw/day. The toxicological implications of the reduction in body-weight gain observed at the high dose are unclear, because rabbits are known to be poor models for testing substances with antibiotic activity. The International Conference on Harmonization's Guideline on Detection of Toxicity to Reproduction for Medicinal Products (FDA 1994) notes that, due to rabbits' "susceptibility to some antibiotics and to disturbance of the alimentary tract, clinical signs can be difficult to interpret," and recommends against use of rabbits for testing such substances. Similarly, Palmer (1978) states, "...rabbits are extremely susceptible to agents (antibiotics) affecting gram positive bacteria and should not be used for testing such materials."

No treatment-related effects on fetal growth, fetal development, or fetal survival were observed up to the highest dose of LAE tested in either the rat or rabbit. In rats, the observed fetal NOAEL was the high dose of 1,473 mg/kg bw/day; in rabbits, the observed fetal NOAEL was the high dose of 736 mg/kg bw/day. Finally, there were no treatment-related effects on fetal weight, placental weight, fetal abnormalities, and fetal variants up to the highest dose of LAE tested.

VI. SAFETY ASSESSMENT AND GRAS DETERMINATION

A. Introduction

LAE is safe and is GRAS for direct addition to foods to inhibit microbial growth in the foods to which it has been added. LAE is intended for addition at a maximum use level of 225 ppm, providing 200 ppm of the active ingredient ethyl-N^{α}-lauroyl-L-arginate hydrochloride, in the food categories listed in Table 1.

This safety assessment and GRAS determination includes two steps. In the first step, the safety of LAE under its intended conditions of use is demonstrated by showing that the EDI of LAE under its intended conditions of use is within the ADI based on toxicity studies of LAE. In the second step, LAE is determined to be GRAS by demonstrating that the safety of LAE under its intended conditions of use is based on publicly available information and is generally recognized among scientific experts qualified by scientific education and experience to evaluate the safety of substances added to food.

The regulatory framework for establishing whether a substance is GRAS, in accordance with Section 201(s) of the FDCA, is set forth under 21 CFR §170.30, which states that general recognition of safety based on scientific procedures may be based on the view of qualified experts as described in §170.30(b).

A GRAS determination based on scientific procedures requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive; this is referred to as the "technical element." In addition, a GRAS determination requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination has two components:

1. The data and information relied upon to establish safety must be generally available, although it may be supported by unpublished data; and
2. There must be agreement among qualified experts regarding the safety of the substance for its intended use.

B. Safety Assessment of LAE for Its Intended Use

Safety for ingredients to be added to food is defined (21 CFR §170.3(i)) as "a reasonable certainty in the minds of competent scientists that the substance is not harmful under its intended conditions of use." This same regulation specifies that three factors must be considered in determining safety:

1. The probable consumption of the substance and of any substance formed in or on food because of its use;
2. The cumulative effect of the substance in the diet, taking into account any chemically- or pharmacologically-related substance or substances in such diet;

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3. Safety factors that, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food and food ingredients, are generally recognized as appropriate.

1. EDI of LAE

As described in Chapter IV, LAE is intended for addition at a maximum use level of 225 ppm, providing 200 ppm of the active ingredient, ethyl-N^{α}-lauroyl-L-arginate hydrochloride, in the foods listed in Table 1. The estimated mean 2-day average LAE intake from all intended food uses combined among U.S. consumers age 2 years and older is 2.5 mg/kg bw/day and the 90th percentile 2-day average intake, the EDI, is 4.7 mg/kg bw/day. The estimated mean and 90th percentile intakes of ethyl-N^{α}-lauroyl-L-arginate hydrochloride are 2.2 mg/kg bw/day and 4.2 mg/kg bw/day, respectively. About 99% of the U.S. population age 2 years and older consumes foods in one or more of the LAE target use categories. It should be noted that these intake estimates are likely to be exaggerated because it is assumed that LAE is added at the maximum use level of 225 ppm to all foods in each of the intended LAE use categories.

2. Cumulative Effect of LAE in the Diet

As has been extensively discussed, the primary metabolites of LAE are arginine and lauric acid (Ruckman et al. 2004). One mole of each is formed by each mole of LAE metabolized. Both of these substances are found in a wide variety of foods and, consequently, are part of the diet. In Chapter IV, data were cited from the Institute of Medicine (IOM 2003a) showing that the mean daily dietary intake of arginine in the U.S. population is 4.2 g/day. The estimated mean daily intake of lauric acid is 0.7 g/day for the population 3 years and older (Allison et al. 1999). Expressed on a body-weight basis for a 70-kg individual, these intakes correspond to intakes of approximately 60 mg/kg bw/day for arginine and 10 mg/kg bw/day for lauric acid.

Based on the molecular weights of ethyl-N^{α}-lauroyl-L-arginate hydrochloride, arginine, and lauric acid, it is calculated that each gram of ethyl-N^{α}-lauroyl-L-arginate hydrochloride consumed is metabolized to 0.41 g arginine and 0.47 g lauric acid. Thus, consumption of 4.2 mg/kg bw/day ethyl-N^{α}-lauroyl-L-arginate hydrochloride, the 90th percentile estimated intake, produces 1.7 mg/kg bw/day arginine and 2.0 mg/kg bw/day lauric acid. These amounts represent incremental increases of approximately 3% for arginine and 20% for lauric acid. Both of these figures are well within the ranges of normal variability of individual intakes of these substances and are not believed to be biologically significant.

3. Safety of LAE

LAE and Mirenat-N have been tested in a variety of toxicity studies designed to evaluate the safety of this material. The studies span a wide range of endpoints, including genotoxic, acute, subchronic, and embryo-fetal endpoints (Ruckman et al. 2004). Absorption, metabolism and excretion studies were also conducted (Ruckman et al. 2004). Both LAE and Mirenat-N have a low order of acute toxicity (the LD_{50} for both materials is >2,000 mg/kg bw). The material is not genotoxic, nor is it a developmental toxicant in two species; the rat study with LAE was also negative for neurotoxicity.

A systemic NOAEL for subchronic exposure to LAE can be derived from the two 13-week feeding studies conducted with the LAE-containing formulation, Mirenat-N, or with crystalline LAE. The study using Mirenat-N investigated concentrations of 3,200, 12,800, and 50,000 ppm (equivalent to LAE concentrations of 691, 2,765, and 10,800 ppm). The study with LAE studied the toxicity of 5,000, 15,000, and 50,000 ppm concentrations.

In the study with Mirenat-N, no adverse effects were seen at any concentration (Ruckman et al. 2004). The NOAEL for systemic toxicity of Mirenat-N was thus the highest level tested, 50,000 ppm (3,324 and 3,927 mg/kg bw/day for males and females, respectively). Since Mirenat-N contained 21.6% LAE (w/w), these intakes correspond to LAE doses of 718 mg/kg bw/day for males and 848 mg/kg bw/day for females. The NOAEL for systemic toxicity of LAE was the highest dose tested in both sexes, 718 mg/kg bw/day.

In the study with crystalline LAE added to the feed, marked effects attributed to irritancy (microscopic changes in the forestomach) and unpalatability (body-weight reduction and reduced feed consumption) of the material were seen at the highest concentration tested (50,000 ppm) and to a lesser extent at the mid-level concentration of 15,000 ppm (Ruckman et al. 2004). Body weights were reduced during the first week and reduced feed consumption was noted. Increased feed scatter indicated unpalatability. In comparison, no effects on stomach histopathology, body weight, or feed consumption were noted in the study with Mirenat-N, corroborating the finding that when the crystalline LAE is tested without the accompanying propylene glycol matrix, the effects at this concentration level are probably associated with local irritancy and an adverse effect on palatability. The effects seen at 15,000 and 50,000 ppm LAE are, therefore, considered unrelated to systemic toxicity, but rather to the irritancy and unpalatability of the compound, since no adverse effects are seen when these properties are masked by the propylene glycol matrix. There were no other observations that would indicate systemic toxicity at these levels of dietary concentration. The highest dietary concentration of 50,000 ppm corresponds with achieved intakes of 3,714 mg/kg bw/day for males and 3,915 mg/kg bw/day for females. The NOAEL for systemic toxicity in this study was the highest achieved dose tested in both sexes, 3,714 mg/kg bw/day.

The metabolism of LAE has also been well characterized and further substantiates its safety in mammals. In the single oral dose study with radiolabeled LAE (Ruckman et al. 2004), radioactivity was well absorbed and eliminated slowly. The main route of elimination was as exhaled CO_2, with much less in the urine and feces. Similar results were obtained by Schwartz et al. (1997) with L-N^G-methylarginine hydrochloride in rats and dogs. The single major metabolite in urine co-chromatographed with ^{14}C-urea, which suggests that ethyl-N^{α}-lauroyl-L-arginate hydrochloride is rapidly metabolized by hydrolysis of the ethyl ester and lauroyl amide to arginine. This radiolabeled arginine subsequently undergoes natural amino acid catabolism via the urea and citric acid cycles, with ultimate elimination as $^{14}CO_2$ in expired air and ^{14}C-urea in urine. Furthermore, entry into these biochemical pathways would result in the formation of other radiolabeled amino acids and subsequent incorporation into many endogenous products. This would account for the high retention of radioactivity in the carcasses after 5 days. Finally, since the results suggest that LAE is converted to arginine, it is likely that the toxicity would be similar to that of the amino acid arginine. Additional *in vitro* experiments confirmed this proposed metabolism. *In vitro* incubation of [arginine-[U-^{14}C]]LAE with preparations of rat liver S9

fractions and control rat plasma have shown that ethyl-N^{α}-lauroyl-L-arginate hydrochloride is rapidly metabolized by hydrolysis of the ethyl ester and lauroyl amide to the naturally occurring amino acid arginine, which undergoes further catabolism to ornithine and urea (Ruckman et al. 2004). *In vitro* experiments with ^{14}C-LAE in plasma have also demonstrated that both LAE and N^{α}-lauroyl-L-arginine would be quantitatively extracted from rat plasma (Ruckman et al. 2004). This suggests that the increase in the non-extractable radioactivity observed in the *in vivo* (and to a lesser extent *in vitro*) experiments is due to incorporation of naturally-occurring LAE metabolites into plasma proteins. Given the rapid hydrolytic degradation of LAE and LAS in both liver and plasma *in vitro* and the results from analysis of *in vitro* plasma samples, it may be concluded that exposure to these compounds *in vivo* is likely to be short. Thus, taken together, the metabolism data demonstrate that ethyl-N^{α}-lauroyl-L-arginate hydrochloride is primarily and rapidly metabolized to naturally occurring amino acids, mainly arginine and ornithine. These in turn are incorporated into endogenous products and finally degraded to carbon dioxide through the normal mammalian biochemical pathways. This metabolic pathway further substantiates the overall safety in mammals from oral exposure to LAE.

Additionally, the metabolic and toxicokinetic data are used to determine appropriate safety factors to establish an ADI of LAE based on the subchronic toxicity data. The metabolism of LAE has been adequately characterized and follows well-known metabolic pathways that involve only substances of very low toxicity. Furthermore, the data demonstrate that LAE is rapidly and completely converted to lauric acid and arginine, which are in turn metabolized and incorporated into endogenous products or degraded to carbon dioxide and urea through the usual biochemical pathways. Thus, it is appropriate to use a safety factor of 100, allowing for interspecies differences in sensitivity and for variability in sensitivity among humans. Based on this consideration, the NOAEL of 3,714 mg/kg bw/day obtained in the 13-week feeding study with crystalline LAE serves as the basis for deriving an ADI of 37 mg/kg bw/day for LAE in the diet.

Since this ADI is approximately eight-fold higher than the EDI, the estimated 90th percentile daily intake of LAE from its intended use, it may be concluded that LAE is safe for its intended use.

C. General Recognition of the Safety of LAE

The intended use of LAE has been determined to be safe through scientific procedures as set forth in 21 CFR §170.30(b). Because this safety evaluation was based on generally available information, it satisfies the first component of the common knowledge element of a GRAS determination. Determination of the safety and GRAS status of LAE for addition to foods under its intended conditions of use has been made through the deliberations of an Expert Panel comprising Robert J. Nicolosi, Ph.D., Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D., with Joseph F. Borzelleca as Scientific Advisor to the Expert Panel. These individuals are qualified by scientific training and experience to evaluate the safety of substances intended to be added to food. They have critically reviewed and evaluated the publicly available information summarized in this document and have individually and collectively concluded:

Ingestion of LAE, also known as lauric arginate, from the intended uses results in a daily intake that remains within safe limits established by published *in vitro* and animal studies and corroborated by more recent (currently unpublished) animal and human studies. LAE has been sufficiently characterized to ensure that it is food-grade product. Therefore, LAE meeting the specifications described in the GRAS monograph is safe when added to foods listed in Table 1 at a concentration providing not in excess of 200 ppm ethyl-N^{α}-lauroyl-L-arginate hydrochloride.

It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same information would reach the same conclusion. Therefore, LAE, also known as lauric arginate, is safe and is GRAS via scientific procedures, when added to foods listed in Table 1 at up to 225 ppm, providing 200 ppm of the active ingredient ethyl-N^{α}-lauroyl-L-arginate hydrochloride, to inhibit the growth of microorganisms.

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VII. ADDITIONAL SAFETY RESEARCH

A. Introduction

Since LAE was first determined to be GRAS for its then-intended uses in the summer of 2003, additional metabolic and toxicological studies have been completed that corroborate the findings of the published studies that provide the basis for the GRAS determination. Since these newer studies are not yet published, they cannot serve as the basis for GRAS status, but they may appropriately be viewed as data that further support the published information. Furthermore, since GRAS determinations are always liable to re-evaluation if evidence were to come to light that casts doubt on the safety of the intended uses of the substance, it is appropriate that this additional information be shared with the Expert Panel and summarized in this document.

Four additional studies have been completed since LAE was determined to be GRAS in 2003: a two-phase *in vivo* study of the metabolism of LAE in humans, an *in vitro* mouse lymphoma cell mutation assay with LAE, a two-generation reproductive performance study with LAE in the rat, and a 52-week dietary toxicity study with LAE in the rat. These studies are briefly described and their results summarized below.

B. *In Vivo* Investigations into the Metabolism of LAE in Humans

This study was conducted under Good Laboratory Practice (GLP) standards, as specified in the UK (Statutory Instrument 1999 No. 3106, as amended by Statutory Instrument 2004 No. 994), EC (Official Journal No. L 77/8, as amended in Official Journal No. L50/44), and OECD (ENV/MC/CHEM(98)17). It was designed to investigate the rate and extent of metabolism of LAE in humans. In the first phase (CentraLabS Clinical Research 2005a), two adult males received a single oral dose of 2.5 mg/kg bw LAE as a 25% solution in propylene glycol. Their blood was sampled at 0 h (pre-dose), at 5, 10, 15, and 30 minutes, and at 1, 2, 4, 8, 12, and 24 hours post administration. The collected plasma was isolated and extracted with methanol; the extract and residue were analyzed by HPLC with limits of quantification of 1 ng/mL for LAE and its major metabolite, LAS, and 20 ng/mL for arginine.

As shown in Figure 3, LAE was not detected at any point, and the LAS and arginine levels declined to non-detectable levels within 12 hours.

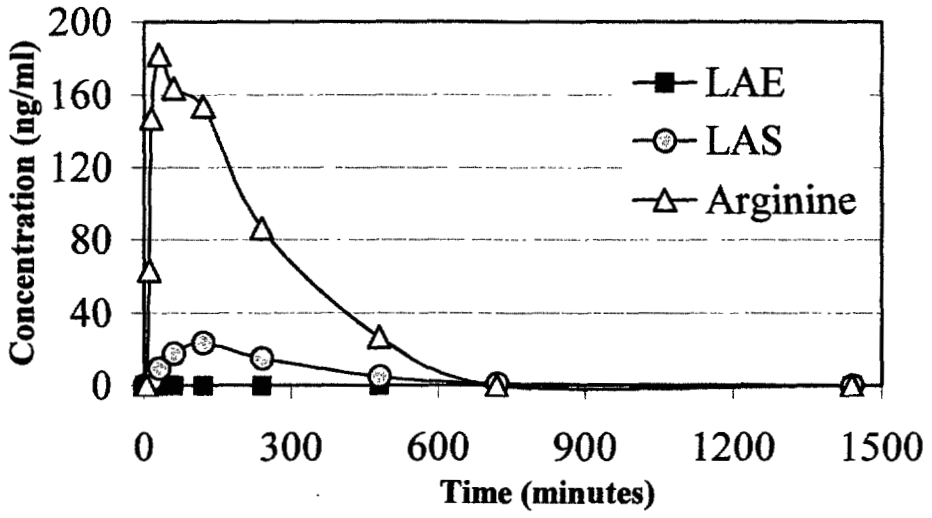

Figure 3. Metabolism of 2.5 mg/kg LAE in Humans

In the second phase (CentraLabS Clinical Research 2005b), four adult males were administered a single dose of 1.5 mg/kg bw LAE as a 25% solution in propylene glycol. The methods were the same as in the first study. As shown in Figure 4, the results were also similar: LAE was not detected, and the concentrations of both LAS and arginine decreased to undetectable levels within 12 hours of administration.

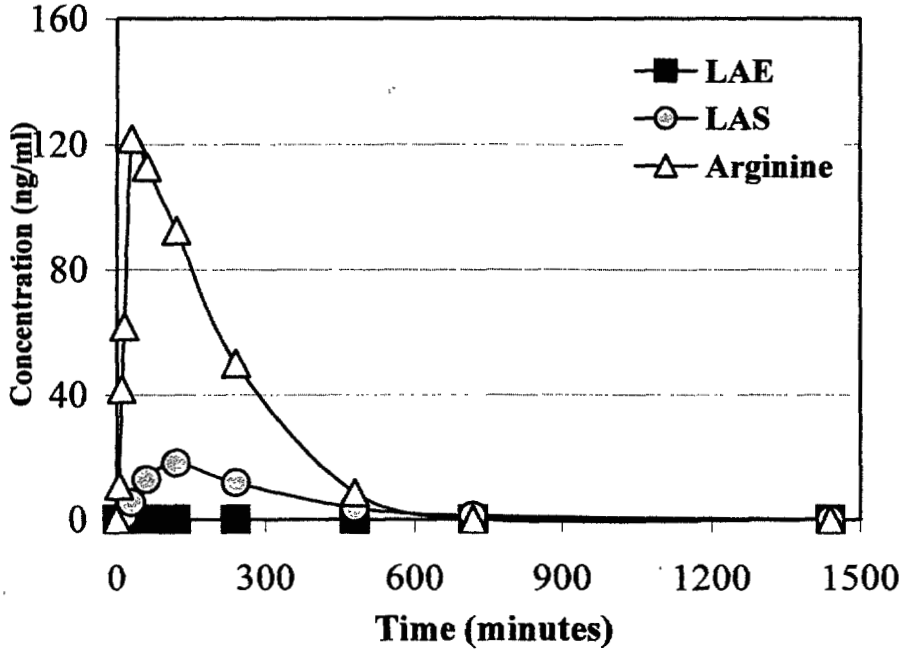

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Figure 4. Metabolism of 1.5 mg/kg LAE in Humans

C. In Vitro Mouse Lymphoma Cell Mutation Assay with LAE

The objective of this study, which was performed in compliance with UK, EC, and OECD GLP regulations and consistent with OECD Guideline No. 476 (*In Vitro* Mammalian Cell Gene Mutation Test) and U.S. EPA Guideline OPPTS 870.5300 (*In Vitro* Mammalian Cell Gene Mutation Test), was to test the mutagenic potential of LAE (Huntingdon Life Sciences 2004). The study comprised a preliminary toxicity test and six independent mutagenicity tests, during which mouse lymphoma L5178Y cells were exposed for 3 or 24 hours in the absence of exogenous metabolic activation (S9) and 3 hours in the presence of S9. The maximum LAE concentration tested in the preliminary toxicity test was 60 mg/ml (final concentration in medium of 600 μg/ml), approximately four times the limit of solubility.

In the preliminary toxicity test, a steep toxicity curve was observed after treatment with LAE in the absence and presence of S9 with a 3-hour exposure, and in the absence of S9 with a 24-hour exposure. The preliminary toxicity test was extended for all conditions using closely spaced concentrations to achieve a range of concentration levels spanning the toxicity range of approximately 10–100% relative suspension growth. In the absence and presence of S9 with a 3-hour exposure and in the absence of S9 with a 24-hour exposure, the concentration ranges observed to reduce relative suspension growth from approximately 100% to 10% were 10–50 μg/ml, 10–50 μg/ml, and 1–50 μg/ml, respectively.

With 3-hour exposures in the absence and presence of S9, the maximum concentrations assessed for determination of mutant frequency were 34 μg/ml and 46 μg/ml, respectively. With a 24-hour exposure in the absence of S9, the maximum concentration assessed was 50 μg/ml. There were no statistically significant increases in mutant frequency at any of the concentrations tested, while the concurrent solvent and positive controls were within the normal historical ranges. It is concluded that LAE does not demonstrate mutagenic potential under the experimental conditions described.

D. Two-Generation Reproductive Performance Study with LAE

The objective of this study, which was designed and executed in compliance with OECD Test Guideline 416, was to assess the influence of LAE on reproductive performance when administered continuously in the diet through two successive generations of CD rats (Huntingdon Life Sciences 2005a). Four groups of Crl:CD (SD)IGS BR rats received diets containing 0, 2,500, 6,000, or 15,000 ppm dietary concentrations of LAE. In the F0 generation, there were 28 animals per sex in each group, and in the F1 generation there were 24 animals per sex per group. The basal diet was a powdered laboratory animal diet (UAR VFRI) supplied by Charles River UK, to which LAE, kept stored under refrigerated conditions (4°C), was admixed in appropriate concentrations. The diets were analyzed at appropriate intervals for concentration of LAE, homogeneity, and stability.

The mean achieved dosages of LAE in the F0 generation were 0, 181, 434, and 1,073 mg/kg bw/day for males and 0, 207, 502, and 1,226 mg/kg bw/day for females. During lactation, the mean female dosages were 0, 402, 1,018, and 2,600 mg/kg bw/day. In the F1 generation, the mean achieved dosages were 0, 224, 537, and 1,356 mg/kg bw/day for males and 0, 246, 582,

and 1,489 mg/kg bw/day for females. During lactation in the F1 generation, the mean female dosages were 0, 409, 898, and 2,353 mg/kg bw/day.

F0 Generation: There were no apparent treatment-related adverse effects, and none of the three unscheduled deaths (one male in the low-dose group and one of each sex in the high-dose groups) was treatment related. There were no statistically significant, consistent, treatment-related adverse effects on feed consumption or body-weight gain, nor on pre-pairing estrous cycles, mating performance or fertility, gestation length or gestation index, litter size, sex ratio, offspring survival, or Day 1 body weights. Body-weight-relative weights of spleen and ovaries among females in the 15,000-ppm group and relative ovary weights among females in the 2,500-ppm group were statistically significantly lower than controls, but these were not dose-dependent and are not toxicologically significant.

F1 Offspring: There were no statistically significant, consistent, treatment-related, dose-dependent adverse effects on implantations, litter size, offspring survival, or sex ratio, nor on surface righting, air righting, or the auditory and visual responses of offspring. Absolute spleen weights were significantly lower than control in the 15,000-ppm groups of both males and females, but these differences were not regarded as toxicologically significant because they were within the range of historical controls and were not associated with histopathology and because relative spleen weights did not significantly differ.

F1 Generation: There were three unscheduled deaths (one female in the low-dose group and one of each sex in the high-dose groups), none treatment related. There were no statistically significant, consistent, treatment-related, dose-dependent adverse effects on body-weight gain of males; body-weight gain of females in the 6,000- and 15,000-ppm groups was significantly lower than controls during the first week, but the difference disappeared during the latter half of lactation. Male sexual maturation was unaffected by treatment with LAE, but a treatment-related effect on vaginal opening was seen in females receiving 15,000 ppm LAE. This effect was transient and occurred at a point when estimated achieved dosage was in excess of 1,900 mg/kg bw/day, and was not regarded as toxicologically significant.

F2 Offspring: There were no statistically significant, consistent, treatment-related, dose-dependent adverse effects on implantations, litter size, offspring survival, or sex ratio, nor on surface righting, air righting, or the auditory and visual responses of offspring. Spleen weights relative to body weight were significantly lower than control in the 15,000-ppm group, but these differences were not regarded as toxicologically significant because they were within the range of historical controls and were not associated with histopathology.

It was concluded that the NOAEL for reproductive performance in the CD rat is 15,000 ppm LAE, the highest concentration tested, equivalent to 1,073 mg/kg bw/day based on the lowest average intake by adult rats before pairing.

E. Toxicity Study by Dietary Administration of LAE to CD Rats for 52 Weeks

The objective of this study was to assess the systemic toxic potential of LAE when administered to rats in the diet for 52 weeks (Huntingdon Life Sciences 2005b). The study was

designed and executed in compliance with OECD Test Guideline 452. Four groups of Crl:CD (SD)IGS BR rats received diets containing 0, 2,000, 6,000, or 18,000 ppm dietary concentrations of LAE. There were 20 animals per sex in each group. The basal diet was a powdered laboratory animal diet (Rat and Mouse #1 Maintenance Diet) supplied by Charles River UK, to which LAE, kept stored under refrigerated conditions (4°C), was admixed in appropriate concentrations. The diets were analyzed at appropriate intervals for concentration of LAE, homogeneity, and stability.

The mean achieved dosages of LAE for the period Week 1 to Week 52 were 0, 106, 307, and 907 mg/kg bw/day for males and 0, 131, 393, and 1,128 mg/kg bw/day for females. During the study, clinical condition, body weight, and feed consumption were recorded for all animals. Ophthalmic examination, hematology, blood chemistry, and urinalysis were carried out at scheduled intervals. In addition, detailed physical examination and arena observations were performed at regular intervals, and sensory reactivity, grip strength, and motor activity were assessed toward the end of the study. Blood samples were withdrawn from animals near the end of the study. Following sacrifice, bone marrow smears were prepared and a full myelogram completed; a macroscopic examination was performed, organ weights were recorded, and tissues were retained for subsequent histopathological examination.

During the first three weeks of treatment, male rats at both the 6,000- and 18,000-ppm levels and female rats at the 18,000-ppm level showed statistically significantly lower gain in body weight than controls. These weight-gain decrements were associated with lower than control feed intake. After the first three weeks, the animals' weight gain was similar in all treatment groups and in the control. Some disturbances were seen in white-cell and blood-chemistry parameters in all dose levels and in both sexes, but the magnitude of the disturbances were minor and were not supported by corroborative histopathological changes; these effects were not considered to be of toxicological importance.

A statistically significantly higher number of lesions in the forestomach were seen in animals in the 18,000-ppm concentration groups. Females tended to be more severely affected than the males; for the males, incidence was related to dosage but severity was not. These effects were confined to a specific area of the forestomach, the esophageal groove, and are thought to represent a local response to irritation. There was no correlation between the animals' stomach lesions and weight gain or other indications of biochemical disturbances. Thus, they are not considered to be indicative of direct systemic toxicity. The NOAEL for systemic toxicity is the highest level tested, 18,000 ppm dietary concentration, resulting in an intake of 907 mg/kg bw/day.

F. Risk Assessment and Confirmation of GRAS Status

Application of a 100-fold safety factor to the NOAEL for systemic toxicity (907 mg/kg bw/day) results in an ADI of 9 mg/kg bw/day. Since this ADI is based on the results of a chronic study, while the ADI used for the GRAS determination is based on a subchronic study, this new lower ADI is regarded as controlling. The intended uses of LAE remain GRAS, because the EDI of 4.7 mg/kg bw/day (the 90th percentile of the estimated intake distribution) is below the new ADI.

The Expert Panel whose deliberations served as the basis for the original GRAS determination of LAE, Robert J. Nicolosi, Ph.D., Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D., with Joseph F. Borzelleca as Scientific Advisor to the Expert Panel, reviewed the more recent data and concluded that they do not call into question the safety of the intended use of LAE, but rather further confirm that LAE is safe for this use. It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same information would reach the same conclusion. Thus, the earlier conclusion that LAE is GRAS for its intended use, based on published information and corroborated by unpublished information, is confirmed.

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Submission End

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Part II of the GRAS Exemption Claim for Lauramide Arginine Ethyl Ester (LAE) Added to Food to Inhibit Microbial Growth (GRN 000164)

Evidence of Antimicrobial Efficacy and Absence of Organoleptic Effects

Prepared for:
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Barcelona, Spain

Prepared by:
JHeimbach LLC
Washington DC

April 2005

Part II of the GRAS Exemption Claim for Lauramide Arginine Ethyl Ester (LAE) Added to Food to Inhibit Microbial Growth (GRN 000164)

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Part II of the GRAS Exemption Claim for Lauramide Arginine Ethyl Ester (LAE) Added to Food to Inhibit Microbial Growth

I. Introduction

Lauramide arginine ethyl ester (LAE), also known as lauric arginate, has been determined to be generally recognized as safe (GRAS) for addition to a variety of food categories, as shown in Table 1.1, at a maximum addition level of 225 parts per million (ppm) to inhibit microbial growth. The active ingredient, which constitutes about 90% of LAE, is ethyl-N^{α}-lauroyl-L-arginate hydrochloride. The addition level of 225 ppm of LAE thus is equivalent to addition of 200 ppm of ethyl-N^{α}-lauroyl-L-arginate hydrochloride. The food categories for which addition of LAE is regarded as GRAS include both fresh and processed meat and poultry products and dishes containing them, as well as the other food categories shown in Table 1.1.

Table 1.1. Intended Food Uses of LAE

Up to 200 ppm ethyl-N^{α}-lauroyl-L-arginate hydrochloride in the following food categories in 21 CFR 170.3(n)
(3) Beverages and beverage bases, not including dairy products, soft drinks, or alcoholic beverages.
(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.
(7) Tea, including regular, decaffeinated, and instant types.
(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.
(11) Egg dishes including egg roll, egg foo young, egg salad, and frozen multicourse egg meals, but not fresh eggs.
(12) Margarine and margarine-like table spreads, mayonnaise, and spoonable and pourable dressings for salads.
(13) Fish products, including all prepared main dishes, salads, appetizers, frozen multicourse meals, and spreads containing fish, shellfish, and other aquatic animals, but not fresh fish.
(15) Fresh fish, including only fresh and frozen fish, shellfish, and other aquatic animals.
(17) Fresh meats, including only fresh or home-frozen beef or veal, pork, lamb or mutton and home-prepared fresh meat-containing dishes, salads, appetizers, or sandwich spreads made therefrom.
(18) Fresh poultry, including only fresh or home-frozen poultry and game birds and home-prepared fresh poultry-containing dishes, salads, appetizers, or sandwich spreads made therefrom.
(22) Pie fillings.
(24) Gravies and sauces, including all meat sauces and gravies, and tomato, milk, buttery, and specialty sauces.

(29) Meat products, including all meats and meat-containing dishes, salads, appetizers, frozen multicourse meat meals, and sandwich ingredients prepared by commercial processing or using commercially processed meats with home preparation.
(34) Poultry products, including all poultry and poultry-containing dishes, salads, appetizers, frozen multicourse poultry meals, and sandwich ingredients prepared by commercial processing or using commercially processed poultry with home preparation.
(35) Processed fruits and fruit juices excluding apple juice, including all commercially processed juices and juice punches, concentrates, dilutions, ades, and drink substitutes made therefrom, and dried fruits; also including strained fruits and fruit juices excluding apple juice as baby or toddler foods.
(36) Processed vegetables and vegetable juices, including potato salads, raw vegetables, vegetable juices and blends, and tomato sauces
(40) Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable, and combination soups and soup mixes.

Part I of the GRAS notice for LAE, designated as GRN 000164 and filed by FDA on March 8, 2005, includes complete identification and physical and chemical characterization of the material; methods of manufacture; product purity, consistency, and stability; estimated consumer exposure resulting from the intended uses; and evidence of safety. Part I states that the intended technical effect of LAE is to inhibit microbial growth in foods to which it is added and indicates that evidence supporting this purpose is submitted in a separate document.

This document, Part II of GRAS notice GRN 000164, focuses on issues regarding LAE's suitability for addition to foods for its stated purpose. The discussion is divided into four sections. In the first section, published research is presented that demonstrates LAE's mode of action in inhibiting the growth of microorganisms. The second section includes a description of tests to establish the minimum inhibitory concentration (MIC) of LAE against a variety of Gram positive and Gram negative bacteria, as well as yeasts and molds. The third section presents studies of the efficacy of LAE in inhibiting growth of pathogenic bacteria and aerobic bacteria in a number of meat and poultry products as well as other foods. Finally, the fourth section presents data showing that use of LAE does not alter the appearance or organoleptic characteristics of foods to which it is added.

As was noted in the information regarding the identity and use of the product, LAE is not directly added to food in its solid crystalline form. Rather, it is dissolved in propylene glycol and this solution is referred to as Mirenat-N. Most of the studies of antimicrobial efficacy have been based on applications of Mirenat-N to deliver the appropriate concentration of LAE.

II. Mode of Action

The active ingredient in LAE, ethyl-N^{α}-lauroyl-L-arginate hydrochloride, is a cationic surfactant that inhibits the proliferation of a wide variety of microorganisms, including both Gram positive and Gram negative bacteria, molds, and yeasts. This inhibitory effect is a direct consequence of its chemical properties as a surface-active compound that leads to changes in the conductivity and solubility of solutions. Negatively charged materials, such as bacterial enzymes and membrane proteins, attract cationic surfactants, which degrade or solubilize cell membranes, leading to loss of membrane potential, altering cell permeability and leaking cellular constituents. These changes result in the arrest of growth or cell lysis (Kanazawa et al. 1995).

During the 1980s, a number of studies were conducted on this class of compounds—that is, long-chain N^{α}-acyl di-amino acids and their derivatives (Infante et al. 1984, Infante et al. 1985). These studies included compounds with a variety of amino acids, including both lysine and arginine, non-esterified compounds, and esters of both methane and ethane, and served to elucidate the influence of the chain length and the terminal amino acid on the antimicrobial properties of this type of compound. Interestingly, it was found that the activity against bacterial cells is not influenced by the stereochemistry of these compounds.

To document the effect of LAE on bacterial membranes and cellular permeability, LAMIRSA, the manufacturer of the product, commissioned a study at the University of Barcelona (Rodriguez et al. 2004). Bacterial populations were studied using transmission electron microscopy (TEM) and fluorescence microscopy (FM) to observe structural changes to the cellular membranes and cytoplasm of bacteria exposed to LAE. Additionally, flow cytometry (FC) was employed to measure membrane permeability, enzymatic activity, and oxidative metabolism of individual cells. Measures of changes in pH (proton translocation) were employed to measure alterations in membrane permeability, and changes in the concentration of potassium ions in extracellular fluid (ion efflux) were employed to measure loss of cytoplasmic material.

Two bacterial species were used, *Salmonella typhimurium* (ATCC 14028) and *Staphylococcus aureus* (ATCC 6538). The minimum inhibitory concentration (MIC) of LAE for each species was determined, defined as the lowest concentration of LAE that inhibited development of visible growth after 24 hours of incubation at 37°C. For *S. typhimurium*, the MIC was 32 μg/mL; for *S. aureus*, the MIC was 8 μg/mL

Suspensions of the microorganisms were exposed to LAE solutions at concentrations corresponding to the MIC. The contact times were 3 hours for TEM observations; 30 minutes and 3, 6, and 24 hours for FC observations. All experiments were performed in triplicate.

TEM and FM observations of *S. typhimurium* and *S. aureus* found swelling of the outer cell membrane and membrane-enclosed vacuoles, but the cells remained intact: cell

lysis was not observed and the integrity of the cells was evident in the fluorescent micrographs.

While the cell membranes remained intact, the FC observations revealed considerable damage. For *S. typhimurium*, approximately 94% of the cells showed membrane damage after 30 minutes of contact with LAE at the MIC of 32 μg/mL. The proportion of cells showing membrane damage increased over time; after 24 hours, 97% of cells exhibited some degree of membrane damage. For *S. aureus*, 64% of the cells showed membrane damage after 30 minutes of contact with LAE at 8 μg/mL, while 74% showed such damage after 24 hours. Cells of both species were counted to examine the relationship between cell damage and cell viability. For *S. typhimurium*, 99.8% reduction in viability was found after 30 minutes of exposure to LAE. The relationship between damage and viability was less clear for *S. aureus*, but the data suggest that even partially damaged cell membranes resulted in reduced viability.

Intracellular potassium leakage resulting from exposure to LAE was observed in both *S. typhimurium* and *S. aureus*. These results indicate that one effect of LAE is to cause a significant increase in membrane permeability, resulting in a loss of cytoplasmic potassium. Membrane permeability and potassium loss continued to increase over the 3-hour duration of this experiment.

Both *S. typhimurium* and *S. aureus* showed decreases in proton flux after exposure to LAE. Although differences in proton flux were small, they corroborate the findings of the ion efflux studies in suggesting increases in membrane permeability caused by LAE. Based on these results, it is evident that LAE causes increases in the permeability of cell membranes.

In conclusion, the results from this study indicate that, although no disruption of cells was observed, LAE causes structural damage to cell membranes and disturbances in membrane potential, leading to loss of cell viability. This loss of viability, in turn results in inhibition of proliferation.

III. Minimum Inhibitory Concentrations

A series of assays was performed with Gram positive and Gram negative bacteria, as well as yeast and fungal strains, to determine the antimicrobial susceptibility of a variety of microorganisms on the basis of the minimal inhibitory concentration (MIC). The MIC for each microorganism is the lowest level of an antimicrobial agent that inhibits visible growth of that organism.

Methods

The MIC of LAE for each microorganism was determined by the methods described in the sixth edition of the *Manual of Clinical Microbiology* (Murray 1995). Specific assay methods were those described in the following chapters:

- *Antimicrobial Susceptibility Test: Dilution and disk Diffusion Methods* (Woods 1995)
- *Susceptibility Test of Fastidious Bacteria* (Doern 1995)
- *Susceptibility Test of Anaerobic Bacteria* (Wexler and Doern 1995)
- *Antifungal Agents and Susceptibility Testing* (Espinel-Ingroff and Pfaller 1995)

The culture media were as follows:

- General media for bacterial strains:
  - Muller Hinton Broth (Sharlau, 2-1136)
  - Muller Hinton Agar (Oxoid CM 377)
  - Nutrient Agar (Oxoid CM1)

- Specific media for bacterial strains:
  - Media for *Lactobacillus*: MRS Broth (Cultured 413785), MRS Agar (Cultured, 413784)
  - Media for anaerobes: Brain Heart Infusion (Oxoid CM255)
  - Media for *Campylobacter*: Blood Agar Base (Oxoid CM55), for maintenance; test medium, (Difco 211088)

- Media for fungal species:
  - Saboraud Broth (ADSA 2-165)
  - Saboraud Agar (Oxoid, CM41)

The antimicrobial agent (LAE) was used at the following final concentrations: 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg/mL

Results

As shown in Table 3.1, LAE was effective against both Gram positive and Gram negative bacteria, with generally similar MICs, as well as against yeasts and molds. The highest MIC against any tested microorganism was 128 μg/mL.

Table 3.1. Minimum Inhibitory Concentrations of LAE

Microorganism	LAE MIC (µg/mL)
Gram Positive Bacteria	
Arthrobacter oxydans (ATCC 8010)	64
Bacillus cereus var mycoide (ATCC 11778)	32
Bacillus subtilis (ATCC 6633)	16
Clostridium perfringens (ATCC 77454)	16
Clostridium perfringens (ATCC 12917)	16
Clostridium botulinum (ATCC 19397)	64
Listeria monocytogenes (ATCC 15313)	32
Staphylococcus aureus (ATCC 6538)	32
Micrococcus luteus (ATCC9631)	128
Lactobacillus delbrueckkii ssp lactis (ATCC 10705)	16
Lactobacillus plantarum (ATCC 8014)	16
Lactobacillus paracasei (ATCC 25302)	16
Lactobacillus curvatus (ATCC 25601)	16
Gram Negative Bacteria	
Alcaligenes faecalis (ATCC 8750)	64
Bordetella bronchiseptica (ATCC 4617)	128
Campylobacter jejuni (ATCC 29428)	8
Citrobacter freundii (ATCC 22636)	64
Enterobacter aerogenes (ATCC 13048)	32
Enterobacter faecalis (ATCC 27285)	4
Enterobacter sakazakii (ATCC 29544)	32
Escherichia coli (ATCC 8739)	32
Escherichia coli 0157:H7 (ATCC 35150)	32
Proteus mirabilis (CECT 170)	32
Pseudomonas aeruginosa (ATCC 9027)	64
Salmonella cholerasuis (ATCC 13076)	8
Salmonella typhimurium (ATCC 14028)	32
Serratia marcescens (ATCC 10759)	32
Pseudomonas fluorescens (ATCC 13430)	32
Shigella dysentariae (ATCC 13313)	8
Vibrio parahaemoliticus (ATCC 17802)	128
Yersinia enterocolitica (ATCC 27729)	16
Klebsiella pneumoniae var pneumoniae (ATCC 4352)	32
Mycobacterium phlei (ATCC 41423)	2

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Microorganism	LAE MIC (µg/mL)
Yeasts	
Candida albicans (ATCC 10231)	16
Rhodotorula rubra (CECT 1158)	16
Saccharomyces cerevisiae (ATCC 9763)	32
Molds	
Aspergillus niger (ATCC 14604)	32
Aureobasidium pullulans (ATCC 9348)	16
Gliocadium virens (ATCC 4546)	32
Chaetonium globosum (ATCC 6205)	16
Penicillium chrysogenum (ATCC 9480)	128
Penicillium funiculosum (CECT 2914)	16

IV. Studies of Microbial Inhibition in Foods

A. *Introduction*

The antimicrobial efficacy of LAE has been studied extensively, both in Europe and in the U.S. A series of studies of the antimicrobial efficacy of LAE was conducted at ABC Research Corp. in Florida. In these studies, which involved only meats and meat-based products, samples of untreated foods and foods treated with LAE (in the form of Mirenat-N) were inoculated with defined levels of pathogenic bacteria. Specifically, samples of fresh chicken breasts were inoculated with three strains of *Salmonella*, samples of fresh beef were inoculated with three strains of *E. coli* O157:H7, and samples of processed meat and poultry products were inoculated with five strains of *Listeria monocytogenes*. Inoculated treated and control samples were stored at 4.4°C and at 15°C, and tested for bacterial load at times appropriate for the product. Additionally, uninoculated treated and untreated samples were stored and counts of aerobic bacteria were taken at appropriate intervals. The U.S. studies included:

- Fresh chicken breasts (*Salmonella* spp. inoculum; aerobes)
- Fresh beef cuts (*E. coli* O157:H7 inoculum; aerobes)
- Ground beef (*E. coli* O157:H7 inoculum)
- Sliced roast turkey (*Listeria monocytogenes* inoculum; aerobes)
- Sliced smoked turkey (*Listeria monocytogenes* inoculum; aerobes)
- Sliced cured ham (*Listeria monocytogenes* inoculum; aerobes)
- Sliced salami (*Listeria monocytogenes* inoculum; aerobes)
- Frankfurters (*Listeria monocytogenes* inoculum; aerobes)
- Ready-to-eat chicken salad (*Listeria monocytogenes* inoculum; aerobes)

The European studies, which include both meat and non-meat foods, were mostly based on observation of the proliferation of aerobic bacteria and other microorganisms in treated and untreated samples of food during storage. However, two studies of cured ham were based on inoculation of the food with a single strain of *Listeria monocytogenes*. Foods studied, and the microbiological parameters observed, included:

- Fresh tomato sauce (aerobes and yeasts)
- Cooked rice (aerobes and *Bacillus cereus* spp.)
- Marinated beef (aerobes and *Enterobacter* spp.)
- Bratwurst (aerobes, enteric bacteria, *Staphylococcus aureus*, *Clostridium* spp., *Salmonella* spp., *Shigella* spp., and *Lactobacillus* spp.)
- Sliced cured ham (*Listeria monocytogenes* inoculum)
- Diced cured ham (*Listeria monocytogenes* inoculum)

Copies of the complete study reports for all research on the microbiological efficacy of LAE are included in Chapter VII.

B. U.S. Studies

1. Introduction

All of the U.S. research on the antimicrobial efficacy of LAE was performed at ABC Research Corporation in Gainesville FL, and all studies employed similar methodologies. In order to avoid unnecessary repetition, the common elements are listed below:

- In all studies, LAE was added in the form of Mirenat-N, a 20-25% solution of LAE in propylene glycol at a final concentration not exceeding 200 mg/kg of the active ingredient.

- The fresh meat and poultry products (fresh chicken breast, beef cuts, and ground beef) were obtained locally by ABC Research, inoculated with the appropriate strains of bacteria, and held under refrigeration for 20 minutes to allow for bacterial attachment. Test samples were immersed for 30 seconds in a 5% solution of Mirenat-N in distilled water at 22°C. Frankfurters and chicken salad were also prepared at ABC Research, using ingredients obtained locally.

- Processed meat and poultry products (roast turkey, smoked turkey, cured ham, and salami) were manufactured and sliced per commercial practices by the Columbus Salame Company of San Francisco. Test samples had Mirenat-N incorporated into the formulation at a rate of 700 mg/kg. Samples were shipped in insulated containers with refrigerant to ABC Research Corp. via overnight courier; samples were kept refrigerated at 35°F until used (within 96 hours). Samples were immersed for 30 seconds in a 5% solution of Mirenat-N in distilled water at 22°C and excess solution was allowed to drip from the samples before inoculation with the appropriate strains of bacteria.

- Three inocula were used:
    1. *Salmonella*: A three-strain inoculum was prepared, including serotypes *enteritidis* (ATCC 13076), *typhimurium* (ATCC 14028), and *montevideo* (ATCC 8387). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth (TSB). Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 cfu/ml for each strain. The final inoculum cocktail (ca. 20 ml) was prepared by diluting the mixed suspension in sterile BPB to obtain an approximate cell density of 10^6 cfu/ml. This inoculum was used with the fresh chicken breasts.
    2. *E. coli* O157:H7: A three-strain inoculum was prepared: ATCC 43894, ATCC 43895, and ATCC 700599. Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth (TSB). Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2

(BPB). Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1 x 10^8 cfu/ml for each strain. The final inoculum cocktail (ca. 20 ml) was prepared by diluting the mixed suspension in sterile BPB to obtain an approximate cell density of 10^6 cfu/ml. This inoculum was used with the fresh beef cuts and the freshly ground beef.

3. *Listeria monocytogenes*: A five-strain inoculum was prepared: ATCC 19115 (Serotype 4B), "Serotype ½a," "Serotype ½b," ATCC 7644 (Gibson) and ATCC 49594 (Scott A). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth with yeast extract (TSBYE). Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was resuspended in BPB to obtain a cell suspension of approximately 1 x 10^8 cfu/ml. Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1 x 10^8 cfu/ml for each strain. The final inoculum cocktail (ca. 40 ml) was prepared by diluting the mixed suspension in sterile BPB to obtain an approximate cell density of 5 x 10^5 cfu/ml. This inoculum was used with all processed meat and poultry products and ready-to-eat chicken salad.

- Samples of all products were stored at 4.4°C. Beef cuts were stored for 6 days; chicken breasts, ground beef, and RTE chicken salad were stored for 12 days; frankfurters were stored for 6 weeks; and roast turkey, smoked turkey, cured ham, and salami were stored for 8 weeks.

- Except for ground beef, additional samples of all products were also stored at 15°C for 4 days.

- Except for ground beef, additional uninoculated samples of both LAE-treated and control products were stored at both 4.4°C and 15°C to test for proliferation of aerobic bacteria.

2. Fresh Chicken Breasts

The Mirenat-N immersion treatment resulted in a mean initial *Salmonella* reduction of 3.19 log cfu/cm^2 (from 4.09 to 0.90 log cfu/cm^2) on the surface of the chicken breasts.

The *Salmonella* mean counts decreased from a pre-treatment level of 4.09 log cfu/cm^2 to <1.00 and 3.27 log cfu/cm^2, respectively, on treated and untreated samples during the first 3 days of storage at 4.4°C. There was variation in the *Salmonella* counts of treated and untreated samples over 12 days of storage but there was no evidence of a growth trend. It should be noted that the mean *Salmonella* count of the treated samples

remained more than 3 log units lower than that of untreated samples throughout 12 days of storage at 4.4°C.

After the initial 3.19 log unit reduction of *Salmonella* on the samples by the Mirenat-N immersion treatment, the mean counts of the treated samples remained approximately 3.19 and 2.68 log units, respectively, lower than that of untreated samples after 1 and 2 days storage at 15°C. *Salmonella* counts increased by 2.71 and 2.20 log units on the surface of treated and untreated chicken breasts during 2 days of storage at 15°C with an increase of 4.63 and 2.39 log units, respectively, for treated and untreated samples by 4 days of storage.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, fresh chicken breasts were also evaluated. The initial APC levels on treated and untreated samples were 3.17 and 3.94 log cfu/cm^2, respectively. As compared to the untreated samples, the Mirenat-N immersion treatment inhibited the growth of APC bacteria during storage at 4.4°C. APC levels in treated samples and untreated samples were 75,000,000 and 510,000,000 cfu/cm^2, respectively, after 12 days of storage at 4.4°C.

In summary, the Mirenat-N immersion treatment resulted in a significant reduction (3.19 log cfu/cm^2) of *Salmonella* inoculated onto the surface of fresh chicken breasts. The Mirenat-N treatment resulted in inhibition of aerobic plate counts developing on the chicken breasts during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated fresh chicken breasts.

3. Fresh Beef Cuts

The Mirenat-N immersion treatment resulted in a mean initial *E. coli* O157:H7 reduction of 2.34 log cfu/cm^2 (from 3.83 to 1.49 log cfu/cm^2) on the surface of the beef cuts.

As would be expected, there was no evidence of *E. coli* O157:H7 growth on treated or untreated samples stored at 4.4°C. In fact, the counts of *E. coli* O157:H7 gradually decreased during 6 days of storage at 4.4°C. *E. coli* O157:H7 mean counts decreased from a pre-treatment level of 3.83 cfu/cm^2 on beef cut surfaces to 0.55 and 2.24 cfu/cm^2, respectively, on treated and untreated samples after 6 days of storage at 4.4°C.

After the initial 2.34 log cfu/cm^2 reduction of *E. coli* O157:H7 on the samples by the Mirenat-N immersion treatment, the mean counts of the treated samples remained approximately 2 log units lower than that of untreated samples after 2 and 4 days storage at 15°C. *E. coli* O157:H7 counts increased by approximately 4 log units on the surface of both treated and untreated beef cuts during 4 days of storage at 15°C.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, fresh beef cuts subsequently stored at 4.4° or 15°C were also

evaluated. The APC levels on uninoculated beef cut samples were very low initially (<10 cfu/cm^2) on both treated and untreated samples. As compared to the untreated samples, the Mirenat-N immersion treatment inhibited the growth of APC bacteria during storage at 4.4 or 15°C. APC levels in untreated samples and treated samples were 11,8000,000 and 4,000 cfu/cm^2, respectively, after 9 days of storage at 4.4°C. APC levels were 17,000,000 and 6,500,000 cfu/cm^2 in untreated samples and treated samples, respectively, after 4 days of storage at 15°C.

In summary, the Mirenat-N immersion treatment resulted in a significant reduction (2.34 log cfu/cm^2) of *E. coli* O157:H7 inoculated onto the surface of fresh beef cuts. The Mirenat-N treatment resulted in a substantial inhibition of aerobic plate counts developing on the beef cuts during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated fresh beef cuts.

4. Freshly Ground Beef

The Mirenat-N immersion treatment of the beef trim resulted in a mean initial *E. coli* O157:H7 reduction of 0.76 log cfu/g (from 4.82 to 4.06 log cfu/g) in the derived ground beef.

After the initial reduction by the Mirenat-N treatment, *E. coli* O157:H7 counts decreased in treated and untreated ground beef samples during 12 days of storage at 4.4°C but mean counts were consistently much lower in treated than in untreated samples at each storage interval. Mean counts of *E. coli* O157:H7 on treated samples after 3, 9 and 12 days of storage were 1.66, 1.02, and 0.47 log lower, respectively, than those of untreated samples.

In summary, the Mirenat-N treatment of beef trim inoculated with *E. coli* O157:H7 resulted in a significant reduction (0.76 log cfu/g) of *E. coli* O157:H7 in the derived, freshly ground beef. The Mirenat-N also resulted in significantly lower *E. coli* O157:H7 levels in the derived ground beef than in untreated samples during subsequent storage at 4.4°C.

5. Sliced Roast Turkey

The Mirenat-N treatment resulted in a mean initial *L. monocytogenes* reduction of 1.42 log cfu/g on the sliced roast turkey (from 3.42 to 2.00 log cfu/g).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated roast turkey samples after 1 and 2 weeks of storage at 4.4°C were 1.53 and 1.50 log cfu/g lower, respectively, than those of untreated samples. *L. monocytogenes* counts were similar on treated and untreated samples after 4, 6 and 8 weeks of storage at 4.4°C. *L. monocytogenes* counts increased throughout 8 weeks of storage at 4.4°C in treated and untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 4 days of storage at 15°C in treated and untreated roast turkey samples but were consistently lower in treated than in untreated samples at each storage interval. Mean counts of *L. monocytogenes* in the treated sliced roast turkey samples after 1, 2 and 4 days storage at 15°C were 0.95, 0.85 and 0.39 log cfu/g lower, respectively, than those of untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, sliced roast turkey subsequently stored at 4.4° or 15°C were also evaluated. The initial APC levels were very low in all roast turkey samples. As compared to the untreated samples, the Mirenat-N treatment of the roast turkey inhibited the growth of APC bacteria during storage at 4.4°C or 15°C. APC levels in treated samples were remained relatively low and were consistently much lower than those of corresponding untreated samples at each storage interval for either temperature. The APC on the treated sample was <10 cfu /g after 8 weeks of storage at 4.4°C whereas the APC of the untreated sample was 240,000,000 cfu/g after 8 weeks of storage. Since the APC level at all other storage intervals were ≤10 cfu/g, the higher APC (34,000 cfu/g) for the treated sample after 4 weeks of storage at 4.4°C suggests a sample "outlier" and is inconsistent with any actual growth trend as was clearly indicated for the untreated samples during storage.

In summary, the Mirenat-N treatment of the sliced roast turkey resulted in a significant reduction (1.42 log cfu/g) of *L. monocytogenes* inoculated onto sliced roast turkey. The Mirenat-N treatment also inhibited the growth of aerobic plate counts developing on the samples during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated sliced roast turkey.

6. Sliced Smoked Turkey

The Mirenat-N treatment resulted in a mean initial *L. monocytogenes* reduction of greater than 1.94 log cfu/g in the sliced smoked turkey (from 3.04 to <1.10 log cfu/g).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 8 weeks of storage at 4.4°C on treated and untreated smoked turkey samples but were consistently much lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on treated samples after 1, 2, 4, 6 and 8 weeks of storage at 4.4°C were >1.03, 1.48, 1.83, 1.05 and 0.62 log cfu/g lower, respectively, than those of untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 4 days of storage at 15°C in treated and untreated smoked turkey samples but were consistently much lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on the treated sliced smoked turkey samples after 1, 2 and 4 days storage at 15°C were >1.94, 2.32 and 2.55 log cfu/g lower, respectively, than those of untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, sliced smoked turkey subsequently stored at 4.4° or 15°C were also evaluated. The initial APC levels were very low in all smoked turkey samples. As compared to the untreated samples, the Mirenat-N treatment of the smoked turkey inhibited the growth of APC bacteria during storage at 4.4°C or 15°C. APC levels in treated samples were consistently much lower than those of corresponding untreated samples at each storage interval for either temperature. In fact, APC levels on treated samples remained at very low levels (≤10 cfu/g) during 8 weeks of storage at 4.4°C whereas those of untreated samples increased to 1,740,000 cfu/g over 8 weeks of storage.

In summary, the Mirenat-N treatment of the sliced smoked turkey resulted in a significant reduction (>1.94 log units) of *L. monocytogenes* inoculated onto sliced smoked turkey. *L. monocytogenes* levels were consistently much lower on treated than on untreated samples during storage at 4.4° or 15°C. The Mirenat-N treatment also inhibited the growth of aerobic plate counts developing on the samples during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated sliced smoked turkey.

7. Sliced Cured Ham

The Mirenat-N treatment resulted in a mean initial *L. monocytogenes* reduction of 1.62 log cfu/g on the sliced cured ham (from 3.67 to 2.04 log cfu/g).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated cured ham samples after 1, 2, 4 and 6 weeks of storage at 4.4°C were 1.71, 1.96, 1.22 and 0.39 log cfu/g lower, respectively, than those of untreated samples. *L. monocytogenes* counts were similar on treated and untreated sample after 8 weeks of storage at 4.4°C. *L. monocytogenes* counts increased throughout 8 weeks of storage at 4.4°C in treated and untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 4 days of storage at 15°C in treated and untreated cured ham samples but were consistently lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on the treated samples after 1, 2 and 4 days storage at 15°C were 1.75, 1.64 and 1.00 log cfu/g lower, respectively, than those of untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, sliced cured ham subsequently stored at 4.4° or 15°C were also evaluated. The initial APC levels were very low in all cured ham samples. As compared to the untreated samples, the Mirenat-N treatment of the cured ham inhibited the growth of APC bacteria during storage at 4.4°C or 15°C. APC levels in treated samples were remained relatively low and were consistently much lower than those of corresponding untreated samples at each storage interval at 4.4°C. The APC on the treated sample was <10 cfu/g after 8 weeks of storage at 4.4°C whereas the APC of untreated samples evidenced some growth over 8 weeks of storage.

In summary, the Mirenat-N treatment of the sliced cured ham resulted in a significant reduction (1.62 log units) of *L. monocytogenes* inoculated onto sliced cured ham. The Mirenat-N treatment also inhibited the growth of aerobic plate counts developing on the samples during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated sliced cured ham.

8. Sliced Salami

The Mirenat-N treatment resulted in a mean initial *L. monocytogenes* reduction of greater than 2.64 log cfu/g in the sliced salami (from 3.64 to <1.00 log cfu/g).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 8 weeks of storage at 4.4°C on treated and untreated salami samples but were consistently much lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on treated samples after 1, 2, 4, 6 and 8 weeks of storage at 4.4°C were >2.36, 3.94, 2.58, 1.83 and 0.98 log cfu/g lower, respectively, than those of untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 4 days of storage at 15°C in treated and untreated salami samples but were consistently much lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on the treated sliced salami samples after 1, 2 and 4 days storage at 15°C were 2.52, 3.17 and 2.22 log cfu/g lower, respectively, than those of untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, sliced salami subsequently stored at 4.4° or 15°C were also evaluated. The APC levels were relatively low in all salami samples initially and throughout storage with no evidence of growth on treated or untreated samples at either temperature.

In summary, the Mirenat-N treatment of the sliced salami resulted in a significant reduction (>2.64 log units) of *L. monocytogenes* inoculated onto sliced salami. *L. monocytogenes* levels were consistently much lower on treated than on untreated samples during storage at 4.4° or 15°C.

9. Frankfurters

The Mirenat-N treatment resulted in a mean initial *L. monocytogenes* reduction of 3.33 log cfu/ml in the frankfurters (from 4.58 to 1.25 log cfu/ml of sample rinse).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated frankfurters samples after 1, 2 and 4 weeks of storage at 4.4°C were 3.84, 3.29 and 1.55 log cfu/ml lower, respectively, than those of untreated samples. *L. monocytogenes* counts were similar on treated and untreated sample by 6 weeks of

storage at 4.4°C. *L. monocytogenes* counts increased throughout 6 weeks of storage at 4.4°C in treated and untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated frankfurters samples after 1, 2 and 4 days storage at 15°C were 3.34, 2.57 and 1.22 log cfu/ml lower, respectively, than those of untreated samples. *L. monocytogenes* counts increased throughout 4 days of storage at 15°C in treated and untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, frankfurters subsequently stored at 4.4° or 15°C were also evaluated. The Mirenat-N treatment resulted in an initial APC reduction of ca. 1.3 log cfu/ml on the frankfurters (from 2.41 to 1.11 log cfu/ml of rinse). As compared to the untreated samples, the Mirenat-N treatment of the frankfurters inhibited the growth of APC bacteria during storage at 4.4°C. APC levels in treated samples were consistently much lower than those of corresponding untreated samples at each storage interval for either temperature.

In summary, the Mirenat-N treatment of the frankfurters resulted in a significant reduction (3.33 log cfu/ml) of *L. monocytogenes* inoculated onto frankfurters. The Mirenat-N treatment also resulted an initial reduction and inhibition of aerobic plate counts developing on the frankfurters during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated frankfurters.

10. Ready-to-Eat Chicken Salad

The Mirenat-N treatment of the product ingredients resulted in an initial, mean *L. monocytogenes* reduction of greater than 1.61 log cfu/g (from 2.61 to <1.00 log cfu/g) in the chicken salad.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated chicken salad samples after 3, 6, 9 and 12 days storage at 4.4°C were 1.61, 0.54, 0.29 and 0.46 log cfu/g lower, respectively, than those of untreated samples. *L. monocytogenes* counts increased throughout 12 days of storage at 4.4°C in treated and untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated chicken salad samples after 1 and 2 days storage at 15°C were 2.20 and 0.76 log cfu/g lower, respectively, than those of untreated samples. *L. monocytogenes* counts were similar on treated and untreated sample by 4 days of storage at 15°C. *L. monocytogenes* counts increased throughout 4 days of storage at 15°C in treated and untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, chicken salad subsequently stored at 4.4° or 15°C were also evaluated. The Mirenat-N treatment resulted in an initial APC reduction of 2.57 log cfu/g

in the chicken salad (from 6.21 to 3.64 log cfu/g). As compared to the untreated samples, the Mirenat-N treatment of the salad ingredients inhibited the growth of APC bacteria during storage at 4.4°C particularly during the first 3 days of storage. APC levels in treated samples and untreated samples were 42,000 and 4,900,000 cfu/g, respectively, after 3 days of storage at 4.4°C.

In summary, the Mirenat-N treatment of the product ingredients resulted in a significant reduction (>1.61 log cfu/g) of *L. monocytogenes* inoculated into ready-to-eat chicken salad. The Mirenat-N treatment also resulted in inhibition of aerobic plate counts developing in the chicken salad during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated ready to eat chicken salad.

11. Conclusion from the U.S. Studies

Treatment of meat and poultry products with LAE in the form of Mirenat-N, at a maximum level of 200 mg/kg active ingredient, consistently inhibited the growth of both pathogens and aerobic bacteria. The treatment was effective on all products tested, and against all pathogens tested: *Salmonella* spp., *E. coli* O157:H7, and *Listeria monocytogenes*.

C. *European Studies*

1. Fresh Tomato Sauce

Methods

The fresh tomato sauce was prepared by Salaons I Derivats St. (C/Castello, 25-27; 43500 Tortosa; Tarragona) following the usual manufacturing practices used in this sector. Raw tomatoes were stored under refrigeration for 1-2 days, then washed and cut and the seeds removed. The tomatoes were cooked for 1.5-2 hours at low heat and salt and sugar were added. The tomato sauce was sieved to remove skin residues and packaged in 50-g plastic containers under modified atmosphere.

There were three experimental conditions: LAE (added as Mirenat-N) at 200 ppm of the active ingredient, sorbic acid at 1000 ppm, and an untreated control. All samples were stored at a temperature of 0-5°C, but planned "breaks" were introduced each day during which the temperature of the samples was raised to 25°C for 8 hours. These breaks were introduced in order to accelerate the study. Samples for testing were taken after the breaks on Days 1, 4, 7, and 11. Samples were diluted with buffered peptone water and were cultured with TSA agar at 35°C for 48 hours to test for aerobic bacteria and with Rose Bengale agar with chloramfenicol at 25°C for 5 days to test for yeasts.

Results

There was no detectable growth (i.e., less than 2 log cfu/g) in either aerobic bacteria or yeasts in the tomato sauce treated with LAE or that treated with sorbic acid.

The untreated tomato sauce, on the other hand, exhibited rapid proliferation of both aerobic bacteria, which reached 7.7 log cfu/g by Day 11, and yeast, which reached 7.6 log cfu/g by Day 11.

2. Cooked Rice

Methods

The rice was prepared in the Vedeqsa laboratory following the usual manufacturing practices used in this sector. The process was:

1. Mix water with salt (in the treated samples the preservative was added at this point).
2. Add the rice when the water begins to boil. The cooking process was about 12-15 minutes; at the end of the boiling process, there was practically no water.
3. Drain the rice and wash with cold water.
4. Pack the rice in sterile bags and store at either 4°C or 20°C.

There were four experimental conditions: two different concentrations of LAE, one test condition with potassium sorbate, and an untreated control.

1. Treated lot with LAE (100 mg active ingredient/kg water) added as Mirenat-N. Once the rice was boiled, the concentration of the active ingredient was 81 mg/kg.
2. Treated lot with LAE (300 mg active ingredient/kg water) added as Mirenat-N. Once the rice was boiled, the concentration of active ingredient was 267 mg/kg.
3. Treated lot with 1 g/kg potassium sorbate.
4. Control lot with no added preservative.

Samples for microbiological testing were taken on Days 0, 7, 9, and 11 from cooked rice stored at 4°C and on Days 1, 2, and 3 from cooked rice stored at 20°C. Samples were cultured and examined for total aerobic bacteria and *Bacillus cereus*.

Results

At 4°C, there was no proliferation of aerobic bacteria over the 11 days of the study in samples of cooked rice treated with LAE at 300 mg/kg. In samples of rice treated with 100 mg/kg of LAE, aerobic bacteria reached 5 log cfu/g by Day 11, while proliferation in samples treated with potassium sorbate and untreated control samples reached 9 log and 10 log cfu/g, respectively. At 20°C, the proliferation of aerobic bacteria reached 6.5 log cfu/g in the samples treated with either 100 or 300 mg/kg of LAE by Day 3; however, the aerobic bacteria reached 10 log cfu/g in the samples treated with potassium sorbate or left untreated.

There was no proliferation of *Bacillus cereus* in any of the samples at either of the two temperatures during the study.

3. Marinated Beef

Methods

The solid marinade was prepared by Kerry Hungaria Kft (H-1106 Budapest, Feher ut 10, Hungary) following standard manufacturing practices of this sector. The liquid marinade consisted of water, sunflower oil, spices, salt, sucrose, dextrose, diphosphates, xanthan gum, and blue food color. The marinade was added to beef, followed by manual tumbling and vacuum packaging, and the marinated meat was stored at 4-7°C for 24 days.

There were three experimental conditions—treatment with LAE (150 mg/kg active ingredient) as Mirenat-N, treatment with 150 mg/kg sodium nitrite, and untreated control. Five samples from each condition were taken on each of Days 1, 3, 7, 10, 14, 17, 21, and 24 for microbiological analyses of aerobic bacteria and *Enterobacter* spp.

Results

Aerobic bacteria proliferated in marinated beef in all three experimental conditions. However, the proliferation was consistently and statistically significantly greater in the untreated control samples and the samples treated with sodium nitrite than in those treated with LAE. By Day 24, aerobic bacteria were present at 8.5 log cfu/g in the control and sodium-nitrite samples and at 7.4 log cfu/g in the samples treated with LAE.

Some proliferation of *Enterobacter* was observed, especially in the untreated control samples, but it was at such low levels that no significant differences could be identified.

4. Bratwurst

Methods

The bratwurst was manufactured by Subirats S.A. (C/ Reus, number 1, Industrial La Florida, Sta. Perpètua de la Mogoda 08130 Barcelona). The ingredients were minced pork back, underbelly, spices, salt, milk protein, diphosphate, antioxidant, and flavor enhancer. In the test batch, LAE (added as Mirenat-N) was diluted in water and then added to the mass. The final dose of LAE was 100 mg active ingredient/kg in the bratwurst. The regular manufacturing practices that are used in this sector were followed in making both the test and untreated control batches of bratwurst.

The bratwurst was stored at refrigerator temperature (0-5°C) for the 90-day shelf life of this product, and five samples of both treated and control bratwurst were taken for testing at each of Days 0, 15, 30, 45, 60, 75, and 90. Growth was assessed of total aerobic bacteria (both in the interior and on the surface of the bratwurst), enteric bacteria, *Staphylococcus aureus*, *Clostridium* spp., *Salmonella* spp., *Shigella* spp., and *Lactobacillus* spp.

Results

Total aerobic bacteria: There was some growth of aerobic bacteria in the interior of the bratwurst, but at the end of 90 days the concentration was only 3.2 log cfu/g in the untreated bratwurst and 2.7 log cfu/g in the bratwurst treated with LAE. The average differences between the test and control samples were not statistically significant. However, there was greater proliferation of aerobes on the surface of the bratwurst, reaching 5.5 log cfu/g on the surface of the untreated control samples and 4.8 log cfu/g on the surface of the LAE-treated bratwurst. The average difference in counts of aerobic bacteria between the test and control bratwurst samples over the 90 days was statistically significant ($p < 0.05$).

Clostridium spp.: The treated samples of bratwurst exhibited significantly ($p < 0.05$) lower counts than did the controls at the end of 90 days. In the control samples, the counts of *Clostridium* spp. exceeded 2 log cfu/g while they reached only 1.3 log cfu/g in the samples treated with LAE.

Staphylococcus aureus: No growth was observed in the samples treated with LAE; some growth was observed in the control samples, but the average counts were not significantly different from zero.

Salmonella spp.: No growth was observed in either the treated or control samples over the 90 days.

Shigella spp.: No growth was observed in either the treated or control samples over the 90 days.

Lactobacillus spp.: No growth was observed in either the treated or control samples over the 90 days.

Enteric bacteria: No growth was observed in either the treated or control samples over the 90 days.

5. Sliced Cured Ham

Methods

The cured ham was prepared by Subirats S.A. (C/ Reus, number 1, Industrial La Florida, Sta. Perpètua de la Mogoda 08130 Barcelona) following the regular practices employed in this sector, using the standard ingredients added to produce cured ham. The ham was sliced at LAMIRSA, and then the 25-35-g slices were immersed in a bath of 10 mg/ml LAE as Mirenat-N (test samples) or distilled water (control samples). The final level of LAE in the ham was 175 mg/kg active ingredient.

An inoculum was prepared consisting of a single strain of *Listeria monocytogenes* (ATCC 35152) at a concentration of 6 log cfu/ml and distributed as a thin film over the surface of the ham, resulting in a count of 3.48 log cfu/g.

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The sliced cured ham was stored at refrigerator temperature (0-5°C) and two samples of both treated and control sliced ham were taken for testing at each of Days 0, 7, 14, 21, 28, 42, and 56.

Results

Treatment with Mirenat-N resulted in an initial decrease in the plate count of *L. monocytogenes* of 1.65 log cfu/g as compared with the control samples (1.95 v. 3.60 log cfu/g). Over the 8 weeks of storage, the load of *L. monocytogenes* did not increase at all in the samples treated with Mirenat-N, while the counts in the untreated samples increased to a maximum of 4.70 log cfu/g after 6 weeks of storage before declining to 3.48 log cfu/g after 8 weeks.

In summary, Mirenat-N was significantly effective in inhibiting the proliferation of *L. monocytogenes* in sliced cured ham.

6. Diced Cured Ham

Methods

The cured ham was prepared by Subirats S.A. (C/ Reus, number 1, Industrial La Florida, Sta. Perpètua de la Mogoda 08130 Barcelona) following the regular practices employed in this sector, using the standard ingredients added to produce cured ham. The ham was diced at LAMIRSA, and then the diced cubes were immersed in a bath of 10 mg/ml LAE as Mirenat-N (test samples) or distilled water (control samples). The final level of LAE in the ham was 175 mg/kg active ingredient.

An inoculum was prepared consisting of a single strain of *Listeria monocytogenes* (ATCC 35152) at a concentration of 7 log cfu/ml and distributed as a thin film over the surface of the ham cubes, resulting in a count of 5 log cfu/g.

Samples of the diced cured ham were stored either at refrigerator temperature (0-5°C) or room temperature (50-55°C) and two samples of both treated and control sliced ham were taken for testing at each sampling point. For the samples stored at 0-5°C, these were on Day 0 and Day 59. For the samples stored at 50-55°C, these were on Days 0, 1, 2, and 3.

Results

Treatment with Mirenat-N resulted in an initial decrease in the plate count of *L. monocytogenes* of 3.50 log cfu/g as compared with the control samples (2.00 v. 5.50 log cfu/g). The counts did not change significantly over 59 days of storage at 0-5°C in either the treated or untreated samples. In the samples stored at 50-55°C, the counts of *L. monocytogenes* in the treated samples gradually increased to 4.6 log cfu/g over the 3 days, but remained lower than the counts in the untreated samples.

In summary, Mirenat-N was significantly effective in inhibiting the proliferation of *L. monocytogenes* in diced cured ham.

7. Conclusion from the European Studies

Treatment with LAE in the form of Mirenat-N is effective in inhibiting the growth of aerobic bacteria in a variety of both meat and non-meat products. Reductions in the proliferation of aerobes of up to 7 log cfu/g have been shown in tomato sauce and roast turkey, and reductions of 3-6 log cfu/g in other foods. Mirenat-N also significantly reduced the growth of the pathogenic bacterium *Listeria monocytogenes* inoculated onto the surface of both sliced and diced cured ham.

D. Conclusion from the U.S. and European Studies

In tests of a variety of foods, including non-meat products, cured meat products, and uncured meat products, LAE has proven to be an effective antimicrobial treatment to inhibit the growth of *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes*, as well as total aerobic bacteria. With regard to the latter, reductions in the proliferation of aerobes of up to 7 log cfu/g have been shown (in tomato sauce and roast turkey), and reductions of 3-6 log cfu/g were shown on other foods.

In the inoculation studies performed by ABC Research Corporation, LAE was consistently shown to be effective in reducing proliferation of the inoculum. Fresh chicken breasts treated with LAE had lower counts of *Salmonella* spp. than did untreated breasts by about 3 log cfu/cm^2. Fresh cuts of beef treated with LAE had lower counts of *E. coli* O157:H7 by about 2.3 log cfu/cm^2; LAE treatment of freshly ground beef was less successful with reduction in growth of *E. coli* O157:H7 of only about 0.7 log cfu/g. LAE proved to be extremely efficacious against *Listeria monocytogenes* in a variety of cured and uncured processed meat products, with reductions of 1.5-3.3 log cfu/g in roast and smoked turkey, cured ham, salami, and frankfurters, as well as ready-to-eat chicken salad. Further, growth inhibition nearly always persisted over some or all of the storage period at both refrigerator temperature (4.4°C) and room temperature (15°C).

It is clear that LAE provides significant protection for both meat and nonmeat foods against the proliferation of a wide variety of both Gram-positive and Gram-negative pathogenic bacteria.

V. Appearance and Organoleptic Characteristics

A. *Introduction*

The effect—or, rather, lack of effect—of LAE on the appearance and organoleptic characteristics of food products to which it is applied has been addressed in six studies, five in Europe and one in the U.S. All applied LAE to the test foods in the form of Mirenat-N, a solution of LAE in propylene glycol. The European studies employed fresh tomato sauce, cooked rice, marinated beef, fresh sausage, and bratwurst; the U.S. study employed cured ham. The European raters provided qualitative judgments regarding appearance, color, and odor; the U.S. raters used 7-point scales to assess color, aroma, and overall acceptability.

Except for the European study with fresh sausage, these investigations of organoleptic effects were performed in conjunction with studies of the antimicrobial effectiveness of LAE. The full reports of the studies are provided in Chapter VII of this submission.

B. *Fresh Tomato Sauce*

Methods

The fresh tomato sauce was prepared by Salaons I Derivats St. (C/Castello, 25-27; 43500 Tortosa; Tarragona) following the usual manufacturing practices used in this sector. Raw tomatoes were stored under refrigeration for 1-2 days, then washed and cut and the seeds removed. The tomatoes were cooked for 1.5-2 hours at low heat and salt and sugar were added. The tomato sauce was sieved to remove skin residues and packaged in 50-g plastic containers under modified atmosphere.

There were three experimental conditions: LAE (added as Mirenat-N) at 200 ppm, sorbic acid at 1000 ppm, and an untreated control. All samples were stored at a temperature of 0-5°C, but planned "breaks" were introduced each day during which the temperature of the samples was raised to 25°C for 8 hours. These breaks were introduced in order to accelerate the study. Samples were taken for testing after the breaks on Days 1, 4, 7, and 11. One sample from each experimental condition was evaluated for appearance, color, and odor by an experienced tester. Additionally, the pH of each sample was determined.

Results

There were no changes in the appearance, color, or odor of any of the samples between Days 1, 4, and 7; there were no differences on any of these days between the sample treated with LAE, that treated with sorbic acid, or the untreated control. By Day 11, the control sample was causing ballooning of its package, although there was no change in color or odor. The two treated samples were unchanged. There were no significant differences in the pH of any of the samples, either between treatment conditions or over time.

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C. *Cooked Rice*

Methods

The rice was prepared in the Vedeqsa laboratory following the usual manufacturing practices used in this sector. The process was:

5. Mix water with salt (in the treated samples the preservative was added at this point).
6. Add the rice when the water begins to boil. The cooking process was about 12-15 minutes; at the end of the boiling process, there was practically no water.
7. Drain the rice and wash with cold water.
8. Pack the rice in sterile bags and store at either 4°C or 20°C.

There were four experimental conditions: two different concentrations of LAE, one test condition with potassium sorbate, and an untreated control.

5. Treated lot with 100 mg/kg LAE added as Mirenat-N. Once the rice was boiled, the concentration of LAE was 81 mg/kg.
6. Treated lot with 300 mg/kg LAE added as Mirenat-N. Once the rice was boiled, the concentration of LAE was 267 mg/kg.
7. Treated lot with 1 g/kg potassium sorbate.
8. Control lot with no added preservative.

Organoleptic testing was completed on Days 1, 2, 3, and 4 for samples stored at 20°C and on Days 0, 7, and 9 for samples stored at 4°C. Experienced food raters judged the samples on appearance, color, and odor.

Results

At 20°C, there were no changes in the appearance of the cooked rice over the 4 days of the study and no differences between the experimental conditions. There were no color changes and no differences between treatment conditions until Day 4, when the control sample and the sample treated with potassium sorbate began to appear yellowish while the two samples treated with LAE remained white. On Day 1, there were no differences between treatment conditions in the odor of the rice, but the untreated sample began to develop a bad odor beginning on Day 2, while the sample treated with potassium sorbate began to develop a bad odor on Day 3. There was no change in the odor of the two samples treated with LAE throughout the 4-day study. These results are summarized in Table 5.1.

Table 5.1. Effect of LAE on Organoleptic Attributes of Cooked Rice Stored at 20°C

Day	Attribute	Control	LAE (100 mg/kg)	LAE (300 mg/kg)	Potassium sorbate
Day 1	Appear-ance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Color	White	White	White	White
	Odor	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
Day 2	Appear-ance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Color	White	White	White	White
	Odor	Fermented odor	Characteristic of the product	Characteristic of the product	Characteristic of the product
Day 3	Appear-ance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Color	White	White	White	White
	Odor	Bad odor	Characteristic of the product	Characteristic of the product	Slightly bad odor
Day 4	Appear-ance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Color	White-yellow	White	White	White yellow
	Odor	Bad odor	Characteristic of the product	Characteristic of the product	Bad odor

There were no differences on Day 0 in the appearance, color, or odor of the treated and control samples of cooked rice stored at 4°C. By Day 7, the control sample had begun to develop an off-odor, which was pronounced by Day 9. None of the treated samples developed a bad odor, and neither the treated nor control samples exhibited any changes in appearance or color over the 9 days. These results are shown in Table 5.2.

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Table 5.2. Effect of LAE on Organoleptic Attributes of Cooked Rice Stored at 4°C

Day	Attribute	Control	LAE (100 mg/kg)	LAE (300 mg/kg)	Potassium sorbate
Day 0	Appear-ance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Color	White	White	White	White
	Odor	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
Day 7	Appear-ance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Color	White	White	White	White
	Odor	Slightly bad odor	Characteristic of the product	Characteristic of the product	Characteristic of the product
Day 9	Appear-ance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Color	White	White	White	White
	Odor	Bad odor	Characteristic of the product	Characteristic of the product	Characteristic of the product

D. Marinated Beef

Methods

The marinade was prepared by Kerry Hungaria Kft (H-1106 Budapest, Feher ut 10, Hungary) following standard manufacturing practices of this sector. The marinade consisted of water, sunflower oil, spices, salt, sucrose, dextrose, diphosphates, xanthan gum, and blue food color. The marinade was added to beef, followed by manual tumbling and vacuum packaging, and the marinated meat was stored at 4-7°C for 24 days.

There were three experimental conditions—treatment with LAE (as Mirenat-N), treatment with sodium nitrite, and untreated control. Five samples from each condition were evaluated on each of Days 1, 17, and 24 by experienced food testers.

Results

There were no significant differences in the appearance, color, or odor of the samples in any of the three experimental conditions on any of the analysis days. There were no changes in the appearance, color, or odor of any of the samples over the 24 days of the experiment.

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E. *Fresh Sausage*

Methods

The sausages used in this study were manufactured by Subirats S.A (C/ Reus, number 1, Pol. Industrial La Florida, Sta. Perpètua de la Mogoda 08130 Barcelona). The sausage ingredients were minced pork back (85%), streaky bacon (15%), salt, sugar, sodium citrate, sodium ascorbate, and color. In the test batch, LAE (added as Mirenat-N) was diluted in water and then added to the mass. The final dose of LAE was 200 mg/kg in the fresh sausages. The standard manufacturing practices that are used in this sector were followed in making both the test and control batches of sausages.

The organoleptic testing was done by Calitec S.C.P (C/ Rocafort n° 204-Bajos 08029 Barcelona). The sausages were stored at refrigerator temperature (0-5°C) for the ten days of the study, and five samples of both treated and control sausages were taken for organoleptic testing at each of Days 0, 1, 2, 3, 7 and 10. All evaluations were performed by two experienced food testers. The appearance, color, and odor of the fresh sausages were assessed at each sampling point.

Results

For the first two days, all organoleptic properties were characteristic of this food. By Day 3, both the test and control samples showed some darkening. By Day 7, the darkening was pronounced and some of the sausages were beginning to develop a disagreeable odor. Finally, by Day 10, all samples had darkened considerably and had a disagreeable odor. There was no appearance of spoilage in either the control or test sausages.

At no point over the ten days were there significant differences in appearance, color, or odor of the treated and control sausages.

F. *Bratwurst*

Methods

The bratwurst was again manufactured by Subirats S.A. The ingredients were minced pork back, underbelly, spices, salt, milk protein, diphosphate, antioxidant, and flavor enhancer. In the test batch, LAE (added as Mirenat-N) was diluted in water and then added to the mass. The final dose of LAE was 100 mg/kg in the bratwurst. The regular manufacturing practices that are used in this sector were followed in making both the test and untreated control batches of bratwurst.

The organoleptic testing was done by Calitec S.C.P (C/ Rocafort n° 204- Bajos 08029 Barcelona). The bratwurst was stored at refrigerator temperature (0-5°C) for the 90-day shelf life of this product, and five samples of both treated and control bratwurst were taken for organoleptic testing at each of Days 0, 15, 30, 45, 60, 75, and 90. All evaluations were performed by two experienced food testers. The appearance, color, and

odor of the bratwurst were assessed at each sampling point. Additionally, the pH of each sample was determined

Results

Unlike the findings with fresh sausage, no changes in the appearance, color, or odor of the bratwurst in either the test or control samples were noted throughout the 90 days. There were no significant differences between the treated and control bratwurst. There were no significant differences in the pH of any of the samples, either between the two treatment conditions or over time.

G. Cured Ham

Methods

The cured ham product was manufactured and sliced per standard commercial practices by the Columbus Salame Co. (CSC) of San Francisco CA. Sample batches were prepared with LAE (as Mirenat-N) incorporated into the formulation. Corresponding samples of product without LAE (i.e., untreated controls) were also prepared at the CSC facility. The cured ham samples were shipped in insulated containers with refrigerant to ABC Research Corp. in Gainesville FL via overnight courier. Samples were kept refrigerated (35°F) until used in the study (within 96 hrs).

The cured ham slices containing LAE were topically treated by complete immersion (30 sec. @ 22°C) in Mirenat-N solution (5% in distilled water). A 4-liter bath of Mirenat-N solution was prepared for the immersion treatment. Excess solution was allowed to drip from the samples for at least 15 sec. before packaging. The control cured ham slices (without LAE) were not immersion-treated prior to packaging. Product slices (ca. 25-35 g each) were aseptically placed into plastic pouches (3 slices per pouch) and vacuum packaged.

One treated sample and one untreated sample were evaluated on Day 0 and after 1, 2, 4, 6, and 8 weeks of storage at 4.4°C (40°F). The organoleptic evaluations were conducted by a group of 6 panelists experienced in the sensory evaluation of food products. Product was served in sample cups marked with random numbers that corresponded to numbers on the ballots. Water was provided to cleanse the pallet. The product was evaluated on color, aroma, and overall acceptability on a 7-point hedonic scale (i.e., 7 = liked extremely, 4 = neither liked/disliked, 1 = disliked extremely). Panelists' ratings were tabulated and averaged for each sample. The averages were statistically analyzed to determine if there were significant differences ($p < 0.05$) between the means of the treated vs. untreated samples at each sampling point.

Results

Results of organoleptic sample evaluations are presented in Table 5.3. There were no significant differences ($p<0.05$) between treated and untreated cured ham samples at any sampling point in judged color, aroma, or overall acceptability.

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Table 5.3. Effect of LAE on Organoleptic Attributes of Sliced Cured Ham Stored at 4.4°C

Attribute		Storage Time (weeks)											
		0		2		3		4		6		8	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Color	mean	5.17	5.50	4.83	3.83	4.00	3.50	3.67	3.50	4.17	3.17	4.17	4.25
	sd	0.75	0.55	1.47	1.60	1.26	1.97	1.97	1.05	1.17	1.47	1.17	1.60
	min	4	5	3	1	2	1	1	2	3	1	3	2
	max	6	6	7	5	6	7	6	5	6	5	6	6.5
Aroma	mean	4.00	4.50	3.00	2.50	3.17	2.17	3.50	3.33	3.50	3.83	3.25	3.83
	sd	1.55	2.07	1.10	1.38	1.17	0.98	2.07	0.82	0.84	1.33	1.99	1.72
	min	3	1	2	1	2	1	1	2	3	2	1	2
	max	7	7	5	4	5	4	6	4	5	6	6.5	6
Overall Acceptability	mean	4.17	4.50	3.50	3.00	3.33	2.50	3.17	3.33	3.83	3.67	3.50	4.08
	sd	0.98	1.52	1.22	1.26	1.51	1.05	1.60	0.82	1.17	1.37	1.52	1.56
	min	3	2	2	1	2	1	1	2	3	2	2	2
	max	6	6	5	4	6	4	5	4	6	6	6	6.5

H. Conclusion

LAE has been tested for organoleptic effects, including changes in appearance, color, odor or aroma, and general acceptability, in a wide variety of foods. Tested foods include both non-meat products (fresh tomato sauce and cooked rice) and meat products (marinated beef, fresh sausage, bratwurst, and cured ham). In no case was there any evidence of change in any tested attribute of the treated product as compared to untreated controls when the testing was performed prior to the beginning of spoilage.

The only organoleptic differences between LAE-treated food samples and untreated controls were those that emerged as the untreated controls began to exhibit signs of spoilage (such as the development of off-odors) more quickly than did the samples treated with LAE.

Based on this extensive body of research, it is concluded that treatment of foods with LAE is generally likely to be without effect on the organoleptic characteristics of the food.

VI. References

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VII. Copies of Antimicrobial Efficacy Study Reports

A. U.S. Studies

Fresh Chicken Breasts (*Salmonella* spp. inoculum; aerobes)

Fresh Beef Cuts (*E. coli* O157:H7 inoculum; aerobes)

Ground Beef (*E. coli* O157:H7 inoculum)

Sliced Roast Turkey (*Listeria monocytogenes* inoculum; aerobes)

Sliced Smoked Turkey (*Listeria monocytogenes* inoculum; aerobes)

Sliced Cured Ham (*Listeria monocytogenes* inoculum; aerobes)

Sliced Salami (*Listeria monocytogenes* inoculum; aerobes)

Frankfurters (*Listeria monocytogenes* inoculum; aerobes)

Ready-to-Eat Chicken Salad (*Listeria monocytogenes* inoculum; aerobes)

B. European Studies

Fresh Tomato Sauce (Aerobes and yeasts)

Cooked Rice (Aerobes and *Bacillus cereus* spp.)

Marinated Meat (Aerobes and *Enterobacter* spp.)

Fresh Sausage (Organoleptic data only)

Bratwurst (Aerobes, enteric bacteria, *Staphylococcus aureus*, *Clostridium* spp., *Salmonella* spp., *Shigella* spp., and *Lactobacillus* spp.)

Sliced Cured Ham (*Listeria monocytogenes* inoculum)

Diced Cured Ham (*Listeria monocytogenes* inoculum)

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A FOOD TESTING LABORATORY SINCE 1967

PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *Salmonella* spp. on Fresh Chicken Breasts.

OBJECTIVE: To evaluate the antimicrobial efficacy of a Mirenat-N immersion treatment for inactivating or inhibiting *Salmonella* spp. on fresh, skinless chicken breasts packaged in retail display packages during subsequent storage at 4.4 or 15°C. The effect of the Mirenat-N treatment on the microbial shelf-life of uninoculated chicken breasts was also evaluated.

METHODS:

A. Products and Materials

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided by the client and prepared as a 5% solution in distilled water for treatment of skinless chicken breasts. ABC Research obtained fresh, skinless chicken breasts locally in the Gainesville area. Product was stored at 35°F for up to 96 h until used in the study.

B. Test Microorganisms

The following three strains of *Salmonella* were used in this study: *Salmonella* serotype *enteritidis* (ATCC 13076), *Salmonella* serotype *typhimurium* (ATCC 14028), and *Salmonella* serotype *montevideo* (ATCC 8387). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth (TSB) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 20 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 10^6 CFU/ml.

C. PRODUCT PREPARATION AND INOCULATION

Designated chicken breasts were placed in sterile trays and inoculated by adding 0.1 ml of the final *Salmonella* inoculum in 0.01 ml droplets across a designated surface area (i.e., 10 cm^2) of the chicken breast and distributing it as a thin film over the designated site. The inoculation technique was designed to obtain a target *Salmonella* count of ca. 10,000 CFU/cm^2 on the designated inoculation site. After inoculation, the samples were held under refrigeration for approximately 20 min. to allow for bacterial attachment before Mirenat-N treatment, packaging or any microbial analyses. Uninoculated samples were also prepared to monitor the effect of the Mirenat-N on indigenous spoilage microflora (i.e., APC).

Three chicken breasts (10 cm^2) were sampled (using the surface swab technique) prior to treatment (i.e., time-0, untreated control samples) and 3 chicken breasts were sampled just after treatment (i.e., time-0, treated samples). One uninoculated chicken breast was likewise sampled prior to treatment (i.e., time-0, untreated control sample) and one chicken breast was sampled just after treatment (i.e., time-0, treated samples).

D. PRODUCT ANTIMICROBIAL TREATMENT

Inoculated and uninoculated chicken breast samples were treated by complete immersion (i.e., 30 sec. at 22°C) in the 5% Mirenat-N solution (in distilled water). Two 4 L baths of Mirenat-N solution (i.e., one bath for inoculated samples and another for uninoculated samples) were prepared. Samples were completely immersed one at a time in the designated Mirenat-N solution. Excess solution was allowed to drip from treated samples for ca. 15 sec. prior to analysis or packaging. Within 15 min. of the immersion treatment in Mirenat-N solution (i.e., time-0, treated samples), triplicate inoculated samples and 1 uninoculated sample were analyzed (Section F). As untreated control samples, a set of inoculated and uninoculated samples were also prepared with no treatment. Extra inoculated and uninoculated samples of each treatment variable were prepared.

E. PRODUCT PACKAGING AND STORAGE

Using sanitary procedures, the treated and untreated chicken breast samples were individually packaged in a retail foam trays and over-wrapped with saran wrap for storage. One set of treated and untreated samples was stored at 4.4°C and another set was stored at 15°C. Samples were collected after 3, 6, 9 and 12 days storage at 4.4°C and after 1, 2 and 4 days storage at 15°C. At each storage interval, three inoculated samples were analyzed for *Salmonella* and one uninoculated sample analyzed for APC from each treatment/temperature combination using the swab method (Section F).

F. SAMPLE ANALYSES

For inoculated samples, the designated inoculation sites (10-cm^2) were sampled using the swab technique. The designated site was thoroughly swabbed and the swab placed in 10 mL of sterile Difco neutralizing broth. At time-0 and after 3 and 6 days of storage, the *Salmonella* was enumerated/analyzed in respective swab rinsates using a direct plating

method (XLT4 agar). The XLT4 plates were incubated at 35°C for 24 h. Due to the inherent difficulties posed by background bacteria interference with the direct plating enumeration method, *Salmonella* was enumerated in respective swab sample rinsates after 9 and 12 days of storage at 4.4°C using a (3-tube) Most-Probable-Number (MPN) procedure using PCR analysis (BAX® System, DuPont Qualicon).

For uninoculated samples, a 10 sq. cm area was likewise swabbed and the swab rinsate analyzed for Aerobic Plate Counts (APC) incubated at 22-25°C for 5 days (to include both mesophilic and psychrotrophic spoilage bacteria) using standard methods agar.

All results were expressed as colony forming units (CFU's) per sq. cm and converted to $\log_{10}$ transforms. Sample means and corresponding standard deviations were calculated for each sample set at each time interval. The initial treatment reduction was calculated from the initial mean count of untreated samples and the initial mean count of treated samples. Changes in $\log_{10}$ counts (increase or decrease) at each time interval were also calculated for the treated and untreated samples against respective time-0 counts.

RESULTS:

The results of the Mirenat-N immersion treatment on *Salmonella* inoculated onto fresh chicken breasts subsequently stored at 4.4° or 15°C are presented in Table 1. The Mirenat-N immersion treatment resulted in an initial, mean *Salmonella* reduction of 3.19 $\log_{10}$ CFU/cm^2 (i.e., from 4.09 to 0.90 $\log_{10}$ CFU/cm^2) on the surface of the chicken breasts.

The *Salmonella* mean counts decreased from a pre-treatment level of 4.09 CFU/cm^2 to <1.00 and 3.27 CFU/cm^2, respectively, on treated and untreated samples during the first 3 days of storage at 4.4°C. There was variation in the *Salmonella* counts of treated and untreated samples over 12 days of storage but there was no evidence of a growth trend. It should be noted that the mean *Salmonella* count of the treated samples remained more than 3 $\log_{10}$ units lower than that of untreated samples after throughout 12 days of storage at 4.4°C.

After the initial 3.19 $\log_{10}$ unit reduction of *Salmonella* on the samples by the Mirenat-N immersion treatment, the mean counts of the treated samples remained approximately 3.19 and 2.68 $\log_{10}$ units, respectively, lower than that of untreated samples after 1 and 2 days storage at 15°C. *Salmonella* counts increased by 2.71 and 2.20 $\log_{10}$ units on the surface of treated and untreated chicken breasts during 2 days of storage at 15°C with an increase of 4.63 and 2.39 $\log_{10}$ units, respectively, for treated and untreated samples by 4 days of storage.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, fresh chicken breasts subsequently stored at 4.4° or 15°C are presented in Table 2. The initial APC levels on treated and untreated samples were 3.17 and 3.94 $\log_{10}$ CFU/cm^2, respectively. As compared to the untreated samples, the Mirenat-N immersion treatment inhibited the growth of APC bacteria during storage at 4.4°C. APC levels in treated samples and untreated samples were 75,000,000 and 510,000,000 CFU/cm^2, respectively, after 12 days of storage at 4.4°C.

SUMMARY:

The Mirenat-N immersion treatment resulted in a significant reduction (i.e., 3.19 $\log_{10}$ CFU/cm^2) of *Salmonella* inoculated onto the surface of fresh chicken breasts. The Mirenat-N treatment resulted in inhibition of aerobic plate counts developing on the chicken breasts during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated fresh chicken breasts.

(b) (6)

PREPARED BY

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Table 1. Effect of Mirenat-N on *Salmonella* Inoculated onto Fresh Chicken Breasts Stored at 4.4° and 15°C.

Storage Time (days)		4.4°C Storage				15°C Storage			
		Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
		CFU/cm²	Log CFU/cm²	CFU/cm²	Log CFU/cm²	CFU/cm²	Log CFU/cm²	CFU/cm²	Log CFU/cm²
0	rep. 1	10	1.00	13,600	4.13	10	1.00	13,600	4.13
	rep. 2	10	1.00	14,900	4.17	10	1.00	14,900	4.17
	rep. 3	5	0.70	8,900	3.95	5	0.70	8,900	3.95
	Mean		**0.90**		**4.09**		**0.90**		**4.09**
	Std. Dev.		0.17		0.12		0.17		0.12
Initial Reduction			**3.19**				**3.19**		
1	rep. 1	--	--	--	--	27	1.43	11,200	4.05
	rep. 2	--	--	--	--	18	1.26	14,100	4.15
	rep. 3	--	--	--	--	<1	<0.00	11,800	4.07
	Mean	--	--	--	--		**0.90**		**4.09**
	Std. Dev.	--	--	--	--		0.78		0.05
	Change	--	--	--	--		**0.00**		**0.00**
2	rep. 1	--	--	--	--	7,300	3.86	1,760,000	6.25
	rep. 2	--	--	--	--	1,730	3.24	1,590,000	6.20
	rep. 3	--	--	--	--	5,500	3.74	2,600,000	6.41
	Mean	--	--	--	--		**3.61**		**6.29**
	Std. Dev.	--	--	--	--		0.33		0.11
	Change	--	--	--	--		**+2.71**		**+2.20**
3	rep. 1	<1	<0.00	1,320	3.12	--	--	--	--
	rep. 2	<1	<0.00	2,360	3.37	--	--	--	--
	rep. 3	<1	<0.00	2,080	3.32	--	--	--	--
	Mean		**<0.00**		**3.27**	--	--	--	--
	Std. Dev.		0.00		0.13	--	--	--	--
	Change		**< -0.90**		**-0.81**	--	--	--	--
4	rep. 1	--	--	--	--	154,000	5.19	3,000,000	6.48
	rep. 2	--	--	--	--	650,000	5.81	4,400,000	6.64
	rep. 3	--	--	--	--	400,000	5.60	1,980,000	6.30
	Mean	--	--	--	--		**5.53**		**6.47**
	Std. Dev.	--	--	--	--		0.32		0.17
	Change	--	--	--	--		**+4.63**		**+2.39**
6	rep. 1	<1	<0.00	2,800	3.45	--	--	--	--
	rep. 2	<1	<0.00	5,000	3.70	--	--	--	--
	rep. 3	<1	<0.00	8,200	3.91	--	--	--	--
	Mean		**<0.00**		**3.69**	--	--	--	--
	Std. Dev.		0.00		0.23	--	--	--	--
	Change		**< -0.90**		**-0.40**	--	--	--	--
9	rep. 1	0.4	-0.40	>1,100	>3.04	--	--	--	--
	rep. 2	<0.3	<-0.52	>1,100	>3.04	--	--	--	--
	rep. 3	0.4	-0.40	>1,100	>3.04	--	--	--	--
	Mean		**-0.44**		**>3.04**	--	--	--	--
	Std. Dev.		0.07		0.00	--	--	--	--
	Change		**-1.34**		**-1.04**	--	--	--	--
12	rep. 1	110	2.04	46,000	4.66	--	--	--	--
	rep. 2	9.3	0.97	4,300	3.63	--	--	--	--
	rep. 3	4.3	0.63	15,000	4.18	--	--	--	--
	Mean		**1.21**		**4.16**	--	--	--	--
	Std. Dev.		0.74		0.51	--	--	--	--
	Change		**+0.31**		**+0.07**	--	--	--	--

Notes: 1) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples)
2) Change = (mean count at subject time) – (respective mean count at Time-0)

Table 2. Effect of Mirenat-N Treatment on Aerobic Plate Counts of Uninoculated, Fresh Chicken Breasts Stored at 4.4° and 15°C.

Storage Time (days)	4.4°C Storage				15°C Storage			
	Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
	CFU/cm^2	Log CFU/cm^2	CFU/cm^2	Log CFU/cm^2	CFU/cm^2	Log CFU/cm^2	CFU/cm^2	Log CFU/cm^2
0	1,480	3.17	8,800	3.94	1,480	3.17	8,800	3.94
1	--	--	--	--	1	0.00	3,000,000	6.48
2	--	--	--	--	194,000	5.29	150,000,000	8.18
3	189	2.28	191	2.28	--	--	--	--
4	--	--	--	--	36,000,000	7.56	34,000,000	7.53
6	8,200,000	6.91	75,000,000	7.88	--	--	--	--
9	246,000,000	8.39	660,000,000	8.82	--	--	--	--
12	75,000,000	7.88	510,000,000	8.71				

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A FOOD TESTING LABORATORY SINCE 1967

PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *E. coli* O157:H7 on Fresh Beef Cuts.

OBJECTIVE: To evaluate the antimicrobial efficacy of a Mirenat-N immersion treatment for inactivating or inhibiting *E. coli* O157:H7 on fresh, raw beef cuts packaged in retail display packages during subsequent storage at 4.4 or 15°C. The effect of the Mirenat-N treatment on the microbial shelf-life of uninoculated beef cuts was also evaluated.

METHODS:

A. PRODUCTS AND MATERIALS

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided by the client and prepared as a 5% solution in distilled water for treatment of beef cuts. ABC Research obtained fresh beef flank steaks locally in the Gainesville area. Product was stored at 35°F for up to 96 h until used in the study.

B. TEST MICROORGANISMS

The following three strains of *E. coli* O157:H7 were used in this study: ATCC #43894, ATCC #43895, and ATCC #700599. Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth (TSB) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 20 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 10^6 CFU/ml.

C. PRODUCT PREPARATION AND INOCULATION

Flank steaks were portioned into sample units/cuts (ca. 10 x 10 cm.) for the study. Designated sample cuts were placed in sterile trays and inoculated by adding 0.1 ml of the final *E. coli*

O157:H7 inoculum in 0.01 ml droplets across a designated surface area (i.e., 10 cm^2) of the sample surface and distributing it as a thin film over the designated site. The inoculation technique was designed to obtain a target *E. coli* O157:H7 count of ca. 10,000 CFU/cm^2 on the designated inoculation site. After inoculation, the beef pieces were held under refrigeration for approximately 20 min. to allow for bacterial attachment before the Mirenat-N treatment, packaging or any microbial analyses. Uninoculated sample cuts were likewise fabricated to monitor the effect of the Mirenat-N on indigenous spoilage microflora (i.e., aerobic plate counts).

Three inoculated beef cuts (10 cm^2) were sampled (using the surface swab technique) prior to treatment (i.e., time-0, untreated control samples) and 3 cuts were sampled just after treatment (i.e., time-0, treated samples) per Section F (below). One uninoculated beef cut was likewise sampled prior to treatment (i.e., time-0, untreated control sample) and one sample cut was sampled just after treatment (i.e., time-0, treated samples per Section F (below).

D. PRODUCT ANTIMICROBIAL TREATMENT

Inoculated and uninoculated samples were treated by complete immersion (i.e., 30 sec. @ 22°C) in the Mirenat-N solution (5% in deionized or distilled water). Two 4 L baths of Mirenat-N solution (i.e., one bath for inoculated samples and another for uninoculated samples) were prepared. Samples (i.e., ca. 32 inoculated and 10 uninoculated) were completely immersed one at a time in the designated Mirenat-N solution. Excess solution was allowed to drip from treated samples (e.g., 15 sec.) prior to analysis or packaging. Within 15 min. of the immersion treatment in Mirenat-N solution, triplicate inoculated samples and 1 uninoculated sample were analyzed as described previously (i.e., time-0, treated samples). As untreated control samples, a set of inoculated and uninoculated samples were also prepared with no treatment. Extra inoculated and uninoculated samples of each treatment variable were prepared.

E. PRODUCT PACKAGING AND STORAGE

Using sanitary procedures, the treated and untreated samples were each packaged in a retail foam tray and over-wrapped with saran wrap for storage. One set of treated and untreated samples was stored at 4.4°C and sampled after 3 and 6 days. Another set was stored at 15°C and sampled after 1, 2 and 4 days. At each storage interval, three inoculated samples were analyzed for *E. coli* O157:H7 and one uninoculated sample analyzed for APC from each treatment/temperature combination using the swab method (Section F, below).

F. SAMPLE ANALYSES

For inoculated samples, the designated inoculation sites (10-cm^2) were sampled using the swab technique. The designated site was thoroughly swabbed and the swab placed in 10 mL of sterile Difco neutralizing buffer. The *E. coli* O157:H7 was enumerated/analyzed in respective swab rinsates after the designated storage times using a direct plating method

MSBCIG agar). The MSCBCIG plates were incubated at 35°C for 24 h. Although *E. coli* O157:H7 was to be enumerated after 9 and 12 days of storage in the original protocol, selective enumeration of the organism via the direct plating method was not feasible at those times due to overgrowth of background enteric bacteria on the MSCBCIG plates.

For uninoculated sample, a 10-cm^2 area was likewise sampled and the swab rinsate analyzed for Aerobic Plate Counts (APC) incubated at 22-25°C for 5 days (to include both mesophilic and psychrotrophic spoilage bacteria) using standard methods agar. Although the original protocol was to monitor APC through 9 and 12 days of storage, testing was discontinued after 9 days since *E. coli* O157:H7 testing was also discontinued.

All results were expressed as colony forming units (CFU's) per cm^2 and converted to log_{10} transforms. Sample means and corresponding standard deviations were calculated for each sample set at each time interval. The initial treatment reduction was calculated from the initial mean count of untreated samples and the initial mean count of treated samples. Changes in log_{10} counts (increase or decrease) at each time interval were also calculated for the treated and untreated samples against respective time-0 counts.

RESULTS:

The results of the Mirenat-N immersion treatment on *E. coli* O157:H7 inoculated onto fresh beef cuts subsequently stored at 4.4° or 15°C are presented in Table 1. The Mirenat-N immersion treatment resulted in an initial, mean *E. coli* O157:H7 reduction of 2.34 log_{10} CFU/cm^2 (i.e., from 3.83 to 1.49 log_{10} CFU/cm^2) on the surface of the beef cuts.

As would be expected, there was no evidence of *E. coli* O157:H7 growth on treated or untreated samples stored at 4.4°C. In fact, the counts of *E. coli* O157:H7 inoculum gradually decreased during 6 days of storage at 4.4°C. *E. coli* O157:H7 mean counts had decreased from a pre-treatment level of 3.83 CFU/cm^2 on beef cut surfaces to 0.55 and 2.24 CFU/cm^2, respectively, on treated and untreated samples after 6 days of storage at 4.4°C.

After the initial 2.34 log_{10} CFU/cm^2 reduction of *E. coli* O157:H7 on the samples by the Mirenat-N immersion treatment, the mean counts of the treated samples remained approximately 2 log_{10} units lower than that of untreated samples after 2 and 4 days storage at 15°C. *E. coli* O157:H7 counts increased by approximately 4 log_{10} units on the surface of both treated and untreated beef cuts during 4 days of storage at 15°C.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, fresh beef cuts subsequently stored at 4.4° or 15°C are presented in Table 2. The APC levels on uninoculated beef cut samples were very low initially (i.e., <10 CFU/cm^2) on both treated and untreated samples. As compared to the untreated samples, the Mirenat-N immersion treatment inhibited the growth of APC bacteria during storage at 4.4 or 15°C. APC levels in untreated samples and treated samples were 11,8000,000 and 4,000 CFU/cm^2, respectively, after 9 days of storage at 4.4°C. APC levels were 17,000,000 and 6,500,000 CFU/cm^2,in untreated samples and treated samples, respectively, after 4 days of storage at 15°C.

SUMMARY:

The Mirenat-N immersion treatment resulted in a significant reduction (i.e., 2.34 $\log_{10}$ CFU/cm^2) of *E. coli* O157:H7 inoculated onto the surface of fresh beef cuts. The Mirenat-N treatment resulted in a substantial inhibition of aerobic plate counts developing on the beef cuts during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated fresh beef cuts.

(b) (6)

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Table 1. Effect of Mirenat-N Treatment on *E. coli* O157:H7 Inoculated onto Fresh Beef Cuts Subsequently Stored at 4.4° and 15°C.

Storage Time (days)		4.4°C Storage				15°C Storage			
		Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
		CFU/cm²	Log CFU/cm²	CFU/cm²	Log CFU/cm²	CFU/cm²	Log CFU/cm²	CFU/cm²	Log CFU/cm²
0	rep. 1	27	1.43	8,600	3.93	27	1.43	8,600	3.93
	rep. 2	30	1.48	3,600	3.56	30	1.48	3,600	3.56
	rep. 3	37	1.57	10,000	4.00	37	1.57	10,000	4.00
	Mean		**1.49**		**3.83**		**1.49**		**3.83**
	Std. Dev.		0.07		0.24		0.07		0.24
Initial Reduction			**2.34**				**2.34**		
1	rep. 1	--	--	--	--	6,200	3.79	1,230,000	6.09
	rep. 2	--	--	--	--	4,900	3.69	1,420,000	6.15
	rep. 3	--	--	--	--	710	2.85	560,000	5.75
	Mean	--	--	--	--		**3.44**		**6.00**
	Std. Dev.	--	--	--	--		0.52		0.22
	Change	--	--	--	--		**+1.95**		**+2.17**
2	rep. 1	--	--	--	--	182,000	5.26	67,000,000	7.83
	rep. 2	--	--	--	--	82,000	4.91	24,000,000	7.38
	rep. 3	--	--	--	--	96,000	4.98	23,000,000	7.36
	Mean	--	--	--	--		**5.05**		**7.52**
	Std. Dev.	--	--	--	--		0.18		0.26
	Change	--	--	--	--		**+3.56**		**+3.69**
3	rep. 1	19	1.28	2,370	3.37	--	--	--	--
	rep. 2	11	1.04	2,270	3.36	--	--	--	--
	rep. 3	11	1.04	2,170	3.34	--	--	--	--
	Mean		**1.12**		**3.36**	--	--	--	--
	Std. Dev.		0.14		0.02	--	--	--	--
	Change		**-0.37**		**-0.47**	--	--	--	--
4	rep. 1	--	--	--	--	860,000	5.93	88,000,000	7.94
	rep. 2	--	--	--	--	1,270,000	6.10	96,000,000	7.98
	rep. 3	--	--	--	--	720,000	5.86	90,000,000	7.95
	Mean	--	--	--	--		**5.97**		**7.96**
	Std. Dev.	--	--	--	--		0.13		0.02
	Change	--	--	--	--		**+4.47**		**+4.13**
6	rep. 1	4	0.60	420	2.62	--	--	--	--
	rep. 2	11	1.04	36	1.56	--	--	--	--
	rep. 3	1	0.00	350	2.54	--	--	--	--
	Mean		**0.55**		**2.24**	--	--	--	--
	Std. Dev.		0.52		0.59	--	--	--	--
	Change		**-0.94**		**-1.59**	--	--	--	--

Notes: 1) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples)
2) Change = (mean count at subject time) – (respective mean count at Time-0)

Table 2. Effect of Mirenat-N Treatment on Aerobic Plate Counts of Uninoculated, Fresh Beef Cuts Subsequently Stored at 4.4° and 15°C.

Storage Time (days)	4.4°C Storage				15°C Storage			
	Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
	CFU/cm^2	Log CFU/cm^2	CFU/cm^2	Log CFU/cm^2	CFU/cm^2	Log CFU/cm^2	CFU/cm^2	Log CFU/cm^2
0	8	0.90	3	0.48	8	0.90	3	0.48
1	--	--	--	--	192	2.28	9,900,000	7.00
2	--	--	--	--	1,200,000	6.08	50,000,000	7.70
3	6	0.78	226,000	5.35	--	--	--	--
4	--	--	--	--	6,500,000	6.81	17,000,000	7.23
6	34,000	4.53	590,000	5.77	--	--	--	--
9	4,000	3.60	11,800,000	7.07	--	--	--	--

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PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA) and Sergi Fegueras (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *E. coli* O157:H7 in Freshly Ground Beef.

OBJECTIVE: To evaluate the antimicrobial effect of Mirenat-N as an antimicrobial immersion application in reducing levels of *E. coli* O157:H7 on fresh beef trim and derived freshly ground beef subsequently stored at 4.4°C.

METHODS:

A. PRODUCTS AND MATERIALS

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided by the client and prepared as a 5% solution in distilled water for treatment of beef trim prior to grinding. ABC Research obtained fresh beef flank steaks locally in the Gainesville area to use as beef trim for fabrication of the ground beef. Product was held frozen until used in the study.

B. TEST MICROORGANISMS

The following three strains of *E. coli* O157:H7 were used in this study: ATCC #43894, ATCC #43895, and ATCC #700599. Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth (TSB) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 20 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 10^7 CFU/ml.

C. TRIM PREPARATION AND INOCULATION

Flank steaks were portioned into (trim) pieces weighing 100 g each. Fifty (50) trim pieces were fabricated and inoculated. Trim pieces were placed in sterile trays and inoculated by adding 0.2 ml of the final *E. coli* O157:H7 inoculum in 0.01 ml droplets across the surface and widely distributing it as a thin film over trim surface. The inoculation technique resulted in an *E. coli* O157:H7 count of ca. 60,000 CFU/g of derived ground beef. After inoculation,

the beef pieces were held at 4.4°C for 20-30 min. to allow for bacterial attachment before the Mirenat-N treatment and/or before being ground.

D. TRIM TREATMENT AND GRINDING

One set of inoculated trim pieces (n=25) were treated by complete immersion (i.e., 30 sec. @ 22°C) in the Mirenat-N solution (5% in distilled water). A 6 L bath of Mirenat-N solution was prepared for treatment. Trim pieces were completely immersed one at a time in the Mirenat-N solution. Excess solution was allowed to drip from treated samples (e.g., 15 sec.) prior to being combined and ground. All 25 treated trim pieces were combined, mixed, and ground into a batch of ground beef (i.e., treated ground beef).

The grinding equipment was thoroughly cleaned, sanitized and rinsed before grinding the untreated beef trim. Another set of inoculated, untreated trim pieces (n=25) were combined, mixed, and ground into another batch of ground beef (i.e., untreated ground beef).

E. PRODUCT PACKAGING AND STORAGE

Using sanitary procedures, portions (ca. 100 g each) from the untreated and the treated batch of ground beef were formed into patties and packaged in retail foam trays (one patty to a tray) and over-wrapped with a saran type wrap for storage. Three treated and 3 untreated patty samples were analyzed at time-0 and following 3, 6, 9 and 12 days of storage at 4.4°C.

F. SAMPLE ANALYSES

Samples were microbiologically analyzed by aseptically placing a representative 25 g aliquot into a sterile stomacher bag, diluting the aliquot 1:10 in sterile Difco neutralizing broth, and stomaching for 1 min. At time-0 and after 3 and 6 days of storage, the *E. coli* O157:H7 was enumerated in respective sample homogenates using a direct plating method (MSA-BCIG agar). The MSA-BCIG plates were incubated at 42°C for 24 h. For some samples stored for 3 and 6 days, selective *E. coli* O157:H counts could not be obtained due to numerous interfering background colonies on the MSA-BCIG plates. After 9 and 12 days of storage, *E. coli* O157:H7 was enumerated in respective sample homogenates using a (3-tube) Most-Probable-Number (MPN) procedure via PCR analysis (BAX® System, DuPont Qualicon).

All results were expressed as colony forming units (CFU's) or MPN counts per gram and converted to $\log_{10}$ transforms. Sample means and corresponding standard deviations were also calculated for each treatment/storage time/storage temperature sample set.

RESULTS:

The results of the Mirenat-N treatment of beef trim inoculated with *E. coli* O157:H7 and derived ground beef subsequently stored at 4.4° are presented in Table 1. The Mirenat-N immersion treatment of the beef trim resulted in an initial, mean *E. coli* O157:H7 reduction of 0.76 $\log_{10}$ CFU/g (i.e., from 4.82 to 4.06 $\log_{10}$ CFU/g) in the derived ground beef.

After the initial reduction by the Mirenat-N treatment, *E. coli* O157:H7 counts decreased in treated and untreated ground beef samples during 12 days of storage at 4.4°C but mean counts were consistently much lower in treated than in untreated samples at each storage interval. Mean counts of *E. coli* O157:H7 on treated samples after 3, 9 and 12 days of storage were 1.66, 1.02, and 0.47 $\log_{10}$ units lower, respectively, than those of untreated samples.

SUMMARY:

In summary, the Mirenat-N treatment of beef trim inoculated with *E. coli* O157:H7 resulted in a significant reduction (i.e., 0.76 $\log_{10}$ CFU/g) of *E. coli* O157:H7 in the derived, freshly ground beef. The Mirenat-N also resulted in significantly lower *E. coli* O157:H7 levels in the derived ground beef than in untreated samples during subsequent storage at 4.4°C.

(b) (6)

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Table 1. Effect of Mirenat-N on *E. coli* O157:H7 in Fresh Ground Beef Stored at 4.4°C.

Storage Time (days)		Mirenat-N Treated		Untreated	
		CFU/g	Log CFU/g	CFU/g	Log CFU/g
0	rep. 1	12,000	4.08	78,000	4.89
	rep. 2	11,000	4.04	63,000	4.80
	rep. 3	11,500	4.06	57,000	4.76
	Mean		**4.06**		**4.82**
	Std. Dev.		0.02		0.07
Initial Reduction			**0.76**		n/a
3	rep. 1	2,000	3.30	44,000	4.64
	rep. 2	*	*	180,000	5.26
	rep. 3	*	*	99,000	5.00
	Mean		**3.30**		**4.96**
	Std. Dev.		0.00		0.31
9	rep. 1	2,300	3.36	46,000	4.66
	rep. 2	4,300	3.63	46,000	4.66
	rep. 3	2,300	3.36	12,000	4.08
	Mean		**3.45**		**4.47**
	Std. Dev.		0.16		0.34
12	rep. 1	230	2.36	920	2.96
	rep. 2	230	2.36	360	2.56
	rep. 3	230	2.36	920	2.96
	Mean		**2.36**		**2.83**
	Std. Dev.		0.00		0.24

Notes: 1) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples)
2) Counts could not be estimated due to numerous background colonies on MSA-BCIG plates

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A FOOD TESTING LABORATORY SINCE 1967

PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *Listeria monocytogenes* on Sliced Roast Turkey.

OBJECTIVE: To evaluate the antimicrobial efficacy of Mirenat-N in reducing or inhibiting levels of *Listeria monocytogenes* on sliced roast turkey during subsequent storage at 4.4 or 15°C. The effect of the Mirenat-N treatment on the microbial shelf-life of uninoculated roast turkey was also evaluated.

METHODS:

A. PRODUCTS AND MATERIALS

The roast turkey product was manufactured and sliced per standard commercial practices by the Columbus Salami Co. (CSC). Samples batches with Mirenat-N (Batch JMR-438; Exp. Date: 9/30/05) incorporated into the formulation (i.e., treated) at the rate of 1.0 g per Kg. Corresponding samples of product without Mirenat-N (i.e., untreated controls) were also prepared at the CSC facility. The roast turkey samples were shipped in insulated containers with refrigerant to ABC Research Corp. via overnight courier. Samples were kept refrigerated (35°F) until used in the study (within 96 hrs).

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided to ABC Research by the client for an immersion treatment (i.e., 5% Mirenat-N solution in distilled water) of roast turkey slices prior to inoculation and/or packaging.

B. PRODUCT TREATMENT

Just prior to inoculation, the roast turkey slices (containing Mirenat-N) were topically treated by complete immersion (i.e., 30 sec. @ 22°C) in the Mirenat-N solution (5% in distilled water). A six liter bath of Mirenat-N solution was prepared for the immersion treatment of inoculated slices and a 4 liter bath for uninoculated slices. Excess solution was allowed to drip from the samples at least 15 sec. before inoculation and/or packaging. The control roast turkey slices (without Mirenat-N) were not immersion treated prior to inoculation and/or packaging.

C TEST MICROORGANISMS

The following 5 strains of *Listeria monocytogenes* were used: ATCC 19115 (Serotype 4B), "Serotype ½a", "Serotype ½b", ATCC 7644 (Gibson) and ATCC 49594 (Scott A). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth with yeast extract (TSBYE) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was resuspended in BPB to obtain a cell suspension of approx. 1×10^8 CFU/ml. Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 40 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 5×10^5 CFU/ml.

D. PRODUCT INOCULATION AND PACKAGING

After being allowed to drip from the immersion treatment for at least 15 sec., individual slices (ca. 25-35g each) were aseptically placed into plastic pouches (one slice per pouch) for inoculation. Each slice was inoculated in the package by adding 0.1 ml of the *L. monocytogenes* cocktail inoculum in 0.01 ml droplets across the surface and then distributing it as a thin film over the surface. The inoculation technique resulted in an *L. monocytogenes* count of ca. 3,000 CFU/g. For each treatment set (treated or untreated/control), each inoculated slice was vacuum packaged in the pouch. For each treatment variable, another set of samples was left uninoculated, placed in pouches (one slice per pouch), and vacuum packaged to monitor the effect of the Mirenat-N on aerobic plate counts (APC).

E. PRODUCT STORAGE

For each treatment batch, three inoculated sample packages were analyzed for *L. monocytogenes* immediately after inoculation (i.e., time-0) and one uninoculated sample was analyzed for APC at time-0 (Section F). One set of remaining treated and untreated samples were stored at 4.4°C (40°F) and sampled after 1, 2, 4, 6, and 8 weeks of storage. Another set was stored at 15°C (59°F) and sampled after 1, 2, and 4 days. At each storage interval, three inoculated samples were analyzed for *L. monocytogenes* and one uninoculated sample analyzed for APC from each treatment/temperature combination (Section F).

F. SAMPLE ANALYSES

Sample packages were opened aseptically and the samples along with any liquid placed in a sterile stomacher bag with in sterile Difco neutralizing broth to effect a 1:10 dilution. Samples were stomached for 1 min. and the homogenate serially diluted as required. *L. monocytogenes* was enumerated in inoculated sample homogenates via surface plating on MOX agar. The MOX plates were incubated at 35°C for 48 h and counted. For uninoculated samples, the samples homogenate was analyzed for Aerobic Plate Counts (APC) incubated at 22-24°C for 5 days (to include both mesophilic and psychrotrophic spoilage bacteria) using standard methods agar.

All results were expressed as colony forming units (CFU's) per gram of sample and converted to $\log_{10}$ transforms. Sample means and corresponding standard deviations were calculated for each sample set at each time interval. The initial treatment reduction was calculated from the initial mean counts of treated and untreated samples.

RESULTS:

The results of the Mirenat-N treatment on *Listeria monocytogenes* inoculated onto sliced roast turkey stored at 4.4° or 15°C are presented in Table 1. The Mirenat-N treatment resulted in an initial, mean *L. monocytogenes* reduction of 1.42 $\log_{10}$ units on the sliced roast turkey (i.e., from 3.42 to 2.00 $\log_{10}$ CFU/g).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated roast turkey samples after 1 and 2 weeks of storage at 4.4°C were 1.53 and 1.50 $\log_{10}$ units lower, respectively, than those of untreated samples. *L. monocytogenes* counts were similar on treated and untreated sample after 4, 6 and 8 weeks of storage at 4.4°C. *L. monocytogenes* counts increased throughout 8 weeks of storage at 4.4°C in treated and untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 4 days of storage at 15°C in treated and untreated roast turkey samples but were consistently lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on the treated sliced roast turkey samples after 1, 2 and 4 days storage at 15°C were 0.95, 0.85 and 0.39 $\log_{10}$ units lower, respectively, than those of untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, sliced roast turkey subsequently stored at 4.4° or 15°C are presented in Table 2. The initial APC levels were very low in all roast turkey samples. As compared to the untreated samples, the Mirenat-N treatment of the roast turkey inhibited the growth of APC bacteria during storage at 4.4°C or 15°C. APC levels in treated samples were remained relatively low and were consistently much lower than those of corresponding untreated samples at each storage interval for either temperature. The APC on the treated sample was <10 CFU/g after 8 weeks of storage at 4.4°C whereas the APC of the untreated sample was 240,000,000 CFU/g after 8 weeks of storage. Since the APC level at all other storage intervals were ≤10 CFU/g, the higher APC (i.e., 34,000 CFU/g) for the treated sample after 4 weeks of storage at 4.4°C suggests a sample "outlier" and is inconsistent with any actual growth trend as was clearly indicated for the untreated samples during storage.

SUMMARY:

Mirenat-N treatment of the sliced roast turkey resulted in a significant reduction (i.e., 1.42 $\log_{10}$ units) of *L. monocytogenes* inoculated onto sliced roast turkey. The Mirenat-N treatment also inhibited the growth of aerobic plate counts developing on the samples during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated sliced roast turkey.

(b) (6)

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Table 1. Effect of Mirenat-N on *L. monocytogenes* inoculated onto Sliced Roast Turkey Stored at 4.4° and 15°C.

Storage Time (days)		4.4°C Storage				15°C Storage			
		Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
		CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0	rep. 1	140	2.15	3,200	3.51	140	2.15	3,200	3.51
	rep. 2	140	2.15	2,500	3.40	140	2.15	2,500	3.40
	rep. 3	50	1.70	2,200	3.34	50	1.70	2,200	3.34
	Mean		**2.00**		**3.42**		**2.00**		**3.42**
	Std. Dev.		0.26		0.08		0.26		0.08
Initial Reduction			**1.42**				**1.42**		
1 (day)	rep. 1	--	--	--	--	3,800	3.58	31,000	4.49
	rep. 2	--	--	--	--	1,630	3.21	23,000	4.36
	rep. 3	--	--	--	--	4,200	3.62	25,000	4.40
	Mean	--	--	--	--		**3.47**		**4.42**
	Std. Dev.	--	--	--	--		0.23		0.07
2 (days)	rep. 1	--	--	--	--	46,000	4.66	870,000	5.94
	rep. 2	--	--	--	--	65,000	4.81	710,000	5.85
	rep. 3	--	--	--	--	310,000	5.49	520,000	5.72
	Mean	--	--	--	--		**4.99**		**5.84**
	Std. Dev.	--	--	--	--		0.44		0.11
4 (days)	rep. 1	--	--	--	--	20,500,000	7.31	57,000,000	7.76
	rep. 2	--	--	--	--	24,900,000	7.40	63,000,000	7.80
	rep. 3	--	--	--	--	21,700,000	7.34	46,000,000	7.66
	Mean	--	--	--	--		**7.35**		**7.74**
	Std. Dev.	--	--	--	--		0.04		0.07
1 (week)	rep. 1	280	2.45	19,000	4.28	--	--	--	--
	rep. 2	1,280	3.11	35,000	4.54	--	--	--	--
	rep. 3	940	2.97	19,000	4.28	--	--	--	--
	Mean		**2.84**		**4.37**	--	--	--	--
	Std. Dev.		0.35		0.15	--	--	--	--
2 (weeks)	rep. 1	114,000	5.06	1,290,000	6.11	--	--	--	--
	rep. 2	56,000	4.75	2,090,000	6.32	--	--	--	--
	rep. 3	24,900	4.40	1,810,000	6.26	--	--	--	--
	Mean		**4.73**		**6.23**	--	--	--	--
	Std. Dev.		0.33		0.11	--	--	--	--
4 (weeks)	rep. 1	105,000,000	8.02	30,000,000	7.48	--	--	--	--
	rep. 2	79,000,000	7.90	65,000,000	7.81	--	--	--	--
	rep. 3	74,000,000	7.87	73,000,000	7.86	--	--	--	--
	Mean		**7.93**		**7.72**	--	--	--	--
	Std. Dev.		0.08		0.21	--	--	--	--
6 (weeks)	rep. 1	157,000,000	8.20	67,000,000	7.83	--	--	--	--
	rep. 2	180,000,000	8.26	78,000,000	7.89	--	--	--	--
	rep. 3	260,000,000	8.41	91,000,000	7.96	--	--	--	--
	Mean		**8.29**		**7.89**	--	--	--	--
	Std. Dev.		0.11		0.07	--	--	--	--
8 (weeks)	rep. 1	76,000,000	7.88	68,000,000	7.83	--	--	--	--
	rep. 2	41,000,000	7.61	90,000,000	7.95	--	--	--	--
	rep. 3	190,000,000	8.28	140,000,000	8.15	--	--	--	--
	Mean		**7.92**		**7.98**	--	--	--	--
	Std. Dev.		0.34		0.16	--	--	--	--

Notes: 1) Counts expressed as CFU per gram of sample.
2) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples).

Table 2. Effect of Mirenat-N on Aerobic Plate Counts of Uninoculated onto Sliced Roast Turkey Stored at 4.4° and 15°C.

Storage Time	4.4°C Storage				15°C Storage			
	Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0 (day)	<10	<1.00	<10	<1.00	<10	<1.00	<10	<1.00
1 (day)	--	--	--	--	20	1.30	20	1.30
2 (days)	--	--	--	--	10	1.00	4,500	3.65
4 (days)	--	--	--	--	<10	<1.00	4,700,000	6.67
1 (week)	<10	<1.00	101,000	5.00	--	--	--	--
2 (weeks)	<10	<1.00	330,000	5.52	--	--	--	--
4 (weeks)	34,000	4.53	75,000,000	7.88	--	--	--	--
6 (weeks)	10	1.00	31,000,000	7.49				
8 (weeks)	<10	<1.00	240,000,000	8.38				

Notes: 1) Counts expressed as CFU per gram of sample.

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A FOOD TESTING LABORATORY SINCE 1967

PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *Listeria monocytogenes* on Sliced Smoked Turkey.

OBJECTIVE: To evaluate the antimicrobial efficacy of Mirenat-N in reducing or inhibiting levels of *Listeria monocytogenes* on sliced smoked turkey during subsequent storage at 4.4 or 15°C. The effect of the Mirenat-N treatment on the microbial shelf-life of uninoculated smoked turkey was also evaluated.

METHODS:

A. PRODUCTS AND MATERIALS

The smoked turkey product was manufactured and sliced per standard commercial practices by the Columbus Salami Co. (CSC). Samples batches with Mirenat-N (Batch JMR-438; Exp. Date: 9/30/05) incorporated into the formulation (i.e., treated) at the rate of 1.0 g per Kg. Corresponding samples of product without Mirenat-N (i.e., untreated controls) were also prepared at the CSC facility. The smoked turkey samples were shipped in insulated containers with refrigerant to ABC Research Corp. via overnight courier. Samples were kept refrigerated (35°F) until used in the study (within 96 hrs).

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided to ABC Research by the client for an immersion treatment (i.e., 5% Mirenat-N solution in distilled water) of smoked turkey slices prior to inoculation and/or packaging.

B. PRODUCT TREATMENT

Just prior to inoculation, the smoked turkey slices (containing Mirenat-N) were topically treated by complete immersion (i.e., 30 sec. @ 22°C) in the Mirenat-N solution (5% in distilled water). A six liter bath of Mirenat-N solution was prepared for the immersion treatment of inoculated slices and a 4 liter bath for uninoculated slices. Excess solution was allowed to drip from the samples at least 15 sec. before inoculation and/or packaging. The control smoked turkey slices (without Mirenat-N) were not immersion treated prior to inoculation and/or packaging.

C TEST MICROORGANISMS

The following 5 strains of *Listeria monocytogenes* were used: ATCC 19115 (Serotype 4B), "Serotype ½a", "Serotype ½b", ATCC 7644 (Gibson) and ATCC 49594 (Scott A). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth with yeast extract (TSBYE) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was resuspended in BPB to obtain a cell suspension of approx. 1×10^8 CFU/ml. Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 40 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 5×10^5 CFU/ml.

D. PRODUCT INOCULATION

After being allowed to drip from the immersion treatment for at least 15 sec., individual slices (ca. 25-35g each) were aseptically placed into plastic pouches (one slice per pouch) for inoculation. Each slice was inoculated in the package by adding 0.1 ml of the *L. monocytogenes* cocktail inoculum in 0.01 ml droplets across the surface and then distributing it as a thin film over the surface. The inoculation technique resulted in an *L. monocytogenes* count of ca. 1,000 CFU/g.

E. PRODUCT STORAGE AND PACKAGING

For each treatment set (treated or untreated/control), each inoculated slice was vacuum packaged in the pouch. For each treatment variable, another set of samples was left uninoculated, placed in pouches (one slice per pouch), and vacuum packaged to monitor the effect of the Mirenat-N on aerobic plate counts (APC). For each treatment batch, three inoculated sample packages were analyzed for *L. monocytogenes* immediately after inoculation (i.e., time-0) and one uninoculated sample was analyzed for APC at time-0 (Section F). One set of remaining treated and untreated samples were stored at 4.4°C (40°F) and sampled after 1, 2, 4, 6, and 8 weeks of storage. Another set was stored at 15°C (59°F) and sampled after 1, 2, and 4 days. At each storage interval, three inoculated samples were analyzed for *L. monocytogenes* and one uninoculated sample analyzed for APC from each treatment/temperature combination (Section F).

F. SAMPLE ANALYSES

Sample packages were opened aseptically and the samples along with any liquid placed in a sterile stomacher bag with in sterile Difco neutralizing broth to effect a 1:10 dilution. Samples were stomached for 1 min. and the homogenate serially diluted as required. *L. monocytogenes* was enumerated in inoculated sample homogenates via surface plating on MOX agar. The MOX plates were incubated at 35°C for 48 h and counted. For uninoculated samples, the

samples homogenate was analyzed for Aerobic Plate Counts (APC) incubated at 22-24°C for 5 days (to include both mesophilic and psychrotrophic spoilage bacteria) using standard methods agar.

All results were expressed as colony forming units (CFU's) per gram of sample and converted to $\log_{10}$ transforms. Sample means and corresponding standard deviations were calculated for each sample set at each time interval. The initial treatment reduction was calculated from the initial mean counts of treated and untreated samples.

RESULTS:

The results of the Mirenat-N treatment on *Listeria monocytogenes* inoculated onto sliced smoked turkey stored at 4.4° or 15°C are presented in Table 1. The Mirenat-N treatment resulted in an initial, mean *L. monocytogenes* reduction of greater than 1.94 $\log_{10}$ units on the sliced smoked turkey (i.e., from 3.04 to <1.10 $\log_{10}$ CFU/g).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 8 weeks of storage at 4.4°C on treated and untreated smoked turkey samples but were consistently much lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on treated samples after 1, 2, 4, 6 and 8 weeks of storage at 4.4°C were >1.03, 1.48, 1.83, 1.05 and 0.62 $\log_{10}$ units lower, respectively, than those of untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 4 days of storage at 15°C in treated and untreated smoked turkey samples but were consistently much lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on the treated sliced smoked turkey samples after 1, 2 and 4 days storage at 15°C were >1.94, 2.32 and 2.55 $\log_{10}$ units lower, respectively, than those of untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, sliced smoked turkey subsequently stored at 4.4° or 15°C are presented in Table 2. The initial APC levels were very low in all smoked turkey samples. As compared to the untreated samples, the Mirenat-N treatment of the smoked turkey inhibited the growth of APC bacteria during storage at 4.4°C or 15°C. APC levels in treated samples were consistently much lower than those of corresponding untreated samples at each storage interval for either temperature. In fact, APC levels on treated samples remained at very low levels (i.e., ≤10 CFU/g) during 8 weeks of storage at 4.4°C whereas those of untreated samples increased to 1,740,000 CFU/g over 8 weeks of storage.

SUMMARY:

Mirenat-N treatment of the sliced smoked turkey resulted in a significant reduction (i.e., >1.94 $\log_{10}$ units) of *L. monocytogenes* inoculated onto sliced smoked turkey. *L. monocytogenes* levels were consistently much lower on treated than on untreated samples during storage at 4.4° or 15°C. The Mirenat-N treatment also inhibited the growth of aerobic plate counts developing on the samples during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated sliced smoked turkey.

(b) (6)

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ABC Research Corp.

Table 1. Effect of Mirenat-N on *L. monocytogenes* inoculated onto Sliced Smoked Turkey Stored at 4.4° and 15°C.

Storage Time (days)		4.4°C Storage				15°C Storage			
		Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
		CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0	rep. 1	<10	<1.00	1,440	3.16	<10	<1.00	1,440	3.16
	rep. 2	<10	<1.00	1,340	3.13	<10	<1.00	1,340	3.13
	rep. 3	20	1.30	700	2.85	20	1.30	700	2.85
	Mean		**<1.10**		**3.04**		**1.10**		**3.04**
	Std. Dev.		0.17		0.17		0.17		0.17
Initial Reduction			**>1.94**				**>1.94**		
1 (day)	rep. 1	--	--	--	--	<10	<1.00	770	2.89
	rep. 2	--	--	--	--	<10	<1.00	1,140	3.06
	rep. 3	--	--	--	--	<10	<1.00	740	2.87
	Mean	--	--	--	--		**<1.00**		**2.94**
	Std. Dev.	--	--	--	--		0.00		0.10
2 (days)	rep. 1	--	--	--	--	<10	<1.00	2,700	3.43
	rep. 2	--	--	--	--	50	1.70	3,500	3.54
	rep. 3	--	--	--	--	<10	<1.00	4,800	3.68
	Mean	--	--	--	--		**1.23**		**3.55**
	Std. Dev.	--	--	--	--		0.40		0.13
4 (days)	rep. 1	--	--	--	--	1,330	3.12	640,000	5.81
	rep. 2	--	--	--	--	320	2.51	227,000	5.36
	rep. 3	--	--	--	--	2,600	3.41	248,000	5.39
	Mean	--	--	--	--		**3.01**		**5.52**
	Std. Dev.	--	--	--	--		0.46		0.25
1 (week)	rep. 1	<10	<1.00	1,370	3.14	--	--	--	--
	rep. 2	10	1.00	1,070	3.03	--	--	--	--
	rep. 3	<10	<1.00	830	2.92	--	--	--	--
	Mean		**1.00**		**3.03**	--	--	--	--
	Std. Dev.		0.00		0.11	--	--	--	--
2 (weeks)	rep. 1	170	2.23	4,600	3.66	--	--	--	--
	rep. 2	150	2.18	4,600	3.66	--	--	--	--
	rep. 3	750	2.88	24,900	4.40	--	--	--	--
	Mean		**2.43**		**3.91**	--	--	--	--
	Std. Dev.		0.39		0.42	--	--	--	--
4 (weeks)	rep. 1	6,800	3.83	76,000	4.88	--	--	--	--
	rep. 2	5,400	3.73	390,000	5.59	--	--	--	--
	rep. 3	2,220	3.35	840,000	5.92	--	--	--	--
	Mean		**3.64**		**5.47**	--	--	--	--
	Std. Dev.		0.26		0.53	--	--	--	--
6 (weeks)	rep. 1	410,000	5.61	9,900,000	7.00	--	--	--	--
	rep. 2	580,000	5.76	2,800,000	6.45	--	--	--	--
	rep. 3	390,000	5.59	4,800,000	6.68	--	--	--	--
	Mean		**5.66**		**6.71**	--	--	--	--
	Std. Dev.		0.09		0.28	--	--	--	--
8 (weeks)	rep. 1	39,000,000	7.59	300,000,000	8.48	--	--	--	--
	rep. 2	59,000,000	7.77	211,000,000	8.32	--	--	--	--
	rep. 3	92,000,000	7.96	250,000,000	8.40	--	--	--	--
	Mean		**7.78**		**8.40**	--	--	--	--
	Std. Dev.		0.19		0.08	--	--	--	--

Notes: 1) Counts expressed as CFU per gram of sample.
2) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples).

Table 2. Effect of Mirenat-N on Aerobic Plate Counts of Uninoculated onto Sliced Smoked Turkey Stored at 4.4° and 15°C.

Storage Time	4.4°C Storage				15°C Storage			
	Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0 (day)	<10	<1.00	10	1.00	<10	<1.00	10	1.00
1 (day)	--	--	--	--	<10	<1.00	<10	<1.00
2 (days)	--	--	--	--	10	1.00	<10	<1.00
4 (days)	--	--	--	--	10	1.00	1,280	3.11
1 (week)	<10	<1.00	<10	<1.00	--	--	--	--
2 (weeks)	<10	<1.00	<10	<1.00	--	--	--	--
4 (weeks)	<10	<1.00	4,500	3.65	--	--	--	--
6 (weeks)	<10	<1.00	360,000	5.56				
8 (weeks)	10	1.00	1,740,000	6.24				

Notes: 1) Counts expressed as CFU per gram of sample.

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PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *Listeria monocytogenes* on Sliced Cured Ham.

OBJECTIVE: To evaluate the antimicrobial efficacy of Mirenat-N in reducing or inhibiting levels of *Listeria monocytogenes* on sliced cured ham during subsequent storage at 4.4 or 15°C. The effect of the Mirenat-N treatment on the microbial shelf-life of uninoculated cured ham was also evaluated.

METHODS:

A. PRODUCTS AND MATERIALS

The cured ham product was manufactured and sliced per standard commercial practices by the Columbus Salami Co. (CSC). Samples batches with Mirenat-N (Batch JMR-438; Exp. Date: 9/30/05) incorporated into the formulation (i.e., treated) at the rate of 1.0 g per Kg. Corresponding samples of product without Mirenat-N (i.e., untreated controls) were also prepared at the CSC facility. The cured ham samples were shipped in insulated containers with refrigerant to ABC Research Corp. via overnight courier. Samples were kept refrigerated (35°F) until used in the study (within 96 hrs).

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided to ABC Research by the client for an immersion treatment (i.e., 5% Mirenat-N solution in distilled water) of cured ham slices prior to inoculation and/or packaging.

B. PRODUCT TREATMENT

Just prior to inoculation, the cured ham slices (containing Mirenat-N) were topically treated by complete immersion (i.e., 30 sec. @ 22°C) in the Mirenat-N solution (5% in distilled water). A six liter bath of Mirenat-N solution was prepared for the immersion treatment of inoculated slices and a 4 liter bath for uninoculated slices. Excess solution was allowed to drip from the samples at least 15 sec. before inoculation and/or packaging. The control cured ham slices (without Mirenat-N) were not immersion treated prior to inoculation and/or packaging.

C TEST MICROORGANISMS

The following 5 strains of *Listeria monocytogenes* were used: ATCC 19115 (Serotype 4B), "Serotype ½a", "Serotype ½b", ATCC 7644 (Gibson) and ATCC 49594 (Scott A). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth with yeast extract (TSBYE) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was resuspended in BPB to obtain a cell suspension of approx. 1×10^8 CFU/ml. Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 40 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 5×10^5 CFU/ml.

D. PRODUCT INOCULATION

After being allowed to drip from the immersion treatment for at least 15 sec., individual slices (ca. 25-35g each) were aseptically placed into plastic pouches (one slice per pouch) for inoculation. Each slice was inoculated in the package by adding 0.1 ml of the *L. monocytogenes* cocktail inoculum in 0.01 ml droplets across the surface and then distributing it as a thin film over the surface. The inoculation technique resulted in an *L. monocytogenes* count of ca. 3,000 CFU/g.

E. PRODUCT STORAGE AND PACKAGING

Microbiological Samples.
For each treatment set (treated or untreated/control), each inoculated slice was vacuum packaged in the pouch. For each treatment variable, another set of samples was left uninoculated, placed in pouches (one slice per pouch), and vacuum packaged to monitor the effect of the Mirenat-N on aerobic plate counts (APC). For each treatment batch, three inoculated sample packages were analyzed for *L. monocytogenes* immediately after inoculation (i.e., time-0) and one uninoculated sample was analyzed for APC at time-0 (Section F). One set of remaining treated and untreated samples were stored at 4.4°C (40°F) and sampled after 1, 2, 4, 6, and 8 weeks of storage. Another set was stored at 15°C (59°F) and sampled after 1, 2, and 4 days. At each storage interval, three inoculated samples were analyzed for *L. monocytogenes* and one uninoculated sample analyzed for APC from each treatment/temperature combination (Section F).

Organoleptic Samples.
Uninoculated product slices (ca. 25-35g each) were aseptically placed into plastic pouches (three slices per pouch) and vacuum packaged. One sample each of treated and untreated samples were evaluated at time-0 and after 1, 2, 4, 6, and 8 weeks of storage at 4.4°C (40°F).

F. SAMPLE ANALYSES

Sample packages were opened aseptically and the samples along with any liquid placed in a sterile stomacher bag with in sterile Difco neutralizing broth to effect a 1:10 dilution. Samples were stomached for 1 min. and the homogenate serially diluted as required. *L. monocytogenes* was enumerated in inoculated sample homogenates via surface plating on MOX agar. The MOX plates were incubated at 35°C for 48 h and counted. For uninoculated samples, the samples homogenate was analyzed for Aerobic Plate Counts (APC) incubated at 22-24°C for 5 days (to include both mesophilic and psychrotrophic spoilage bacteria) using standard methods agar.

All results were expressed as colony forming units (CFU's) per gram of sample and converted to $\log_{10}$ transforms. Sample means and corresponding standard deviations were calculated for each sample set at each time interval. The initial treatment reduction was calculated from the initial mean counts of treated and untreated samples.

Organoleptic Samples.
Uninoculated treated and untreated samples were evaluated organoleptically at time-0 and after the designated storage times at 4.4°C. The organoleptic evaluations were conducted by a group of 6 panelists who were familiar with sensory evaluation of food products. Product was served in sample cups marked with random numbers that corresponded to numbers on the ballots. Water was provided to cleanse the pallet. The product was evaluated on color, aroma, and overall acceptability on a 7-point hedonic scale (i.e., 7 = liked extremely, 4 = neither liked/disliked, 1 = disliked extremely). Panelists' ratings were tabulated and averaged for each sample. The averages were statistically analyzed via Analysis of Variance to determine if there were significant differences (alpha = 0.05) between the means of the treated vs. untreated samples at each sampling point.

RESULTS:

Microbiological.
The results of the Mirenat-N treatment on *Listeria monocytogenes* inoculated onto sliced cured ham stored at 4.4° or 15°C are presented in Table 1. The Mirenat-N treatment resulted in an initial, mean *L. monocytogenes* reduction of 1.62 $\log_{10}$ units on the sliced cured ham (i.e., from 3.67 to 2.04 $\log_{10}$ CFU/g).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated cured ham samples after 1, 2, 4 and 6 weeks of storage at 4.4°C were 1.71, 1.96, 1.22 and 0.39 $\log_{10}$ units lower, respectively, than those of untreated samples. *L. monocytogenes* counts were similar on treated and untreated sample after 8 weeks of storage at 4.4°C. *L. monocytogenes* counts increased throughout 8 weeks of storage at 4.4°C in treated and untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 4 days of storage at 15°C in treated and untreated cured ham samples but were consistently lower on treated than on untreated samples at each

storage interval. Mean counts of *L. monocytogenes* on the treated samples after 1, 2 and 4 days storage at 15°C were 1.75, 1.64 and 1.00 $\log_{10}$ units lower, respectively, than those of untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, sliced cured ham subsequently stored at 4.4° or 15°C are presented in Table 2. The initial APC levels were very low in all cured ham samples. As compared to the untreated samples, the Mirenat-N treatment of the cured ham inhibited the growth of APC bacteria during storage at 4.4°C or 15°C. APC levels in treated samples were remained relatively low and were consistently much lower than those of corresponding untreated samples at each storage interval at 4.4°C. The APC on the treated sample was <10 CFU/g after 8 weeks of storage at 4.4°C whereas the APC of untreated samples evidenced some growth over 8 weeks of storage.

Organoleptic.
Results of organoleptic sample evaluations are presented in Table 3. There were no significant differences ($p<0.05$) between treated and untreated ham samples at any sampling point over 8 weeks of storage at 4.4°C.

SUMMARY:

Mirenat-N treatment of the sliced cured ham resulted in a significant reduction (i.e., 1.62 $\log_{10}$ units) of *L. monocytogenes* inoculated onto sliced cured ham. The Mirenat-N treatment also inhibited the growth of aerobic plate counts developing on the samples during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated sliced cured ham.

(b) (6)

PREPARED BY

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Table 1. Effect of Mirenat-N on *L. monocytogenes* inoculated onto Sliced Cured Ham Stored at 4.4° and 15°C.

Storage Time (days)		4.4°C Storage				15°C Storage			
		Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
		CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0	rep. 1	160	2.20	5,400	3.73	160	2.20	5,400	3.73
	rep. 2	120	2.08	4,300	3.63	120	2.08	4,300	3.63
	rep. 3	70	1.85	4,300	3.63	70	1.85	4,300	3.63
	Mean		**2.04**		**3.67**		**2.04**		**3.67**
	Std. Dev.		0.18		0.06		0.18		0.06
Initial Reduction			**1.62**				**1.62**		
1 (day)	rep. 1	--	--	--	--	140	2.15	6,800	3.83
	rep. 2	--	--	--	--	170	2.23	9,100	3.96
	rep. 3	--	--	--	--	100	2.00	7,200	3.86
	Mean	--	--	--	--		**2.13**		**3.88**
	Std. Dev.	--	--	--	--		0.12		0.07
2 (days)	rep. 1	--	--	--	--	1,360	3.13	74,000	4.87
	rep. 2	--	--	--	--	1,740	3.24	87,000	4.94
	rep. 3	--	--	--	--	3,100	3.49	99,000	5.00
	Mean	--	--	--	--		**3.29**		**4.93**
	Std. Dev.	--	--	--	--		0.18		0.06
4 (days)	rep. 1	--	--	--	--	2,300,000	6.36	28,000,000	7.45
	rep. 2	--	--	--	--	2,600,000	6.41	29,000,000	7.46
	rep. 3	--	--	--	--	4,300,000	6.63	31,000,000	7.49
	Mean	--	--	--	--		**6.47**		**7.47**
	Std. Dev.	--	--	--	--		0.14		0.02
1 (week)	rep. 1	50	1.70	2,400	3.38	--	--	--	--
	rep. 2	10	1.00	2,700	3.43	--	--	--	--
	rep. 3	230	2.36	2,400	3.38	--	--	--	--
	Mean		**1.69**		**3.40**	--	--	--	--
	Std. Dev.		0.68		0.03	--	--	--	--
2 (weeks)	rep. 1	260	2.41	22,700	4.36	--	--	--	--
	rep. 2	120	2.08	38,000	4.58	--	--	--	--
	rep. 3	660	2.82	19,000	4.28	--	--	--	--
	Mean		**2.44**		**4.40**	--	--	--	--
	Std. Dev.		0.37		0.16	--	--	--	--
4 (weeks)	rep. 1	5,700,000	6.76	24,100,000	7.38	--	--	--	--
	rep. 2	3,200,000	6.51	30,000,000	7.48	--	--	--	--
	rep. 3	420,000	5.62	48,000,000	7.68	--	--	--	--
	Mean		**6.29**		**7.51**	--	--	--	--
	Std. Dev.		0.59		0.15	--	--	--	--
6 (weeks)	rep. 1	43,000,000	7.63	134,000,000	8.13	--	--	--	--
	rep. 2	72,000,000	7.86	159,000,000	8.20	--	--	--	--
	rep. 3	67,000,000	7.83	137,000,000	8.14	--	--	--	--
	Mean		**7.77**		**8.16**	--	--	--	--
	Std. Dev.		0.12		0.04	--	--	--	--
8 (weeks)	rep. 1	160,000,000	8.20	25,000,000	7.40	--	--	--	--
	rep. 2	120,000,000	8.08	35,000,000	7.54	--	--	--	--
	rep. 3	93,000,000	7.97	34,000,000	7.53	--	--	--	--
	Mean		**8.08**		**7.49**	--	--	--	--
	Std. Dev.		0.12		0.08	--	--	--	--

Notes: 1) Counts expressed as CFU per gram of sample.
2) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples).

Table 2. Effect of Mirenat-N on Aerobic Plate Counts of Uninoculated onto Sliced Cured Ham Stored at 4.4° and 15°C.

Storage Time	4.4°C Storage				15°C Storage			
	Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0 (day)	<10	<1.00	<10	<1.00	<10	<1.00	<10	<1.00
1 (day)	--	--	--	--	20	1.30	<10	<1.00
2 (days)	--	--	--	--	20	1.30	<10	<1.00
4 (days)	--	--	--	--	<10	<1.00	<10	<1.00
1 (week)	<10	<1.00	<10	<1.00	--	--	--	--
2 (weeks)	<10	<1.00	<10	<1.00	--	--	--	--
4 (weeks)	20	1.30	9,600	3.98	--	--	--	--
6 (weeks)	440	2.64	19,800	4.30				
8 (weeks)	<10	<1.00	20	1.30				

Notes: 1) Counts expressed as CFU per gram of sample.

Table 3. Effect of Mirenat-N on Organoleptic Attributes of Sliced Cured Ham Stored at 4.4°C.

Attribute		Storage Time (weeks)											
		0		2		3		4		6		8	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Color	Average	5.17	5.50	4.83	3.83	4.00	3.50	3.67	3.50	4.17	3.17	4.17	4.25
	STD (s)	0.75	0.55	1.47	1.60	1.26	1.97	1.97	1.05	1.17	1.47	1.17	1.60
	min	4	5	3	1	2	1	1	2	3	1	3	2
	max	6	6	7	5	6	7	6	5	6	5	6	6.5
Aroma	Average	4.00	4.50	3.00	2.50	3.17	2.17	3.50	3.33	3.50	3.83	3.25	3.83
	STD (s)	1.55	2.07	1.10	1.38	1.17	0.98	2.07	0.82	0.84	1.33	1.99	1.72
	min	3	1	2	1	2	1	1	2	3	2	1	2
	max	7	7	5	4	5	4	6	4	5	6	6.5	6
Overall Acceptability													
	Average	4.17	4.50	3.50	3.00	3.33	2.50	3.17	3.33	3.83	3.67	3.50	4.08
	STD (s)	0.98	1.52	1.22	1.26	1.51	1.05	1.60	0.82	1.17	1.37	1.52	1.56
	min	3	2	2	1	2	1	1	2	3	2	2	2
	max	6	6	5	4	6	4	5	4	6	6	6	6.5

Note: asterisk (*) indicates significant difference ($p<0.05$).

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PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *Listeria monocytogenes* on Sliced Salami.

OBJECTIVE: To evaluate the antimicrobial efficacy of Mirenat-N in reducing or inhibiting levels of *Listeria monocytogenes* on sliced salami during subsequent storage at 4.4 or 15°C. The effect of the Mirenat-N treatment on the microbial shelf-life of uninoculated salami was also evaluated.

METHODS:

A. Products and Materials

The salami product was manufactured and sliced per standard commercial practices by the Columbus Salami Co. (CSC). Samples batches with Mirenat-N (Batch JMR-438; Exp. Date: 9/30/05) incorporated into the formulation (i.e., treated) at the rate of 1.0 g per Kg. Corresponding samples of product without Mirenat-N (i.e., untreated controls) were also prepared at the CSC facility. The salami samples were shipped in insulated containers with refrigerant to ABC Research Corp. via overnight courier. Samples were kept refrigerated (35°F) until used in the study (within 96 hrs).

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided to ABC Research by the client for an immersion treatment (i.e., 5% Mirenat-N solution in distilled water) of salami slices prior to inoculation and/or packaging.

B. Product Treatment

Just prior to inoculation, the salami slices (containing Mirenat-N) were topically treated by complete immersion (i.e., 30 sec. @ 22°C) in the Mirenat-N solution (5% in distilled water). A six liter bath of Mirenat-N solution was prepared for the immersion treatment of inoculated slices and a 4 liter bath for uninoculated slices. Excess solution was allowed to drip from the samples at least 15 sec. before inoculation and/or packaging. The control salami slices (without Mirenat-N) were not immersion treated prior to inoculation and/or packaging.

C TEST MICROORGANISMS

The following 5 strains of *Listeria monocytogenes* were used: ATCC 19115 (Serotype 4B), "Serotype ½a", "Serotype ½b", ATCC 7644 (Gibson) and ATCC 49594 (Scott A). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth with yeast extract (TSBYE) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was resuspended in BPB to obtain a cell suspension of approx. 1×10^8 CFU/ml. Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 40 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 5×10^5 CFU/ml.

D. PRODUCT INOCULATION AND PACKAGING

After being allowed to drip from the immersion treatment for at least 15 sec., individual slices (ca. 25-35g each) were aseptically placed into plastic pouches (one slice per pouch) for inoculation. Each slice was inoculated in the package by adding 0.1 ml of the *L. monocytogenes* cocktail inoculum in 0.01 ml droplets across the surface and then distributing it as a thin film over the surface. The inoculation technique resulted in an *L. monocytogenes* count of ca. 5,000 CFU/g. For each treatment set (treated or untreated/control), each inoculated slice was vacuum packaged in the pouch. For each treatment variable, another set of samples was left uninoculated, placed in pouches (one slice per pouch), and vacuum packaged to monitor the effect of the Mirenat-N on aerobic plate counts (APC).

E. PRODUCT STORAGE

For each treatment batch, three inoculated sample packages were analyzed for *L. monocytogenes* immediately after inoculation (i.e., time-0) and one uninoculated sample was analyzed for APC at time-0 (Section F). One set of remaining treated and untreated samples were stored at 4.4°C (40°F) and sampled after 1, 2, 4, 6, and 8 weeks of storage. Another set was stored at 15°C (59°F) and sampled after 1, 2, and 4 days. At each storage interval, three inoculated samples were analyzed for *L. monocytogenes* and one uninoculated sample analyzed for APC from each treatment/temperature combination (Section F).

F. SAMPLE ANALYSES

Sample packages were opened aseptically and the samples along with any liquid placed in a sterile stomacher bag with in sterile Difco neutralizing broth to effect a 1:10 dilution. Samples were stomached for 1 min. and the homogenate serially diluted as required. *L. monocytogenes* was enumerated in inoculated sample homogenates via surface plating on MOX agar. The MOX plates were incubated at 35°C for 48 h and counted. For uninoculated samples, the samples homogenate was analyzed for Aerobic Plate Counts (APC) incubated at 22-24°C for 5 days (to include both mesophilic and psychrotrophic spoilage bacteria) using standard methods agar.

All results were expressed as colony forming units (CFU's) per gram of sample and converted to $\log_{10}$ transforms. Sample means and corresponding standard deviations were calculated for each sample set at each time interval. The initial treatment reduction was calculated from the initial mean counts of treated and untreated samples.

RESULTS:

The results of the Mirenat-N treatment on *Listeria monocytogenes* inoculated onto sliced salami stored at 4.4° or 15°C are presented in Table 1. The Mirenat-N treatment resulted in an initial, mean *L. monocytogenes* reduction of greater than 2.64 $\log_{10}$ units on the sliced salami (i.e., from 3.64 to <1.00 $\log_{10}$ CFU/g).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 8 weeks of storage at 4.4°C on treated and untreated salami samples but were consistently much lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on treated samples after 1, 2, 4, 6 and 8 weeks of storage at 4.4°C were >2.36, 3.94, 2.58, 1.83 and 0.98 $\log_{10}$ units lower, respectively, than those of untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, *L. monocytogenes* counts increased during 4 days of storage at 15°C in treated and untreated salami samples but were consistently much lower on treated than on untreated samples at each storage interval. Mean counts of *L. monocytogenes* on the treated sliced salami samples after 1, 2 and 4 days storage at 15°C were 2.52, 3.17 and 2.22 $\log_{10}$ units lower, respectively, than those of untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, sliced salami subsequently stored at 4.4° or 15°C are presented in Table 2. The APC levels were relatively low in all salami samples initially and throughout storage with no evidence of growth on treated or untreated samples at either temperature.

SUMMARY:

Mirenat-N treatment of the sliced salami resulted in a significant reduction (i.e., >2.64 $\log_{10}$ units) of *L. monocytogenes* inoculated onto sliced salami. *L. monocytogenes* levels were consistently much lower on treated than on untreated samples during storage at 4.4° or 15°C.

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Table 1. Effect of Mirenat-N on *L. monocytogenes* inoculated onto Sliced Salami Stored at 4.4° and 15°C.

Storage Time (days)		4.4°C Storage				15°C Storage			
		Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
		CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0	rep. 1	<10	<1.00	4,400	3.64	<10	<1.00	4,400	3.64
	rep. 2	<10	<1.00	3,700	3.57	<10	<1.00	3,700	3.57
	rep. 3	10	1.00	5,000	3.70	10	1.00	5,000	3.70
	Mean		**<1.00**		**3.64**		**<1.00**		**3.64**
	Std. Dev.		0.24		0.10		0.24		0.10
Initial Reduction			**>2.64**				**>2.64**		
1 (day)	rep. 1	--	--	--	--	<10	<1.00	3,300	3.52
	rep. 2	--	--	--	--	20	1.30	3,300	3.52
	rep. 3	--	--	--	--	<10	<1.00	6,800	3.83
	Mean	--	--	--	--		**1.10**		**3.62**
	Std. Dev.	--	--	--	--		0.17		0.18
2 (days)	rep. 1	--	--	--	--	530	2.72	199,000	5.30
	rep. 2	--	--	--	--	120	2.08	460,000	5.66
	rep. 3	--	--	--	--	230	2.36	530,000	5.72
	Mean	--	--	--	--		**2.39**		**5.56**
	Std. Dev.	--	--	--	--		0.32		0.23
4 (days)	rep. 1	--	--	--	--	1,500,000	6.18	178,000,000	8.25
	rep. 2	--	--	--	--	410,000	5.61	171,000,000	8.23
	rep. 3	--	--	--	--	980,000	5.99	91,000,000	7.96
	Mean	--	--	--	--		**5.93**		**8.15**
	Std. Dev.	--	--	--	--		0.29		0.16
1 (week)	rep. 1	<10	<1.00	2,020	3.31	--	--	--	--
	rep. 2	<10	<1.00	2,140	3.33	--	--	--	--
	rep. 3	<10	<1.00	2,800	3.45	--	--	--	--
	Mean		**<1.00**		**3.36**	--	--	--	--
	Std. Dev.		0.00		0.08	--	--	--	--
2 (weeks)	rep. 1	10	1.00	520,000	5.72	--	--	--	--
	rep. 2	120	2.08	570,000	5.76	--	--	--	--
	rep. 3	210	2.32	550,000	5.74	--	--	--	--
	Mean		**1.80**		**5.74**	--	--	--	--
	Std. Dev.		0.70		0.02	--	--	--	--
4 (weeks)	rep. 1	46,000	4.66	32,000,000	7.51	--	--	--	--
	rep. 2	32,000	4.51	41,000,000	7.61	--	--	--	--
	rep. 3	610,000	5.79	37,000,000	7.57	--	--	--	--
	Mean		**4.98**		**7.56**	--	--	--	--
	Std. Dev.		0.70		0.05	--	--	--	--
6 (weeks)	rep. 1	6,000,000	6.78	290,000,000	8.46	--	--	--	--
	rep. 2	3,400,000	6.53	260,000,000	8.41	--	--	--	--
	rep. 3	4,100,000	6.61	330,000,000	8.52	--	--	--	--
	Mean		**6.64**		**8.47**	--	--	--	--
	Std. Dev.		0.13		0.05	--	--	--	--
8 (weeks)	rep. 1	22,600,000	7.35	300,000,000	8.48	--	--	--	--
	rep. 2	25,000,000	7.40	290,000,000	8.46	--	--	--	--
	rep. 3	49,000,000	7.69	280,000,000	8.45	--	--	--	--
	Mean		**7.48**		**8.46**	--	--	--	--
	Std. Dev.		0.18		0.01	--	--	--	--

Notes: 1) Counts expressed as CFU per gram of sample.
2) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples).

Table 2. Effect of Mirenat-N on Aerobic Plate Counts of Uninoculated onto Sliced Salami Stored at 4.4° and 15°C.

Storage Time	4.4°C Storage				15°C Storage			
	Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0 (day)	630	2.80	<10	<1.00	630	2.80	<10	<1.00
1 (day)	--	--	--	--	30	1.48	10	1.00
2 (days)	--	--	--	--	50	1.70	10	1.00
4 (days)	--	--	--	--	160	2.20	10	1.00
1 (week)	<10	<1.00	10	1.00	--	--	--	--
2 (weeks)	<10	<1.00	20	1.30	--	--	--	--
4 (weeks)	<10	<1.00	20	1.30	--	--	--	--
6 (weeks)	<10	<1.00	<10	<1.00				
8 (weeks)	<10	<1.00	<10	<1.00				

Notes: 1) Counts expressed as CFU per gram of sample.

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PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *Listeria monocytogenes* on Frankfurters.

OBJECTIVE: To evaluate the antimicrobial efficacy of Mirenat-N in reducing or inhibiting levels of *Listeria monocytogenes* on frankfurters during subsequent storage at 4.4 or 15°C. The effect of the Mirenat-N treatment on the microbial shelf-life of uninoculated frankfurters was also evaluated.

METHODS:

A. Products and Materials

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided by the client. ABC Research obtained ingredients for standard frankfurters (e.g., fresh pork, spices, salt and cure) locally in the Gainesville area. Perishable ingredients were kept refrigerated (35°F) for up to 48 h until used in the study. The treated and untreated frankfurter batter was formulated, stuffed into casing and cooked per standard commercial practices at the ABC Research Corporation pilot plant facility. The Mirenat-N was incorporated into the raw frankfurter batter as well as prepared as a 5% solution in distilled water for immersion treatment of finished frankfurters prior to inoculation and/or packaging.

B. Product Preparation and Treatment

For the treated batch of frankfurters, sufficient frankfurter batter was formulated with Mirenat-N to fabricate approximately 100 frankfurters (ca. 1.6 oz. or 45 g finished weight per frank). The Mirenat-N was thoroughly incorporated into the batter at the rate of 0.7 g per Kg (or 0.07%). Another batch of frankfurter batter (sufficient for 100 frankfurters as above) was formulated without Mirenat-N and was designated as the untreated control batch. Frankfurters were stuffed into casing and cooked per standard commercial practice. Cooked franks were chilled and kept refrigerated until subjected to further treatments. The casings were removed from the frankfurters prior to further treatment.

Just prior to inoculation, the frankfurters containing Mirenat-N were topically treated by complete immersion (i.e., 30 sec. @ 22°C) in the Mirenat-N solution (5% in distilled water). A six liter bath of Mirenat-N solution was prepared for the immersion treatment. Excess solution was allowed to drip from the samples at least 15 sec. before inoculation and/or packaging. The control frankfurters (without Mirenat-N) were not immersion treated prior to inoculation and/or packaging.

C TEST MICROORGANISMS

The following 5 strains of *Listeria monocytogenes* were used: ATCC 19115 (Serotype 4B), "Serotype ½a", "Serotype ½b", ATCC 7644 (Gibson) and ATCC 49594 (Scott A). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth with yeast extract (TSBYE) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was resuspended in BPB to obtain a cell suspension of approx. 1×10^8 CFU/ml. Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1×10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 40 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 10^6 CFU/ml.

D. PRODUCT INOCULATION AND PACKAGING

After being allowed to drip from the immersion treatment for at least 15 sec., individual franks were inoculated by adding 0.1 ml of the *L. monocytogenes* cocktail inoculum (ca. 10^6 CFU/ml) in 0.01 ml droplets across the surface and then distributing it as a thin film over the surface. The inoculation technique resulted in an *L. monocytogenes* count of ca. 10,000 CFU/ml of sample rinsate (or ca. 5,000 CFU/g assuming ca. 50 g per frank). For each treatment set (treated or untreated/control), the inoculated frankfurters were then vacuum packaged (two franks per package). For each treatment variable, another set of franks was left uninoculated and likewise vacuum packaged to monitor the effect of the Mirenat-N on aerobic plate counts (APC).

E. PRODUCT STORAGE

For each treatment batch, three inoculated sample packages were analyzed for *L. monocytogenes* immediately after inoculation (i.e., time-0) and one uninoculated sample was analyzed for APC at time-0 (Section F). One set of remaining treated and untreated samples were stored at 4.4°C (40°F) and sampled after 1, 2, 4, and 6 weeks of storage. Another set was stored at 15°C (59°F) and sampled after 1, 2 and 4 days. At each storage interval, three inoculated samples were analyzed for *L. monocytogenes* and one uninoculated sample analyzed for APC from each treatment/temperature combination (Section F)

F. SAMPLE ANALYSES

Frankfurters were sampled using a surface-rinse method basically following USDA procedures. The franks in each package (along with any liquid) were placed in a sterile stomacher bag with 50 ml of in sterile Difco neutralizing broth. Samples were thoroughly shaken and massaged by hand for 1 minute and the rinsate serially diluted in BPB as required. *L. monocytogenes* was enumerated in inoculated sample rinsates via surface plating on MOX agar. The MOX plates were incubated at 35°C for 48 h and counted. For uninoculated samples, the samples rinsate was analyzed for Aerobic Plate Counts (APC) incubated at 22-24°C for 5 days (to include both mesophilic and psychrotrophic spoilage bacteria) using standard methods agar.

All results were expressed as colony forming units (CFU's) per ml of sample rinsate and converted to $\log_{10}$ transforms. Sample means and corresponding standard deviations were calculated for each sample set at each time interval. The initial treatment reduction was calculated from the initial mean counts of treated and untreated samples.

RESULTS:

The results of the Mirenat-N treatment on *Listeria monocytogenes* inoculated onto frankfurters stored at 4.4° or 15°C are presented in Table 1. The Mirenat-N treatment resulted in an initial, mean *L. monocytogenes* reduction of 3.33 $\log_{10}$ units on the frankfurters (i.e., from 4.58 to 1.25 $\log_{10}$ CFU/ml of sample rinse).

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated frankfurters samples after 1, 2 and 4 weeks of storage at 4.4°C were 3.84, 3.29 and 1.55 $\log_{10}$ units lower, respectively, than those of untreated samples. *L. monocytogenes* counts were similar on treated and untreated sample by 6 weeks of storage at 4.4°C. *L. monocytogenes* counts increased throughout 6 weeks of storage at 4.4°C in treated and untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated frankfurters samples after 1, 2 and 4 days storage at 15°C were 3.34, 2.57 and 1.22 $\log_{10}$ units lower, respectively, than those of untreated samples. *L. monocytogenes* counts increased throughout 4 days of storage at 15°C in treated and untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, frankfurters subsequently stored at 4.4° or 15°C are presented in Table 2. The Mirenat-N treatment resulted in an initial APC reduction of ca. 1.3 $\log_{10}$ units on the frankfurters (i.e., from 2.41 to 1.11 $\log_{10}$ CFU/ml of rinsate). As compared to the untreated samples, the Mirenat-N treatment of the frankfurters inhibited the growth of APC bacteria during storage at 4.4°C. APC levels in treated samples were consistently much lower than those of corresponding untreated samples at each storage interval for either temperature.

SUMMARY:

Mirenat-N treatment of the frankfurters resulted in a significant reduction (i.e., 3.33 $\log_{10}$ units) of *L. monocytogenes* inoculated onto frankfurters. The Mirenat-N treatment also resulted an initial reduction and inhibition of aerobic plate counts developing on the frankfurters during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated frankfurters.

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Table 1. Effect of Mirenat-N on *L. monocytogenes* inoculated onto Frankfurters Stored at 4.4° and 15°C.

Storage Time (days)		4.4°C Storage				15°C Storage			
		Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
		CFU/ml	Log_{10} CFU/ml	CFU/ml	Log_{10} CFU/ml	CFU/ml	Log_{10} CFU/ml	CFU/ml	Log_{10} CFU/ml
0	rep. 1	10	1.00	46,000	4.66	10	1.00	46,000	4.66
	rep. 2	30	1.48	41,000	4.61	30	1.48	41,000	4.61
	rep. 3	19	1.28	29,000	4.46	19	1.28	29,000	4.46
	Mean		**1.25**		**4.58**		**1.25**		**4.58**
	Std. Dev.		0.24		0.10		0.24		0.10
Initial Reduction			**3.33**				**3.33**		
1 (day)	rep. 1	--	--	--	--	71	1.85	144,000	5.16
	rep. 2	--	--	--	--	57	1.76	183,000	5.26
	rep. 3	--	--	--	--	179	2.25	280,000	5.45
	Mean	--	--	--	--		**1.95**		**5.29**
	Std. Dev.	--	--	--	--		0.26		0.15
2 (days)	rep. 1	--	--	--	--	44,000	4.64	9,500,000	6.98
	rep. 2	--	--	--	--	21,100	4.32	7,600,000	6.88
	rep. 3	--	--	--	--	8,000	3.90	5,300,000	6.72
	Mean	--	--	--	--		**4.29**		**6.86**
	Std. Dev.	--	--	--	--		0.37		0.13
4 (days)	rep. 1	--	--	--	--	12,400,000	7.09	82,000,000	7.91
	rep. 2	--	--	--	--	3,200,000	6.51	113,000,000	8.05
	rep. 3	--	--	--	--	4,000,000	6.60	75,000,000	7.88
	Mean	--	--	--	--		**6.73**		**7.95**
	Std. Dev.	--	--	--	--		0.32		0.09
1 (week)	rep. 1	4	0.60	71,000	4.85	--	--	--	--
	rep. 2	12	1.08	65,000	4.81	--	--	--	--
	rep. 3	16	1.20	55,000	4.74	--	--	--	--
	Mean		**0.96**		**4.80**	--	--	--	--
	Std. Dev.		0.32		0.06	--	--	--	--
2 (weeks)	rep. 1	260	2.41	1,650,000	6.22	--	--	--	--
	rep. 2	4,400	3.64	2,670,000	6.43	--	--	--	--
	rep. 3	640	2.81	1,200,000	6.08	--	--	--	--
	Mean		**2.95**		**6.24**	--	--	--	--
	Std. Dev.		0.63		0.17	--	--	--	--
4 (weeks)	rep. 1	4,300,000	6.63	75,000,000	7.88	--	--	--	--
	rep. 2	1,280,000	6.11	81,000,000	7.91	--	--	--	--
	rep. 3	3,600,000	6.56	147,000,000	8.17	--	--	--	--
	Mean		**6.43**		**7.98**	--	--	--	--
	Std. Dev.		0.28		0.16	--	--	--	--
6 (weeks)	rep. 1	129,000,000	8.11	35,000,000	7.54	--	--	--	--
	rep. 2	400,000,000	8.60	226,000,000	8.35	--	--	--	--
	rep. 3	88,000,000	7.94	219,000,000	8.34	--	--	--	--
	Mean		**8.22**		**8.08**	--	--	--	--
	Std. Dev.		0.34		0.46	--	--	--	--

Notes: 1) Counts expressed as CFU per ml of sample rinsate.
2) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples).

Table 2. Effect of Mirenat-N on Aerobic Plate Counts of Uninoculated Frankfurters Stored at 4.4° and 15°C.

Storage Time	4.4°C Storage				15°C Storage			
	Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
	CFU/ml	Log_{10} CFU/ml	CFU/ml	Log_{10} CFU/ml	CFU/ml	Log_{10} CFU/ml	CFU/ml	Log_{10} CFU/ml
0 (day)	13	1.11	260	2.41	13	1.11	260	2.41
1 (day)	--	--	--	--	6	0.78	12,100	4.08
2 (days)	--	--	--	--	10	1.00	3,500,000	6.54
4 (days)	--	--	--	--	1,220,000	6.09	89,000,000	7.95
1 (week)	4,100	3.61	80,000	4.90	--	--	--	--
2 (weeks)	8	0.90	17,280,000	7.24	--	--	--	--
4 (weeks)	1,560,000	6.19	106,000,000	8.03	--	--	--	--
6 (weeks)	51,000,000	7.71	122,000,000	8.09				

Notes: 1) Counts expressed as CFU per ml of sample rinsate.

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PROJECT REPORT
RESEARCH MICROBIOLOGY DEPARTMENT

DATE: March 16, 2005

PREPARED FOR: Laboratorios Miret S.A. (LAMIRSA)

CLIENT CONTACT: Xavier Rocabayera (LAMIRSA)

PROJECT TITLE: Antimicrobial Efficacy of Mirenat-N against *Listeria monocytogenes* in Ready-to-Eat Chicken Salad.

OBJECTIVE: To evaluate the antimicrobial efficacy of Mirenat-N in reducing or inhibiting levels of *Listeria monocytogenes* in ready-to-eat chicken salad during subsequent storage at 4.4 or 15°C. The effect of the Mirenat-N treatment on the microbial shelf-life of uninoculated chicken salad was also evaluated.

METHODS:

A. Products and Materials

Mirenat-N (Batch JMR-409; Exp. Date: July 29, 2005) was provided by the client and prepared as a 5% solution in distilled water for immersion treatment of chicken salad ingredients. ABC Research obtained ingredients for the chicken salad (i.e., fresh chicken breasts, fresh lettuce, fresh onion, fresh carrots, and cooked corn) locally in the Gainesville area. Ingredients were kept refrigerated (35°F) for up to 48 h until used in the study.

B. Product Preparation and Treatment

The chicken salad was formulated as follows: 75% cooked/diced chicken, 10% diced lettuce, 5% diced onion, 5% diced carrot and 5% cooked corn. The chicken breasts were cooked via a standard baking procedure, cooled and kept refrigerated (ca. 18 h) prior to preparation of the salad. The ingredients for a 4,000 g batch of chicken salad and derived sub-batches (i.e., treated and control) were as follows.

Ingredient	Fabrication	Total Needed	Treated Batch	Control Batch
cooked chicken	dice into ca. ¼ in. cubes	3,000 g	1,500 g	1,500 g
lettuce	dice into ca. ¼ in. pieces	400 g	200 g	200 g
onion	dice into ca. ¼ in. pieces	200 g	100 g	100 g
carrot	dice into ca. ¼ in. pieces	200 g	100 g	100 g
corn (canned)	drained	200 g	100 g	100 g

A 6 liter bath of Mirenat-N solution was prepared for ingredient treatment. For the treated batch, the designated amount of each ingredient (e.g., 1,500 g of diced chicken, 200 g lettuce, etc.) was treated by complete immersion (i.e., 30 sec. @ 22°C) in the Mirenat-N solution (5% in distilled water) using a sanitized colander. Excess solution was drained from the samples in the colander (e.g., ca. 15 sec.) before further salad fabrication. The treated ingredients were then combined and thoroughly mixed.

For the control (untreated) batch, the ingredients were immersed in distilled water (using the same procedure applied with the Mirenat-N solution) to simulate normal washing of the ingredients. The control ingredients were then drained, combined and thoroughly mixed.

C TEST MICROORGANISMS

The following 5 strains of *Listeria monocytogenes* were used: ATCC 19115 (Serotype 4B), "Serotype ½a", "Serotype ½b", ATCC 7644 (Gibson) and ATCC 49594 (Scott A). Each strain was grown separately via at least 2 serial transfers at 35°C for 24 hr in Tryptic Soy Broth with yeast extract (TSBYE) broth. Bacterial cells for each culture were harvested by centrifugation at 10,000 x g for 10 min. and washed with Butterfield's Phosphate Buffer, pH 7.2 (BPB). Each culture was resuspended in BPB to obtain a cell suspension of approx. 1 x 10^8 CFU/ml. Each culture was then resuspended and combined in BPB to prepare a cocktail with an approximate cell density of 1 x 10^8 CFU/ml for each strain. The final inoculum cocktail (ca. 50 ml) was prepared by diluting that mixed suspension in sterile BPB to obtain an approximate cell density of 10^5 CFU/ml.

D. PRODUCT INOCULATION

For each batch of chicken salad (i.e., treated or untreated), a portion (1,500 g each) was inoculated by adding and thoroughly mixing the final *L. monocytogenes* inoculum into the product at the rate of 1.0 ml per 100 g of salad. This inoculation technique was designed to obtain a target *L. monocytogenes* count of ca. 1,000 CFU/g of product. Another portion (i.e., 500 g each) of each batch of chicken salad (i.e., treated or untreated), was left uninoculated to monitor the effect of the Mirenat-N on aerobic plate counts.

E. PRODUCT PACKAGING AND STORAGE

Using sanitary procedures, inoculated and uninoculated portions (i.e. 40 g each) from each treatment batch were placed in a sterile plastic containers (screw cap) for storage. For each treatment batch, three inoculated samples were analyzed for *L. monocytogenes* immediately after inoculation (i.e., time-0) and one uninoculated sample was analyzed for APC at time-0 (Section F, below). One set of remaining treated and untreated samples were stored at 4.4°C (40°F) and sampled after 3, 6, 9 and 12 days of storage. Another set was stored at 15°C (59°F) and sampled after 1, 2 and 4 days. At each storage interval, three inoculated samples were analyzed for *L. monocytogenes* and one uninoculated sample analyzed for APC from each treatment/temperature combination (Section F, below).

F. SAMPLE ANALYSES

Samples were microbiologically analyzed by aseptically placing a 25 g aliquot in a sterile stomacher bag, diluting the aliquot 1:10 in sterile Difco neutralizing broth, and stomaching for 1 min. The *L. monocytogenes* was enumerated/analyzed in respective sample homogenates using a direct plating method (MOX agar). The MOX plates were incubated at 35°C for 48 h and counted. For uninoculated samples a 25 g aliquot was likewise sampled and analyzed for Aerobic Plate Counts (APC) incubated at 22-24°C for 5 days (to include both mesophilic and psychrotrophic spoilage bacteria) using standard methods agar.

All results were expressed as colony forming units (CFU's) per gram and converted to $\log_{10}$ transforms. Sample means and corresponding standard deviations were calculated for each sample set at each time interval. The initial treatment reduction was calculated from the initial mean count of untreated samples and the initial mean count of treated samples.

RESULTS:

The results of the Mirenat-NT treatment on *Listeria monocytogenes* inoculated into ready-to-eat chicken salad stored at 4.4° or 15°C are presented in Table 1. The Mirenat-N treatment of the product ingredients resulted in an initial, mean *L. monocytogenes* reduction of greater than 1.61 $\log_{10}$ CFU/g (i.e., from 2.61 to <1.00 $\log_{10}$ CFU/g) in the chicken salad.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated chicken salad samples after 3, 6, 9 and 12 days storage at 4.4°C were 1.61, 0.54, 0.29 and 0.46 $\log_{10}$ units lower, respectively, than those of untreated samples. *L. monocytogenes* counts increased throughout 12 days of storage at 4.4°C in treated and untreated samples.

After the initial reduction of *L. monocytogenes* by the Mirenat-N treatment, the mean counts of the treated chicken salad samples after 1 and 2 days storage at 15°C were 2.20 and 0.76 $\log_{10}$ units lower, respectively, than those of untreated samples. *L. monocytogenes* counts were similar on treated and untreated sample by 4 days of storage at 15°C. *L. monocytogenes* counts increased throughout 4 days of storage at 15°C in treated and untreated samples.

The results of the Mirenat-N immersion treatment on the Aerobic Plate Counts (APC) of uninoculated, chicken salad subsequently stored at 4.4° or 15°C are presented in Table 2. The Mirenat-N treatment resulted in an initial APC reduction of 2.57 $\log_{10}$ units in the chicken salad (i.e., from 6.21 to 3.64 $\log_{10}$ CFU/g). As compared to the untreated samples, the Mirenat-N treatment of the salad ingredients inhibited the growth of APC bacteria during storage at 4.4°C particularly during the first 3 days of storage. APC levels in treated samples and untreated samples were 42,000and 4,900,000 CFU/g, respectively, after 3 days of storage at 4.4°C.

SUMMARY:

Mirenat-N treatment of the product ingredients resulted in a significant reduction (i.e., >1.61 $\log_{10}$ CFU/g) of *L. monocytogenes* inoculated into ready-to-eat chicken salad. The Mirenat-N treatment also resulted in inhibition of aerobic plate counts developing in the chicken salad during storage at 4.4°C and thus may have the potential to extend the microbial shelf-life of refrigerated ready to eat chicken salad.

(b) (6)

PREPARED BY

James E. (Ken) Kennedy, Ph.D.
Vice President, Research Microbiology
ABC Research Corp.

Table 1. Effect of Mirenat-N on *L. monocytogenes* in Chicken Salad Stored at 4.4° and 15°C.

Storage Time (days)		4.4°C Storage				15°C Storage			
		Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
		CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0	rep. 1	10	1.00	610	2.79	10	1.00	610	2.79
	rep. 2	<10	<1.00	490	2.69	<10	<1.00	490	2.69
	rep. 3	<10	<1.00	220	2.34	<10	<1.00	220	2.34
	Mean		**<1.00**		**2.61**		**<1.00**		**2.61**
	Std. Dev.		0.00		0.23		0.00		0.23
Initial Reduction			**>1.61**				**>1.61**		
1	rep. 1	--	--	--	--	10	1.00	2,500	3.40
	rep. 2	--	--	--	--	10	1.00	2,800	3.45
	rep. 3	--	--	--	--	50	1.70	2,700	3.43
	Mean	--	--	--	--		**1.23**		**3.43**
	Std. Dev.	--	--	--	--		0.40		0.03
2	rep. 1	--	--	--	--	990	3.00	5,100	3.71
	rep. 2	--	--	--	--	860	2.93	3,400	3.53
	rep. 3	--	--	--	--	490	2.69	4,500	3.65
	Mean	--	--	--	--		**2.87**		**3.63**
	Std. Dev.	--	--	--	--		0.16		0.09
3	rep. 1	<10	<1.00	530	2.72	--	--	--	--
	rep. 2	20	1.30	370	2.57	--	--	--	--
	rep. 3	<10	<1.00	710	2.85	--	--	--	--
	Mean		**1.10**		**2.71**	--	--	--	--
	Std. Dev.		0.17		0.14	--	--	--	--
4	rep. 1	--	--	--	--	267,000	5.43	228,000	5.36
	rep. 2	--	--	--	--	234,000	5.37	132,000	5.12
	rep. 3	--	--	--	--	114,000	5.06	127,000	5.10
	Mean	--	--	--	--		**5.28**		**5.19**
	Std. Dev.	--	--	--	--		0.20		0.14
6	rep. 1	470	2.67	1,580	3.20	--	--	--	--
	rep. 2	240	2.38	1,420	3.15	--	--	--	--
	rep. 3	460	2.66	960	2.98	--	--	--	--
	Mean		**2.57**		**3.11**	--	--	--	--
	Std. Dev.		0.17		0.11	--	--	--	--
9	rep. 1	2,050	3.31	3,100	3.49	--	--	--	--
	rep. 2	1,220	3.09	2,500	3.40	--	--	--	--
	rep. 3	1,260	3.10	3,000	3.48	--	--	--	--
	Mean		**3.17**		**3.46**	--	--	--	--
	Std. Dev.		0.13		0.05	--	--	--	--
12	rep. 1	2,600	3.41	7,600	3.88	--	--	--	--
	rep. 2	10,000	4.00	11,900	4.08	--	--	--	--
	rep. 3	2,100	3.32	14,200	4.15	--	--	--	--
	Mean		**3.58**		**4.04**	--	--	--	--
	Std. Dev.		0.37		0.14	--	--	--	--

Notes: 1) Initial Reduction = (mean count of untreated samples) – (mean count of treated samples).
2) Undetectable counts (i.e., <1 CFU/g) were converted to 1 CFU, respectively, for conversion to log_{10} values.

Table 2. Effect of Mirenat-N Treatment on Aerobic Plate Counts of Uninoculated, Chicken Salad Stored at 4.4° and 15°C.

Storage Time (days)	4.4°C Storage				15°C Storage			
	Mirenat-N Treated		Untreated		Mirenat-N Treated		Untreated	
	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0	4,400	3.64	1,640,000	6.21	4,400	3.64	1,640,000	6.21
1	--	--	--	--	1,100,000	6.04	64,000,000	7.81
2	--	--	--	--	8,600,000	6.93	17,000,000	7.23
3	42,000	4.62	4,900,000	6.69	--	--	--	--
4	--	--	--	--	92,000,000	7.96	47,000,000	7.67
6	61,000,000	7.79	104,000,000	8.02	--	--	--	--
9	1,250,000,000	9.10	1,280,000,000	9.11	--	--	--	--
12	690,000,000	8.84	980,000,000	8.99				

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27/01/05 Rev. 3

TECHNICAL REPORT OF EFFICACY TESTS

LAURIC ARGINATE AS A PRESERVATIVE FOR FRESH TOMATO SAUCE

Internal study reference: VED-EC-09

Es conforme con las exigencias de la Norma Española UNE EN ISO 9001 2000

000137

Grupo LAMIRSA

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Quality Assurance Department:

Roger Segret Principal Auditor	Date

Experimental Start Date: 24/11/03

Experimental Completion Date: 24/03/04

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Terms Glossary

- **LAE** : Lauric arginate (Chemical name: Ethyl-N^{α}-lauroyl-*L*-arginine HCl).
- **LAS** : N^{α}-lauroyl-*L*-arginine. The hydrolysis product of LAE.
- **MIRENAT-N** : is a formulation of LAE with propylene glycol and citric acid.
- **n.d.** : not detected.

Introduction

Nowadays ready-to-eat products are having an increasing demand and consequently the food industry has raised the production of these new type of products. Fresh tomato sauce is one of the most common ingredients in ready-to-eat products. Specifically, it is the essential ingredient in most Savoury Toppings or Fillings for ready-to-eat products. These products require that the tomato sauce is obtained in the most traditional way as possible in order to maintain the freshness of the original product, avoiding taste problems as high acidity, high level of salt, etc. Other taste problems may come related to very strong heat treatments (as sterilization in cans or glass containers). Lately fresh tomato sauce is being stored at refrigerating temperatures and with modified atmosphere so final organoleptic characteristics are improved.

All these changes imply as it is logical a loss in the capacity of tomato sauce to be self-preserved. Fresh tomato sauce must be stored at refrigerating temperatures and this is one of the hazard points where fresh tomato sauce can be spoiled. This spoilage implies a microbiological alteration that cannot be tolerated by the consumer. To minimize microbiological risk, producer must have the assurance of a preservative.

In accordance with the directive 95/2/EC the use of sorbic acid and benzoic acid is permitted in non emulsionated sauces at doses up to 1000 mg/kg as a whole.

Objective of application of Lauric arginate

The aim of this study is to demonstrate how LAE could preserve fresh tomato sauce without affecting its technological characteristics.

Experimental procedure

Collaborator companies

The company that collaborated in this study was:

SALAONS I DERIVATS SL
C/Castelló, 25-27
43500 TORTOSA
TARRAGONA
Tlf. 977.50.43.64
NIF: B-43 542 620
The contact person: Sr. Ferran Loras.

SALAONS I DERIVATS SL produced and packaged fresh tomato sauces with different treatments.

Industrial process and ingredients

The industrial process was carried out according to the regular manufacture practises that are used in this sector.
Raw tomato was stored in refrigerating containers for 1-2 days. After that period, tomatoes were washed externally with water. Then, they were cut in two parts and seeds were removed manually. Tomato was then cooked for 1.5-2 hours at low heat. During the cooking, salt and sugar were added in the appropriate doses depending on the type of tomato. Finally, tomato sauce was sieved to eliminate skin residues. The final product was packaged in plastic containers (50 g of product per container) in modified atmosphere.
Different treatments were carried out at the end of the cooking process. These treatments were as follow:

Treatments

Three lots of fresh tomato sauce were produced.

1. The control lot, wasn't treated **BI**
2. The second lot was treated with 200 ppm of LAE **MN1**
3. While the third lot was treated with 1000 mg/kg of sorbic acid **S1**

Sorbic acid dose was the maximum level permitted by law.

Details of the Lauric arginate used

MIRENAT-N is a formulation of LAE with propylene glycol and citric acid. The LAE used was batch Nº 6420 and the MIRENAT-N was referenced as JMR-314. The analytical results were:

LAE (batch Nº 6420):

LAE	74.9%	ID-11-1697, rev.4
LAS	1.1%	ID-11-1697, rev.4
Lauric acid	2.1 %	ID-11-2004, rev.2
Ethyl laurate	1.4 %	ID-11-2004, rev.2
Ashes (700 ºC)	1.7 %	ID-11-083, rev.2
Water	15.3%	ID-11-020, rev.4

MIRENAT-N (ref. JMR-314)

LAE	20.26 %	ID-11-1697, rev.4
Aspect	Clear liquid	
Density	1.047 g/cm^3	ID-11-030
Viscosity (Brookfield, spindle RV2, 50 rpm, 20ºC)	88 mPa·s	ID-11-029
Index of refraction (20ºC)	1.443	
pH (solution 1% H_2O deionized, 20ºC)	3.33	

The methods ID-11-1697 rev.4 and ID-11-2004, rev.2 have been validated by an external laboratory, NOTOX.

Storage conditions

Storage temperature for the three lots was 0-5ºC during 25 days that was the period of study. In order to accelerate the study some breaks of refrigerating temperatures were applied. This graphic/calendar shows when refrigeration breaks were produced:

Mon	Tue	Wed	Thu	Fry	Sat	Sun
	1			4		
		7				
		11				

Breaks of refrigeration, 8 h 25ºC

Shaded areas are days on which there were breaks in refrigeration when the temperature of the lots was raised to 25ºC for eight hours. Samples were taken after the 1st, 4th, 7th and 11th breaks of refrigeration.

Parameters analysed and frequency

Parameters analysed were:

- ✓ organoleptic (general aspect, odour and colour).
- ✓ pH
- ✓ aerobes mesophiles (ID-11-1031, buffered peptone water was used to make dilutions, growth was carried out with TSA agar at 35ºC for 48 hours).
- ✓ yeasts (ID-11-1531, buffered peptone water was used to make dilutions, growth was carried out with Rose Bengale agar with chloramfenicol at 25ºC for 3-5 days).
- ✓ concentration of LAE (ID-11-2493)

All these parameters were analysed in LAMIRSA laboratories.
One sample per treatment was analysed per day of analysis (JMR-318).

Results

Aspect results

8 h, 25ºC	General Aspect
BI	1st break. Fresh tomato sauce has good aspect, red color, good flavour and it shows a little sineresys. 4th break. Idem. 7th break. Idem. 11th break. Package is inflated. There is no bad odour.
MN1	1st break. Fresh tomato sauce has good aspect, red color, good flavour and it shows a little sineresys. 4th break. Idem. 7th break. Idem. 11th break. As during all the study, fresh tomato sauce has good aspect, red color, good flavour and it shows a little sineresys.
S1	1st break. Fresh tomato sauce has good aspect, red color, good flavour and it shows a little sineresys. 4th break. Idem. 7th break. Idem. 11th break. As during all the study, fresh tomato sauce has good aspect, red color, good flavour and it shows a little sineresys.

pH

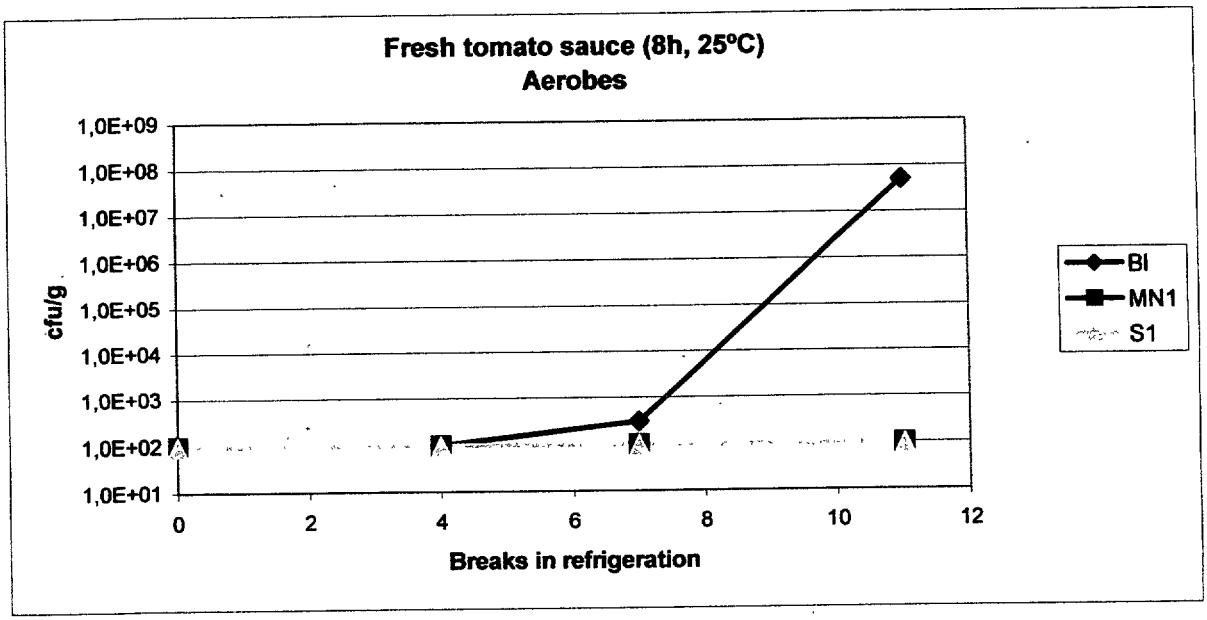
	BI	MN1	S1	Break No.
pH at 25ºC	4.06	4.02	4.06	4th break
	3.98	4.02	3.96	7th break
	4.17	4.13	3.99	11th break

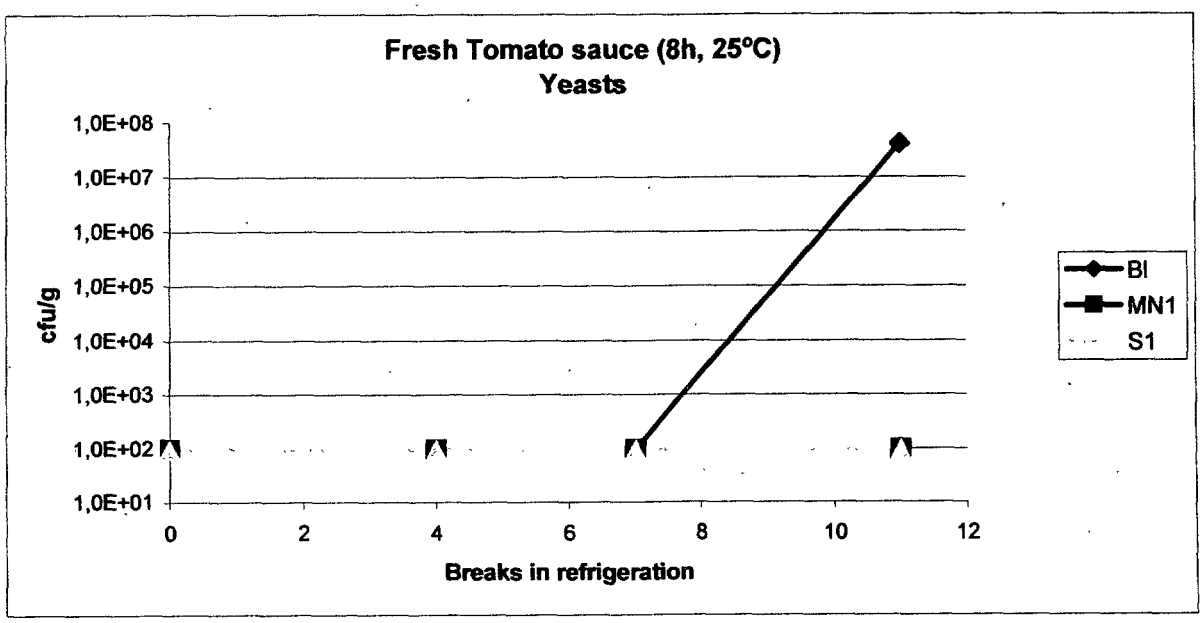
It can be concluded that there are no major differences between treatments.

Microbiological results

	BI	MN1	S1	Break No.
Aerobes ID-11-1031	1.0e2	<1.0e2	<1.0e2	4th break
	3.0e2	<1.0e2	<1.0e2	7th break
	5.1e7	<1.0e2	<1.0e2	11th break
Yeasts ID-11-1531	1.0e2	<1.0e2	<1.0e2	4th break
	<1.0e2	<1.0e2	<1.0e2	7th break
	4.1e7	<1.0e2	<1.0e2	11th break

Results expressed as cfu/g





It can be concluded that treatments S1 and MN1 can help to prevent microbiological degradation of tomato fresh sauce.

Evolution of Lauric arginate concentration along the time

LAE stability in fresh tomato sauce was analysed by HPLC (LI-402). Samples were analysed initially and after 25 days of study (samples were stored at 0-5ºC from break number 11th until the last day of study).

Samples	LAS (mg/L)	LAE (mg/L)
BI	n.d	n.d
MN1 Initial	n.d	254
MN1 25 days	n.d	221

As it is possible to observe on the above table, at the start of the experiment, the sample treated with Mirenat-N (MN1) had a concentration of LAE of 254 mg/L and this concentration was of 221 mg/L after 25 days.
It can be concluded that LAE is stable in fresh tomato sauce.

Conclusions

Lauric arginate is a good preservative for fresh tomato sauce as a basic component of Savoury Toppings or Fillings

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21/02/05 Rev. 3

TECHNICAL REPORT OF EFFICACY TESTS

LAURIC ARGINATE AS A PRESERVATIVE FOR COOKED RICE

Internal study reference: VED-EC-13

Es conforme con las exigencias de la Norma Española UNE - EN ISO 9001 2000

000147

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Experimental Completion Date: 05.12.03

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Terms Glossary

- **LAE :** Lauric arginate (Ethyl-N$^{\alpha}$-lauroyl-*L*-arginate HCl)
- **LAS :** N$^{\alpha}$-lauroyl-*L*-arginine. Hydrolysis product of LAE
- **MIRENAT-N :** is a formulation of LAE with propylene glycol and citric acid.

Introduction

Cooked rice is used as ingredient in many ready-to-eat meals. These products are very perishable so it is important to extend their shelf life and organoleptic qualities, for this reason, the different components of the prepared meals has to be preserved.
Other reason why is important to preserve this product is that cooked rice starts to discolour within the stored life and consumers could avoid to buy this product or the products where the rice acts an ingredient.

Objective of application of Lauric arginate

The aim of this study is to demonstrate that LAE could preserve cooked rice for use in ready-to eat meals, due to its perishable nature.

Experimental procedure

Industrial process and ingredients

The ingredients for all studied treatments were three: tap water, salt and rice. The difference between them was the preservative used or not, to preserve the rice.

The industrial process was carried out in Vedeqsa laboratory according to the regular manufacture practices that are used in this sector.

The elaboration process was:

- To mix water with salt (in the treated samples the preservative is added at this point but in the control samples there was not addition of preservative).
- When the water started to boil, the rice was added. The cooking process was about 12-15 minutes (at the end of the boiling process, there was practically no water).
- The rice was drained and washed with cold water.

- The rice was packed in sterile bags and stored at two different temperatures.

Treatments

Four lots of cooked rice were prepared:

1/ Treated lot with 100 mg/kg of LAE which was added in the water prior to boil the rice (once the rice was boiled, there were 81 mg/kg of LAE)
2/ Treated lot with 300 mg/kg of LAE that was added in the water prior to boil the rice (once the rice was boiled, there were 267 mg/kg of LAE)
3/ Treated lot with potassium sorbate 1g/kg that was added in the water prior to boil.
4/ The control lot was not treated (none preservative was added)

Details of the Lauric arginate used

MIRENAT-N is a formulation of LAE with propylene glycol and citric acid. The LAE used was batch No. 6420 and the MIRENAT-N was referenced as JMR-314. The analytical results were:

LAE (batch No. 6420):

LAE	74.9%	ID-11-1697, rev.4
LAS	1.1%	ID-11-1697, rev.4
Lauric acid	2.1 %	ID-11-2004, rev.2
Ethyl laurate	1.4 %	ID-11-2004, rev.2
Ashes (700 °C)	1.7 %	ID-11-083, rev.2
Water	15.3%	ID-11-020, rev.4

MIRENAT-N (ref. JMR-314)

LAE	19.50 %	ID-11-1697, rev.4
Aspect	Clear liquid	
Density	1.046 g/cm^3	ID-11-030
Viscosity (Brookfield, spindle RV2, 50 rpm, 20°C)	88 mPa·s	ID-11-029
Index of refraction (20°C)	1.442	
pH (solution 1% H_2O deionized, 20°C)	3.26	

The methods ID-11-1697 rev.4 and ID-11-2004, rev.2 were validated by an external laboratory, NOTOX.

Analysis conditions

- The storage temperature for the four lots was (4ºC and 20ºC).
- The analysis of each point was made with 1 sample of the appropriated lot.
- Frequency of analysis:
  - Organoleptical:
    - 20ºC: initial, 1st , 2nd, 3rd and 4th day.
    - 4ºC: initial, 7th and 9th .
  - Microbiological
    - 20ºC: initial, 1st , 2nd and 3rd.
    - 4ºC: initial, 7th, 9th and 11th.

Parameters analysed and frequency

Parameters analysed were:

- ✓ Organoleptical: aspect, odor and colour.
- ✓ Microbiological: mesophilic and aerobic bacteria and *Bacillus cereus* (the microbiological parameters for the samples at 20ºC were only analysed until the 3rd day).
- ✓ The content of Lauric arginate in the matrix after cooking.
- ✓ The microbiological and content of Lauric arginate analysis were made in Vedeqsa laboratory.

All of these parameters were analysed at LAMIRSA facilites.

Results

Tables with the organoleptical, microbiological and chromatographical results are in ANNEXE 1.

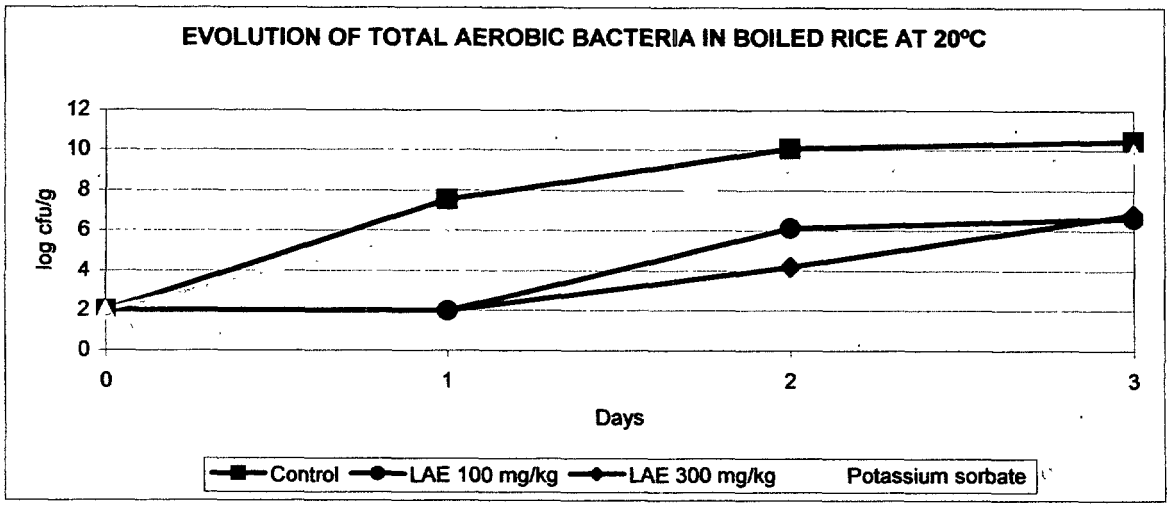
Organoleptical results

- At 20ºC and day 4th there were differences between the four treatments. The control and sorbate samples had a bad odor and the colour of the rice was yellow. However, the samples treated with LAE had the colour and odor characteristic of the rice.
- At 4ºC and day 11th there were differences between the control samples and treated samples . The control samples had a bad odor and the colour of the rice

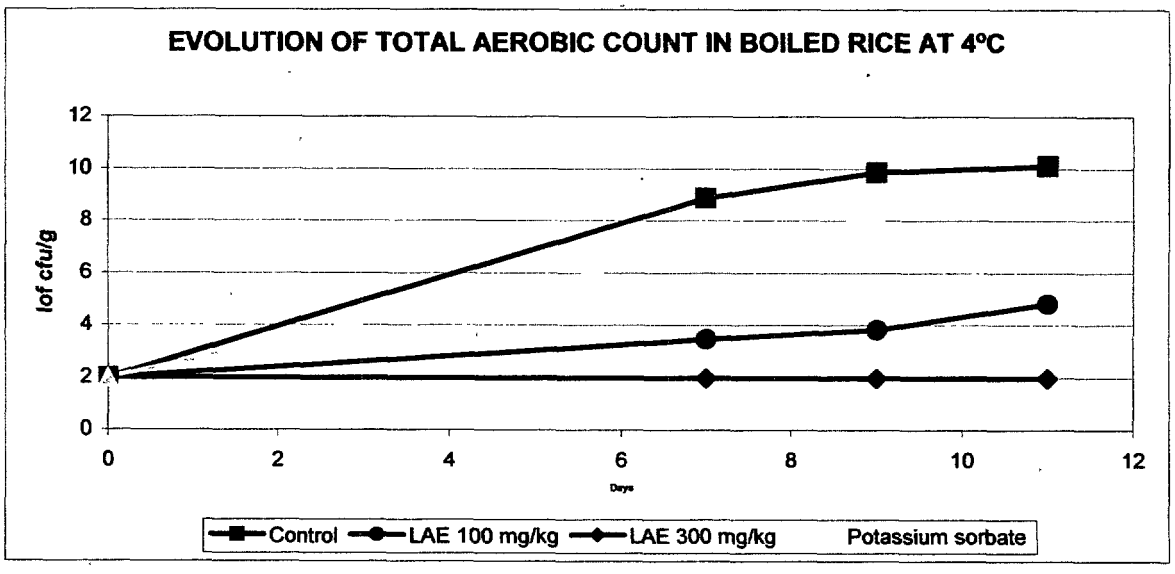
was yellow. However, the samples treated with LAE and sorbate had the colour and odor characteristic of the rice.

Microbiological results

Counts of total aerobic bacteria



Graphic 1. Evolution of aerobic mesophilic bacteria in boiled rice during their storage at 20ºC (3 days of study).



Graphic 2. Evolution of aerobic mesophilic bacteria in boiled rice during their storage at 20ºC (11 days of study).

Growth of *Bacillus sp.*

There was no growth of *Bacillus sp* during the days of the study at any of the two temperatures (4ºC and 20ºC).

Chromatographic results

The content of LAE on the rice was analysed by HPLC:

- In the lot treated with 100 mg LAE/kg there were detected 81 mg LAE/kg.
- In the lot treated with 300 mg LAE/kg there were detected 267 mg LAE/kg.

Conclusions

Lauric arginate:

- Improves the microbiological characteristics of cooked rice.
- Retards the degradation of coked rice without affecting the organoleptical evolution of the product.
- Has a better effectiveness than potassium sorbate.

ANNEXE 1

- **Organoleptical results**

4ºC

		Control	LAE 100 mg/kg	LAE 300 mg/kg	Potassium sorbate
Initial	Appearance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Colour	White	White	White	White
	Odor	Characteristic of the product. Good odor.	Characteristic of the product. Good odor.	Characteristic of the product. Good odor.	Characteristic of the product. Good odor.
7th day	Appearance	Characteristic of the product	Characteristic of the product	Characteristic of the product	Characteristic of the product
	Colour	White	White	White	White
	Odor	The product has a slightly bad odor.	Characteristic of the product. Good odor	Characteristic of the product. Good odor.	Characteristic of the product. Good odor.
9th day	Appearance	Characteristic of the rice	Characteristic of the rice	Characteristic of the rice	Characteristic of the rice
	Colour	White	White	White	White
	Odor	The product has a bad odor.	Characteristic of the product. Good odor.	Characteristic of the product. Good odor.	Characteristic of the product. Good odor.

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27/01/05 Rev. 3

TECHNICAL REPORT OF EFFICACY TESTS

LAURIC ARGINATE AS A PRESERVATIVE FOR MARINATED MEAT

Internal study reference: VED-EC-08

000157

Es conforme con las exigencias de la Norma Española
UNE - EN ISO 9001 2000

Grupo LAMIRSA

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Experimental Start Date: 14/09/04

Experimental Completion Date: 10/01/05

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Terms Glossary

- **LAE :** Lauric arginate (Chemical name: Ethyl-N^{α}-lauroyl-*L*-arginine HCl)
- **LAS :** N^{α}-lauroyl-*L*-arginine. Hydrolysis product of LAE
- **MIRENAT-N :** is a formulation of LAE with propylene glycol and citric acid

Introduction

Meat products can be cured using a marinade. The objective to marinate meat products is to modify the taste and savour of the meat achieving an appealing flavour and scent as well as preserving it against microbiologic spoilage.
Preservation in marinades is achieved by combination of some additives and ingredients as well as using physic methods (i.e. refrigerating temperatures and vacuum storage).
In accordance with the directive 95/2/EC the use of nitrites (E-249, E-250) and nitrates (E-251, E-252), as preservatives, is permitted in cured meat products at doses of 150 mg/kg as $NaNO_2$ and 300 mg/kg as $NaNO_3$. Due to some potential toxicological problems related with nitrites and nitrates reducing daily ingest of these additives would be desirable.
Common salt reduces the water activity to values of 0.97-0.95 and sugar helps to select the microorganisms responsible to reduce the nitrates.

Objective of application of Lauric arginate

The aim of this study is to demonstrate how LAE could preserve marinated meat as an example of non-cooked cured meat products. The use of LAE would allow to reduce the dose level of nitrites and nitrates.

Experimental procedure

Collaborator companies

The company that collaborated in this study was:

KERRY HUNGÁRIA KFT
H-1106 BUDAPEST
Fehér út 10
HUNGARY
Tf. ++36-1-264-2066
The contact person was: Mr. Gyula Bajkai.

KERRY produced and packaged dried marinade.

Process and ingredients

The process was carried out according to the regular manufacture practises that are used in this sector.
Nomenclature used in this report:

- Marinated Solid: dry mix provided by Kerry Hungária.
- Marinated Sauce: liquid mix prepared by adding water and vegetable oil to marinated solid according to the recipe.
- Marinated Meat: meat prepared by marinated sauce addition.

The recipe that was used for the marinated meat was:

Mix Kerry solid marinade: MAYAROS MARINÁD OLAJOS (VEDEQSA Rf. 515), composed by spices, salt, sacarose, dextrose, E450 (diphosphates), E133 (Brilliant blue FCF) and E415 (xanthan gum) with tap water and sunflower oil.
Proportions: 90 g marinated solid + 85 g water + 45 g sunflower oil per one kg of beef cut in cubes shape (3x3x3 cm).

The marinated solid was placed (JMR-430) on a sterile recipient and mixed with sunflower oil and water in the correct proportions in order to obtain the marinated sauce.
The marinated sauce is added to the beef, followed by manual tumbling and vacuum packaging (98%) in a glass container. Finally, marinated meat is stored under refrigeration at 4-7ºC.

Treatments

Three lots of marinated meat were produced:

1. The control lot **(BI)**, produced without any preservative.
2. Treatment with nitrites **(NO_2)**. $NaNO_2$ was added at doses of 150 mg/kg of marinated meat. $NaNO_2$ was added as an ingredient more of the marinated solid.
3. Treatment with Lauric arginate **(LAE)**. LAE was added by dipping the fresh beef in a bath at 1% of LAE in deionised water during 30 seconds. Fresh beef treated in this way was used as described in the recipe.

Details of Lauric arginate used

MIRENAT-N is a formulation of LAE with glycerine and citric acid. The LAE used was batch Nº 6420 and the MIRENAT-N was referenced as JMR-431. The analytical results were:

LAE (batch Nº 6420):

LAE	74.9%	ID-11-1697, rev.4
LAS	1.1%	ID-11-1697, rev.4
Lauric acid	2.1 %	ID-11-2004, rev.2
Ethyl laurate	1.4 %	ID-11-2004, rev.2
Ashes (700 ºC)	1.7 %	ID-11-083, rev.2
Water	15.3%	ID-11-020, rev. 4

MIRENAT-N (ref. JMR-431)

LAE	20,5%	ID-11-1697, rev.4
Aspect	Clear liquid	

The method ID-11-1697 rev.4 and ID-11-2004, rev.2 have been validated by an external laboratory, NOTOX.

Parameters analysed and frequency

Parameters analysed were:

- ✓ organoleptic (general aspect, odour and colour).
- ✓ aerobes mesophiles (ID-11-1031, buffered peptone water was used to make dilutions, growth was carried out with TSA agar at 35ºC for 48 hours).
- ✓ enterobacter (ID-11-2325, buffered peptone water was used to make dilutions, growth was carried out with VRBG agar at 35ºC for 24 hours).
- ✓ concentration of LAE (ID-11-2493).

All these parameters were analysed in LAMIRSA laboratories.

Five samples per treatment and per day of analysis (JMR-430) were analysed.

Results

Organoleptic results

Initially all treatments had the same general aspect, colour and odour. The addition of LAE did not modify organoleptic properties of the marinade.
During the 24 days of the study, no appreciable changes on the organoleptic properties were detected.

Evolution of Lauric arginate concentration along the time

The LAE concentration in marinated meat was analysed by HPLC (LI-459):

Days	mg LAS/kg	mg LAE/kg	Equivalent mg LAE/kg	Mean Equivalent mg LAE/kg
1	24.30	164.70	193.39	**183.33**
	31.00	170.30	206.88	
	25.80	119.30	149.74	
17	43.60	110.30	161.75	**118.58**
	0.00	83.30	83.30	
	50.00	51.70	110.70	
31	40.70	12.40	60.43	**71.74**
	26.14	33.50	64.35	
	41.90	41.00	90.44	

Equivalent of mg LAE/kg is the sum of the
mg of LAE/kg and the mg of LAS (expressed as mg of LAE)/kg founded in the marinated meat.

According to the results reported on the previous table, LAE concentration decreased gradually in the marinated meat along the time. This metabolization comes from natural enzymatic processes producing non toxic and natural compounds like LAS that was determined by HPLC. Other natural compounds like lauric acid and arginine, which are known as products of the metabolization of LAE, have not been determined in this experiment. This metabolization must be attributed to the fact that marinated meat has enzymatic activity and also to the natural based structure and properties of LAE.

Microbiological results

Mesophilic aerobes results are shown in Table 1:

Days	Sample	BI	NO_2	LAE	Days	Sample	BI	NO_2	LAE
1	1	4,5	4,3	3,8	14	1	7,2	6,8	4,7
	2	4,3	4,5	3,7		2	7,2	7,1	5,5
	3	4,5	4,7	3,5		3	7,3	6,8	5,9
	4	4,7	4,7	3,9		4	7,4	6,5	4,7
	5	4,8	4,5	3,7		5	7,4	6,1	3,9
	χ	4,5	4,5	3,7		χ	7,3	6,6	4,9
	σ	0,2	0,2	0,1		σ	0,1	0,4	0,8
3	1	4,5	4,3	4,0	17	1	8,4	7,7	6,1
	2	4,9	4,8	3,9		2	8,1	7,8	7,0
	3	4,7	4,2	3,7		3	8,4	7,1	5,9
	4	4,5	4,7	3,7		4	8,4	7,3	5,9
	5	4,5	4,3	3,8		5	7,9	6,6	6,1
	χ	4,6	4,4	3,8		χ	8,2	7,3	6,2
	σ	0,2	0,3	0,1		σ	0,2	0,5	0,4
7	1	4,2	4,1	3,7	21	1	8,4	8,3	5,6
	2	4,8	4,1	3,7		2	8,2	8,2	7,9
	3	4,8	4,7	3,8		3	8,6	7,9	5,9
	4	4,9	4,5	3,5		4	8,7	8,3	6,4
	5	4,5	4,5	3,8		5	8,2	7,3	7,1
	χ	4,6	4,4	3,7		χ	8,4	8,0	6,6
	σ	0,3	0,2	0,1		σ	0,2	0,4	0,9
10	1	6,9	4,8	3,9	24	1	8,5	8,7	7,7
	2	6,8	4,7	4,0		2	8,7	8,6	7,4
	3	6,7	4,9	3,9		3	8,5	8,3	6,9
	4	6,9	4,7	3,9		4	8,7	8,5	7,7
	5	6,8	4,5	4,1		5	8,1	7,9	7,2
	χ	6,8	4,7	4,0		χ	8,5	8,4	7,4
	σ	0,1	0,1	0,1		σ	0,3	0,3	0,4

Table 1
Results expressed as CFU/g

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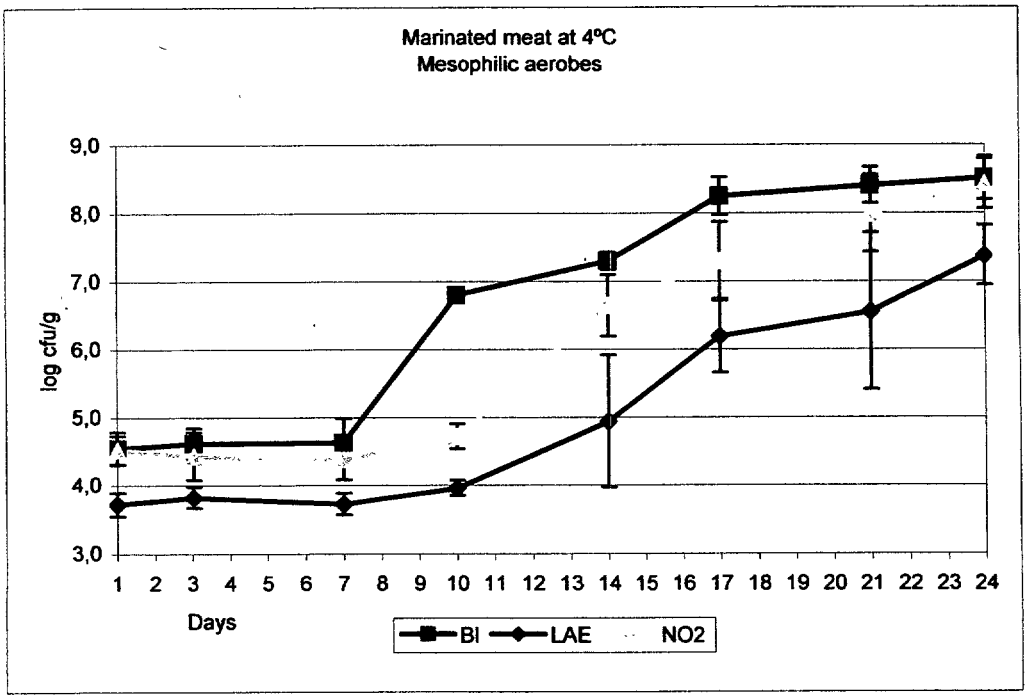
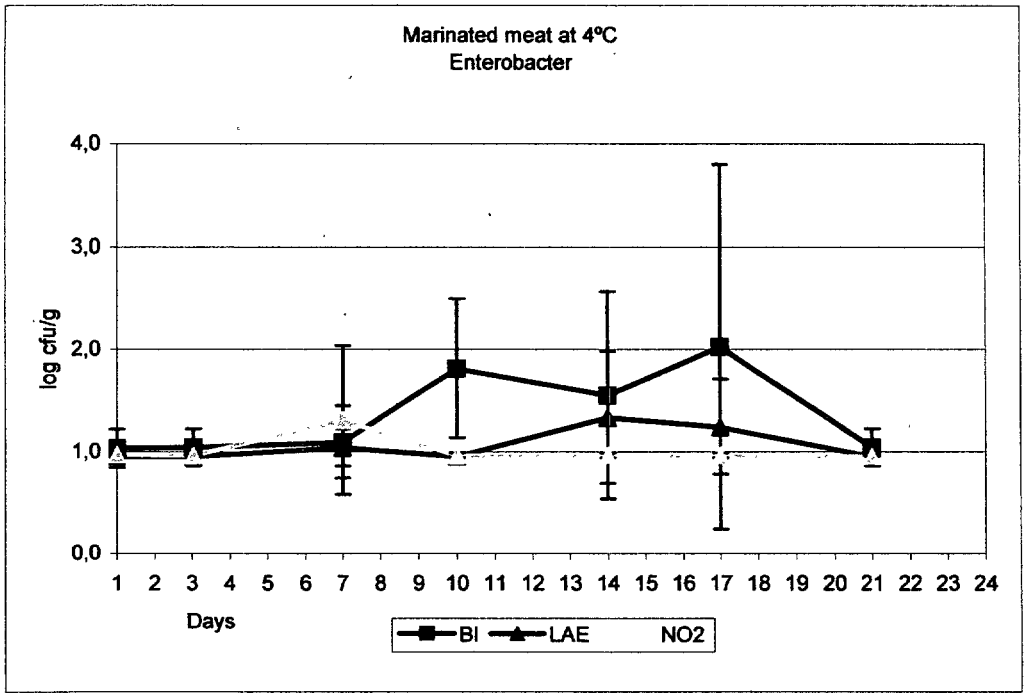
Mesophilic aerobes results are represented in Graphic 1. 95% confidence limits are shown by each day of analysis. Applying t test hypothesis:

- ✓ All day of analysis the averages between LAE and control samples were significantly different (P<0.05).
- ✓ All day of analysis the averages between LAE and nitrite samples were significantly different (P<0.05).



Graphic 1

Enterobacter results are represented in Graphic 2.



Graphic 2

Due to low level of growth no significant differences could be detected.

Conclusions

Microbiological results show that **LAE can reduce significantly the growth of natural occurring flora in the marinated meat.**
According to the previous conclusion, **LAE could improve the microbiological stability of marinated meat** and guarantee the maintenance of all its characteristics once it is consumed.

1. **Lauric arginate is a good preservative for marinated meat.**
2. **Lauric arginate is a good substitute for nitrites and nitrates in the preservation of marinated meat.**

000167

LAURIC ARGINATE AS A PRESERVATIVE FOR FRESH SAUSAGE

000168

Objective

The aim of this study is to demonstrate that LAE could preserve fresh sausages without affecting the technological characteristics of this type of sausages.
In this study only the organoleptic results are shown.

Experimental procedure

Collaborator companies

The companies that collaborated in the fresh sausage study were: Subirats, Calitec, Camil y Camps.

Subirats manufactured the fresh sausages and the samples were stored in Subirats for 10 days:

SUBIRATS S.A
C/ Reus , number 1.
Industrial state La Florida
Sta. Perpètua de la Mogoda. 08130 Barcelona
Tlf: 93.574.31.32
NIF: A 59168112
The contact person: Mr. Miquel Jordá. (Quality responsible)

The organoleptic analysis were made by Calitec:

CALITEC S.C.P
C/ Rocafort nº 204- Bajos
08029 Barcelona
The contact person: Mr- Josep Gou

The company that destroyed the remaining samples:

CAMIL Y CAMPS
C/ Maresme . Factory premises number 2
Polígono Industrial Palaudàries
Lliçà de Vall. 08185 Barcelona
NIF: B-60834967
Tlf: 93.843.78.00
The contact person: Mr. Camil y Camps.(Manager)

Treatments

Two lots of fresh sausages were produced. One lot was treated with 200 ppm of LAE and the other lot, the control lot, wasn't treated.

Industrial process and ingredients

In the control lot the ingredients were: minced pork back (85%), streaky bacon (15%), salt, sugar, sodium citrate (E-331), sodium ascorbate and colorant (E-120). The ingredients for the treated lot with LAE were the same and the only difference was that LAE (added as MIRENAT-N) was diluted in water and then added to the mass. The final dose of LAE was 200 ppm in the fresh sausages.

The industrial process was carried out according to the regular manufacture practices that are used in this sector.

1/ On the mincing machine (LASKA 600) the minced pork back meat and the streaky bacon were minced for 12 minutes.

2/ The rest of the ingredients were added except LAE (in treated samples). The minced meat is mixed (Mixing machine LASKA 800) at 15 rpm for 30 seconds.

3/ The LAE prediluted in water (1:2 p/p) is added. The mixing continues during 30 seconds more.

4/ The mass was vacuum mixed (79%) for 30 seconds.

5/ The mixture was stuffed into natural beef casings (machine stamping VEMAC). The casings were previously soaked in a salted water solution for 30 minutes.

6/ Finally, fresh sausages were labelled and stored at 0-5ºC for 10 days.

Analysis conditions

- The organoleptic analysis were made by two qualified persons and with experience in this type of analysis.
- The analysis of each point was made over 5 samples , of the appropriated lot, that were chosen randomly.
- Frequency of analysis: initial, 1st, 2sd, 3rd, 7th and 10th.
- The stored temperature for both lots was (0-5ºC).

Organoleptic results

		LAE	Control
Initial	Appearance	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass.	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass.
	Colour	Characteristic of the raw and fresh meat.	Characteristic of the raw and fresh meat.
	Odor	Characteristic of the product, fresh spiced meat.	Characteristic of the product, fresh spiced meat.
	Form	Characteristic of the product.	Characteristic of the product.
Day 1	Appearance	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass.	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass.
	Colour	Characteristic of the raw and fresh meat.	Characteristic of the raw and fresh meat.
	Odor	Characteristic of the product, fresh spiced meat.	Characteristic of the product, fresh spiced meat.
	Form	Characteristic of the product.	Characteristic of the product.

000172

		LAE	Control
Day 2	Appearance	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass. A resealed liquid by the product is appreciated.	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass.
	Colour	Characteristic of the raw and fresh meat.	Characteristic of the raw and fresh meat.
	Odor	Characteristic of the product, fresh spiced meat.	Characteristic of the product, fresh spiced meat.
	Form	Characteristic of the product.	Characteristic of the product.
Day 3	Appearance	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass. A resealed liquid by the product is appreciated.	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass.
	Colour	The colour of the samples shows a light darkening respect the characteristic colour of the fresh product.	The colour of the samples shows a light darkening respect the characteristic colour of the fresh product.
	Odor	Characteristic of the product, fresh spiced meat.	Characteristic of the product, fresh spiced meat.
	Form	Characteristic of the product.	Characteristic of the product.

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		LAE	Control
Day 7	Appearance	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass.	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. The external aspect is cleaned and without moulds. A good attachment of the component mass. A regular distribution of the components of the meat mass.
	Colour	The colour of the samples shows a high darkening respect the characteristic colour of the fresh product.	The colour of the samples shows a high darkening respect the characteristic colour of the fresh product.
	Odor	Two of the five samples have a light and disagreeable fermentation odor. The odor of the other samples is characteristic of the product, fresh spiced meat.	Three of the five samples have a light and disagreeable fermentation odor. The odor of the other samples is characteristic of the product, fresh spiced meat.
	Form	Characteristic of the product.	Characteristic of the product.
Day 10	Appearance	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. A good attachment of the component mass. A regular distribution of the components of the meat mass. The aspect of the external part of the sausages is viscous.	Gross grinded mass which is chopped. A good characteristics of raw and fresh meat are appreciated in the central part of the sausage. A good attachment of the component mass. A regular distribution of the components of the meat mass. The aspect of the external part of the sausages is viscous.
	Color	The colour of the samples shows a high darkening respect the characteristic colour of the fresh product.	The colour of the samples shows a high darkening respect the characteristic colour of the fresh product.

		LAE	Control
	Odor	All samples have a high and disagreeable fermentation odor.	All samples have a high and disagreeable fermentation odor.
	Form	Characteristic of the product.	Characteristic of the product.

Conclusions

At day 7th, LAE, retards the fermentation process that occurred in the sausages, but at day 10th, the organoleptic characteristic for control and treated samples are the same.

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21/02/05 Rev. 6

TECHNICAL REPORT OF EFFICACY TESTS

LAURIC ARGINATE AS A PRESERVATIVE FOR BRATWURST SAUSAGE

Internal study reference: VED-EC-07

Es conforme con las exigencias de la Norma Española UNE EN ISO 9001 2000

Grupo LAMIRSA

000176

I+D Department:

Sergi Figueras Study Director	Date
Carmen Codes Contributing Scientist	Date

Quality Assurance Department:

Roger Segret Principal Auditor	Date

Experimental Start Date: 10.10.03

Experimental Completion Date: 28.12.03

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ANNEXE 1 ..... **14**

Terms Glossary

- **LAE :** Lauric arginate (Ethyl-N$^{\alpha}$-lauroyl-*L*-arginate HCl)
- **LAS :** N$^{\alpha}$-lauroyl-*L*-arginine. Hydrolysis product of LAE
- **MIRENAT-N :** is a formulation of LAE with propylene glycol and citric acid.

Introduction

Bratwurst sausages are meat products treated by heat. They are basically constituted by pork meat and spices, and their maximum calibre is about 45 mm.
The composition of Bratwurst sausages is very similar to Frankfurt sausages but the main difference between them is the colour. The first one is grey-brown and the second one is reddish. The difference of the colour between both types of sausages is due to Frankfurt sausage contains nitrites and nitrates as preservatives and they also give the reddish colour to the sausages. Bratwurst sausages don't have any added nitrites and nitrates for technological reasons.

In accordance with the European directive 95/2/EC, the use of nitrites and nitrates is permitted in meat products treated by heat. In reference with BOE 3/1/84 the microbiologic limits for Bratwurst sausage are:

Enteric bacteria (cfu/g)	***Salmonella* and *Shigella* (cfu/g)**	***St. aureus* (cfu/g)**	***Clostridium sp.* (cfu/g)**
$1x10^2$	Absence in 25g	$1x10^2$ in 1 g	$1x10^2$

Objective of application of Lauric arginate

The aim of this study is to demonstrate that LAE could preserve Bratwurst sausages without affecting the technological characteristics of this type of sausages.

Experimental procedure

Collaborator companies

The companies that collaborated in the Bratwurst sausage study were: Subirats, Camil y Camps.

Subirats manufactured the Bratwurst sausages the 10th October of 2003. The samples were stored in Subirats for 3 months (10th on January of 2004, the shelf-life):

SUBIRATS S.A
C/ Reus , number 1.
Industrial state La Florida
Sta. Perpètua de la Mogoda. 08130 Barcelona
Tlf: 93.574.31.32
NIF: A 59168112
The contact person: Mr. Miquel Jordá. (Quality responsible)

The company that destroyed the remaining samples:

CAMIL Y CAMPS
C/ Maresme . Factory premises number 2
Polígono Industrial Palaudàries
Lliçà de Vall. 08185 Barcelona
NIF: B-60834967
Tlf: 93.843.78.00
The contact person: Mr. Camil y Camps.(Manager)

Treatments

Two lots of Bratwurst sausages were produced. One lot was treated with 100 ppm of LAE and the other lot, the control lot, wasn't treated.

Industrial process and ingredients

In the control lot the ingredients were: minced pork back, underbelly, spices, salt and additives (milk protein, diphosphate, antioxidant (E-300) and flavour enhance (E-621)). The ingredients for the treated lot with LAE were the same and the only difference was that LAE (added as MIRENAT-N) was diluted in water and then added to the mass. The final dose of LAE was 100 ppm in the Bratwurst sausages.

The industrial process was carried out according to the regular manufacture practices that are used in this sector.

1/ On the cutter (Cutter SCHEIDMISCHER 120) the minced pork back meat was ground and the rest of the ingredients were added in this order: the additives (except the MIRENAT-N), the underbelly, the spices and MIRENAT-N prediluted in water (1:2 p/p). Ice was also added for controlling the temperature (maximum 5ºC) of the meat. The mixing time was 12 minutes.

2/ The mass was vacuum mixed (92%) (machine that made the vacuum LASKA) for 1 minute.

3/ The mixture was stuffed into natural casings (machine stamping VEMAC). The casings were previously soaked in a salted water solution for 30 minutes.

4/ The Bratwurst sausages were put in a cooking trolley. Cooking took place in a oven (VEMAC AC 504) at 80ºC for 1 hour.

5/ After cooking, the sausages were sprayed with cool water for 5 minutes and put in a cold-storage room (0-5ºC , 24 hours).

6/ The next day, Bratwurst sausages were pasteurised at 80ºC for 30 minutes (boiler KERRES) and then, they were soaked in cool water for 30 minutes.

7/ Finally, Bratwurst sausages were labelled and stored at 5ºC for 3 months.

Details of the Lauric arginate used

MIRENAT-N is a formulation of LAE with propylene glycol and citric acid. The LAE used was batch No. 6420 and the MIRENAT-N was referenced as JMR-314. The analytical results were:

LAE (batch No. 6420):

LAE	74.9%	ID-11-1697, rev.4
LAS	1.1%	ID-11-1697, rev.4
Lauric acid	2.1 %	ID-11-2004, rev.2
Ethyl laurate	1.4 %	ID-11-2004, rev.2
Ashes (700 ºC)	1.7 %	ID-11-083, rev.2
Water	15.3%	ID-11-020, rev.4

MIRENAT-N (ref. JMR-314)

LAE	19.50 %	ID-11-1697, rev.4
Aspect	Clear liquid	
Density	1.046 g/cm^3	ID-11-030
Viscosity (Brookfield, spindle RV2, 50 rpm, 20ºC)	88 mPa·s	ID-11-029
Index of refraction (20ºC)	1.442	
pH (solution 1% H_2O deionized, 20ºC)	3.26	

The methods ID-11-1697 rev.4 and ID-11-2004, rev.2 have been validated by an external laboratory, NOTOX.

Analysis conditions

- The analysis of each point was made over 5 samples , of the appropriated lot, that were chosen randomly.
- Frequency of analysis: initial, each 15 days for 3 months.
- The stored temperature for both lots was (0-5ºC).

Parameters analysed and frequency

Parameters analysed were:

- ✓ Organoleptical: aspect, form, odour and colour.
- ✓ pH.
- ✓ Microbiological: mesophilic and aerobic bacteria (extern and internal), enteric bacteria, *Clostridium sp.*, sporulated bacteria *Salmonella sp.*, *Shigella sp.*, *Staphylococcus aureus* and Lactobacillus.
- ✓ Stability of Lauric arginate in the matrix.

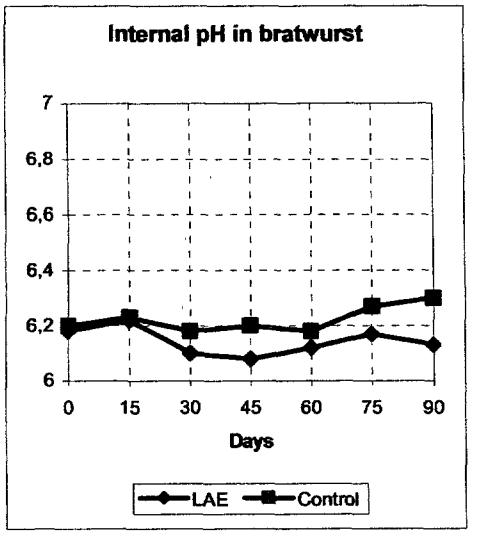
Results

Tables with the results are in ANNEXE 1.

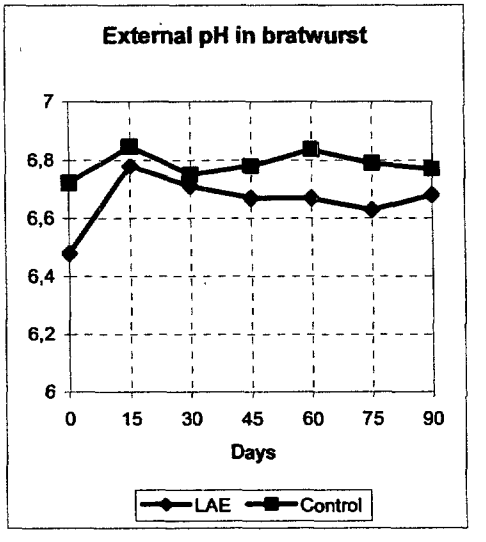
Organoleptical results

Over 90 days there was no change in any of the parameters of control sample and the sample treated with lauric arginate.

pH



Graphic 1.



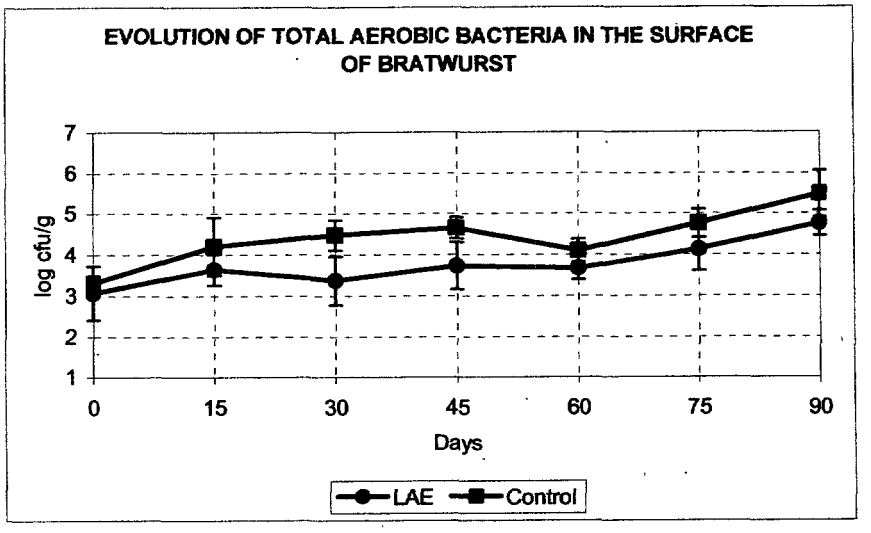
Graphic 2

Graphic 1 and 2. Evolution of external and internal pH in Bratwurst sausages during their shelf-life (90 days).

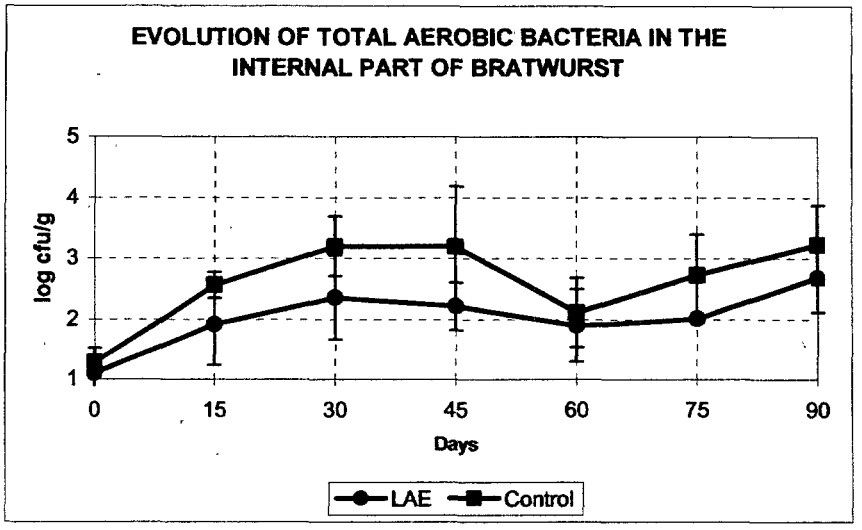
The evolution of the pH in treated and control samples is practically the same. So, the addition of Lauric arginate does not influence the pH characteristics of the Bratwurst sausages.

Microbiological results

Counts of total aerobic bacteria



Graphic 3. Evolution of external aerobic and mesophilic bacteria in Bratwurst sausages during their shelf-life (90 days). (The confidents limits are 95%)

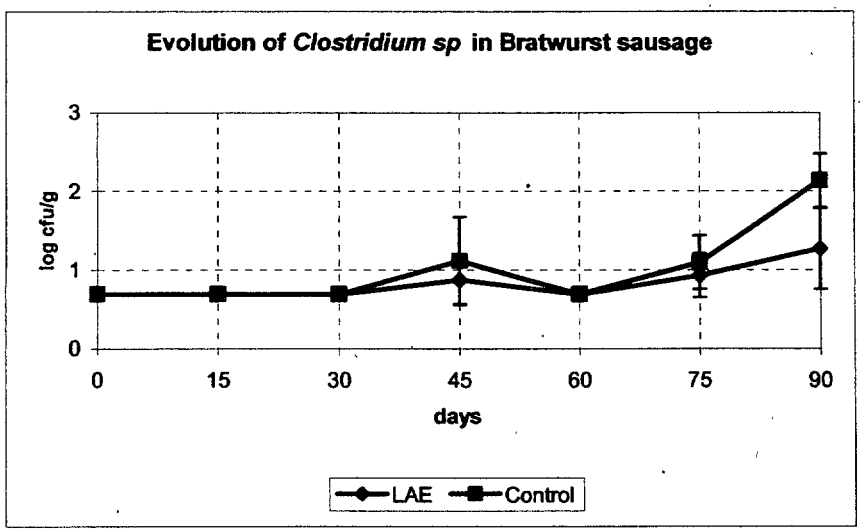


Graphic 4 . Evolution of internal aerobic and mesophilic bacteria in Bratwurst sausages during their shelf-life (90 days). (The confidents limits are 95%)

Applying 95% confidence limits:

On the surface of Bratwurst (where there are more microbiological problems), the averages on days 30, 45, 60, 75 and 90 between treated and control samples were significantly different ($P<0.05$).
In the internal part of the sausages the averages between control and treated samples were not significantly different.

Growth of *Clostridium sp.*



Graphic 5. Evolution *Clostridium sp* in Bratwurst sausages during their shelf-life (90 days). (The confidence limits are 95%).

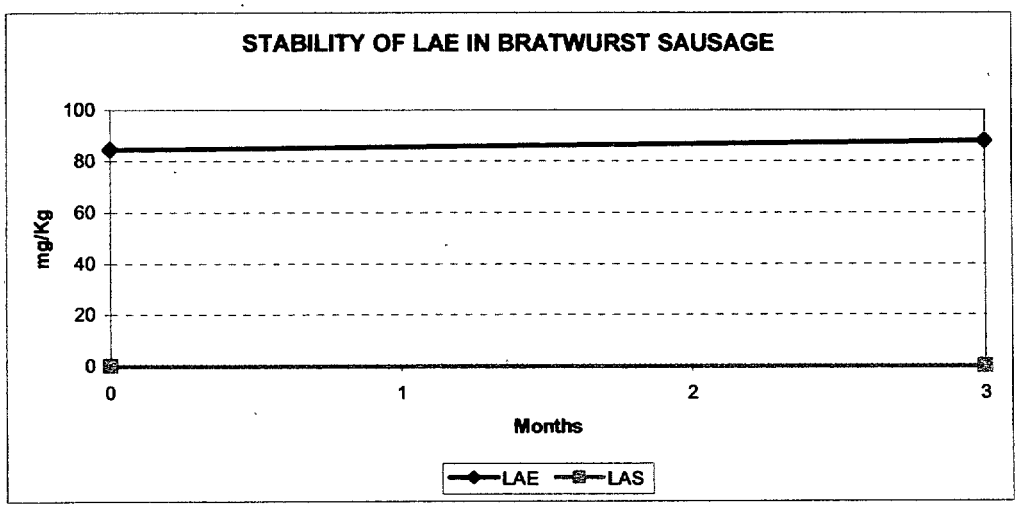
Applying 95% confidence limit:

At day 90 treated and control samples were significantly different ($P<0.05$).

Growth of enteric bacteria, *Staphylococcus aureus* , *Salmonella sp* and *Shigella sp*, and Lactobacillus.

- Enteric bacteria → no growth was observed in treated and control samples for 90 days.
- *Staphylococcus aureus* → in control samples growth was observed on day 90 . Non growth was observed in samples treated with LAE.
- *Salmonella sp* and *Shigella sp* → non growth was observed in treated and control samples for 90 days. They were analysed at initial, 30, 60 and 90 days.
- Lactobacillus → no growth was observed in treated and control samples for 90 days.

Evolution of Lauric arginate concentration along the time



Graphic 6. Stability of Lauric arginate in Bratwurst sausage for three months.

None degradation of Lauric arginate was observed during the three months of the study under the conditions assayed.

Conclusions

- Lauric arginate (formulated as MIRENAT-N) improves the microbiological characteristics of Bratwurst sausages.
- Lauric arginate (formulated as MIRENAT-N) retards the degradation of Bratwurst sausages without affecting the organoleptical evolution of the sausages.
- The concentration of Lauric arginate in the Bratwurst sausage is maintained stable during the study.

ANNEXE 1

- **Organoleptical results**

The organoleptical results observed during the first day of the experiment were the same at the end of the study.

	MIRENAT-N	Control
Appearance	Fine grinded mass which is chopped and treated by heat. The external aspect is cleaned and without moulds. Casing and the total of meat are easily separated. A good attachment of the chopped mass. A regular distribution of the components of the meat mass.	Fine grinded mass which is chopped and treated by heat. The external aspect is cleaned and without moulds. Casing and the total of meat are easily separated. A good attachment of the chopped mass. A regular distribution of the components of the meat mass.
Colour	Homogeneous. Pale and off white.	Homogeneous. Pale and off white.
Odor	Characteristic of the product. Good taste.	Characteristic of the product. Good taste.
Form	Characteristic of the product. Chopped product.	Characteristic of the product. Chopped product.

• **pH results**

Days	Samples	MIRENAT-N		Control	
		External pH	Internal pH	External pH	Internal pH
0 (Initial)	1	6.49	6.12	6.68	6.20
	2	6.48	6.16	6.77	6.21
	3	6.47	6.19	6.76	6.19
	4	6.47	6.20	6.70	6.21
	5	6.49	6.21	6.71	6.20
15	1	6.78	6.26	6.80	6.25
	2	6.81	6.24	6.64	6.23
	3	6.70	6.23	7.09	6.23
	4	6.86	6.16	6.83	6.20
	5	6.76	6.22	6.90	6.22
30	1	6.78	6.08	6.71	6.18
	2	6.69	6.10	6.65	6.19
	3	6.73	6.10	6.80	6.20
	4	6.66	6.09	6.77	6.13
	5	6.68	6.12	6.83	6.21
45	1	6.77	6.15	6.65	6.17
	2	6.68	6.05	6.82	6.22
	3	6.55	6.12	6.75	6.18
	4	6.62	6.08	6.86	6.25
	5	6.71	6.02	6.80	6.18
60	1	6.67	6.10	6.18	6.76
	2	6.60	6.17	6.22	6.89
	3	6.70	6.10	6.12	6.90
	4	6.68	6.10	6.13	6.86
	5	6.71	6.12	6.25	6.80
75	1	6.57	6.14	6.22	6.84
	2	6.54	6.11	6.23	6.79
	3	6.64	6.15	6.31	6.66
	4	6.70	6.21	6.33	6.78
	5	6.68	6.23	6.28	6.88

Days	Samples	MIRENAT-N		Control	
		External pH	Internal pH	External pH	Internal pH
90 (Final)	1	6.62	6.15	6.25	6.76
	2	6.69	6.10	6.30	6.68
	3	6.79	6.12	6.35	6.72
	4	6.72	6.11	6.29	6.82
	5	6.59	6.19	6.31	6.86

- **Microbiological results**

Counts of total aerobic bacteria (AMT) in external part of Bratwurst. (Results expressed in cfu/g)

Days	Samples	MIRENAT-N		Control	
		External AMT	Log (cfu/g)	External AMT	Log (cfu/g)
0 (Initial)	1	$3.5x10^3$	3.54	$2.5x10^3$	3.40
	2	$6.3x10^3$	3.80	$1.1x10^3$	3.04
	3	$3.2x10^2$	2.51	$2.4x10^3$	3.38
	4	$9.3x10^2$	2.97	$2.1x10^3$	3.32
	5	$3.5x10^2$	2.54	$2.7x10^3$	3.43
	χ	-	**3.07**	-	**3.31**
	σ	-	**0.52**	-	**0.14**
15	1	$6.3x10^3$	3.80	$2.5x10^4$	4.40
	2	$3.0x10^3$	3.48	$2.1x10^3$	3.32
	3	$1.7x10^3$	3.23	$6.2x10^3$	3.79
	4	*	*	$4.1x10^4$	4.61
	5	$1.1x10^4$	4.04	$8.0x10^4$	4.90
	χ	-	**3.64**	-	**4.20**
	σ	-	**0.31**	-	**0.57**
30	1	$9.0x10^3$	3.95	$2.5x10^4$	4.40
	2	$3.0x10^3$	3.48	$6.0x10^4$	4.78
	3	$6.0x10^3$	3.78	$9.0x10^3$	3.95
	4	$7.0x10^2$	2.85	$5.2x10^4$	4.72
	5	$6.0x10^2$	2.78	$3.4x10^4$	4.53
	χ	-	**3.37**	-	**4.48**
	σ	-	**0.48**	-	**0.29**

Days	Samples	MIRENAT-N		Control	
		External AMT	Log (cfu/g)	External AMT	Log (cfu/g)
45	1	$2.8x10^4$	4.45	$2.0x10^4$	4.30
	2	$2.8x10^3$	3.45	$3.8x10^4$	4.58
	3	$2.0x10^3$	3.30	$7.1x10^4$	4.85
	4	$1.3x10^4$	4.11	$6.8x10^4$	4.83
	5	$2.2x10^3$	3.34	$5.7x10^4$	4.76
	χ	-	**3.73**	-	**4.66**
	σ	-	**0.46**	-	**0.21**
60	1	$3.9x10^3$	3.59	$1.5x10^4$	4.20
	2	$3.8x10^3$	3.58	$9.1x10^3$	3.96
	3	$3.9x10^3$	3.59	$1.7x10^4$	4.22
	4	$3.0x10^3$	3.48	$5.6x10^3$	3.75
	5	$1.3x10^4$	4.11	$2.5x10^4$	4.44
	χ	-	**3.67**	-	**4.11**
	σ	-	**0.23**	-	**0.24**
75	1	$6.3x10^4$	4.80	$2.7x10^4$	4.43
	2	$3.0x10^4$	4.48	$1.6x10^5$	5.20
	3	$8.0x10^3$	3.90	$5.0x10^4$	4.70
	4	$5.1x10^3$	3.71	$8.4x10^4$	4.92
	5	$6.4x10^3$	3.81	$3.3x10^4$	4.52
	χ	-	**4.14**	-	**4.75**
	σ	-	**0.43**	-	**0.28**
90 (Final)	1	$2.5x10^4$	4.40	$1.0x10^5$	5.00
	2	$4.1x10^4$	4.61	$3.0x10^5$	5.48
	3	$6.2x10^4$	4.79	$9.1x10^5$	5.96
	4	$9.8x10^4$	4.99	$9.0x10^4$	4.95
	5	$1.2x10^5$	5.08	$1.2x10^6$	6.08
	χ	-	**4.77**	-	**5.49**
	σ	-	**0.25**	-	**0.47**

Counts of total aerobic bacteria (AMT) in the internal part of Bratwurst.
(Results expressed in cfu/g)

Days	Samples	MIRENAT-N		Control	
		Internal AMT	Log (cfu/g)	Internal AMT	Log (cfu/g)
0 (Initial)	1	$3.0x10^1$	1.48	$3.0x10^1$	1.48
	2	$<1.0x10^1$	1.00	$<1.0x10^1$	1.00
	3	$<1.0x10^1$	1.00	$<1.0x10^1$	1.00
	4	$<1.0x10^1$	1.00	$8.0x10^1$	1.90
	5	$<1.0x10^1$	1.00	$1.0x10^1$	1.00
	χ	-	**1.01**	-	**1.28**
	σ	-	**0.19**	-	**0.45**
15	1	$3.0x10^2$	2.48	$2.1x10^2$	2.32
	2	$6.0x10^1$	1.78	$4.0x10^2$	2.60
	3	$<1.0x10^1$	1.00	$3.0x10^2$	2.48
	4	$7.0x10^1$	1.85	$3.8x10^2$	2.58
	5	$3.0x10^2$	2.48	$6.7x10^2$	2.83
	χ	-	**1.92**	-	**2.56**
	σ	-	**0.55**	-	**0.17**
30	1	$8.0x10^2$	2.90	$3.8x10^3$	3.58
	2	$4.0x10^2$	2.60	$8.0x10^2$	2.90
	3	$8.0x10^2$	2.90	$8.1x10^2$	2.91
	4	$6.0x10^1$	1.78	$6.0x10^3$	3.78
	5	$4.0x10^1$	1.60	$7.0x10^2$	2.85
	χ	-	**2.36**	-	**3.20**
	σ	-	**0.56**	-	**0.39**
45	1	$3.0x10^2$	2.48	$8.0x10^2$	2.90
	2	8.0x10	1.90	$9.0x10^2$	2.95
	3	$1.0x10^2$	2.00	$9.0x10^3$	3.95
	4	$5.0x10^2$	2.70	$1.0x10^2$	2.00
	5	$1.0x10^2$	2.00	$1.6x10^4$	4.20
	χ	-	**2.22**	-	**3.20**
	σ	-	**0.32**	-	**0.79**

Days	Samples	MIRENAT-N		Control	
		Internal AMT	Log (cfu/g)	Internal AMT	Log (cfu/g)
60	1	$2.0x10^2$	2.30	$2.8x10^2$	2.45
	2	$1.0x10^1$	1.00	$1.9x10^2$	2.28
	3	$9.0x10^1$	1.95	$3.9x10^2$	2.59
	4	$8.0x10^1$	1.90	$2.0x10^1$	1.30
	5	$2.2x10^2$	2.34	$9.0x10^1$	1.95
	χ	-	**1.90**		**2.11**
	σ	-	**0.48**		**0.46**
75	1	$1.0x10^2$	2.00	$2.1x10^2$	2.32
	2	9.0x10	1.95	$7.0x10^2$	2.85
	3	$1.1x10^2$	2.04	$8.7x10^2$	2.94
	4	$1.0x10^2$	2	$3.6x10^3$	3.56
	5	$1.3x10^2$	2.11	$1.0x10^2$	2.00
	χ	-	**2.02**		**2.73**
	σ	-	**0.05**		**0.54**
90 (Final)	1	$2.1x10^2$	2.32	$3.8x10^2$	1.85
	2	$1.0x10^2$	2.00	$9.2x10^2$	2.49
	3	$9.0x10^2$	2.95	$1.0x10^3$	2.45
	4	$8.5x10^2$	2.93	$3.3x10^3$	1.90
	5	$1.8x10^3$	3.26	$1.2x10^4$	2.00
	χ	-	**2.69**	-	**3.23**
	σ	-	**0.46**	-	**0.52**

Counts of *Clostridium sp* (Results expressed in cfu/g)

Days	Samples	MIRENAT-N		Control	
		Clostridium sp	**Log (cfu/g)**	***Clostridium sp***	**Log (cfu/g)**
0 (Initial)	**1**	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	2	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	3	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	4	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	5	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	χ		0.70		0.70
	σ		0.00		0.00
15	**1**	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	2	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	3	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	4	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	5	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	χ		0.70		0.70
	σ		0.00		0.00
30	**1**	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	2	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	3	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	4	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	5	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	χ		0.70		0.70
	σ		0.00		0.00
45	**1**	$2.1x10^1$	1.30	$<5.0x10^1$	0.70
	2	$1.0x10^1$	1.00	$2.1x10^1$	1.30
	3	$<5.0x10^1$	0.70	$8.0x10^1$	1.90
	4	$<5.0x10^1$	0.70	$1.0x10^1$	1.00
	5	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	χ	-	0.88	-	1.12
	σ	-	0.24	-	0.56

Days	Samples	MIRENAT-N		Control	
		Clostridium sp	Log (cfu/g)	*Clostridium sp*	Log (cfu/g)
60	1	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	2	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	3	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	4	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	5	$<5.0x10^1$	0.70	$<5.0x10^1$	0.70
	χ		0.70		0.70
	σ		0.00		0.00
75	1	$1.0x10^1$	1.00	$3.0x10^1$	1.48
	2	$<5.0x10^1$	0.70	$1.0x10^1$	1.00
	3	$2.0x10^1$	1.30	$2.0x10^1$	1.30
	4	$1.0x10^1$	1.00	$<5.0x10^1$	0.70
	5	$<5.0x10^1$	0.70	$1.0x10^1$	1.00
	χ		0.94		1.10
	σ		0.22		0.27
90 (Final)	1	$2.0x10^1$	1.30	$7.0x10^1$	1.85
	2	$1.0x10^1$	1.00	$3.1x10^2$	2.49
	3	$8.0x10^1$	1.90	$2.8x10^2$	2.45
	4	$3.0x10^1$	1.48	$8.0x10^1$	1.90
	5	$<5.0x10^1$	0.70	$1.0x10^2$	2.00
	χ		1.28		2.14
	σ		0.41		0.34

Counts of enteric bacteria and *Staphylococcus aureus* (Results expressed in cfu/g)
Over 90 days there was no growth in any control and LAE treated samples.

Samples	MIRENAT-N		Control	
	Enteric bacteria	*Staphylococcus aureus*	Enteric bacteria	*Staphylococcus aureus*
1	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$
2	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$
3	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$
4	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$
5	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$	$<1.0x10^1$

Counts of *Salmonella sp* and *Shigella sp* and Lactobacillus (results expressed in cfu/g)
Over 90 days there was no growth in any control and LAE treated samples.

Samples	MIRENAT-N		Control	
	Salmonella/ Shigella	Lactobacillus	*Salmonella/ Shigella*	Lactobacillus
1	Absence	$<1.0x10^1$	Absence	$<1.0x10^1$
2	Absence	$<1.0x10^1$	Absence	$<1.0x10^1$
3	Absence	$<1.0x10^1$	Absence	$<1.0x10^1$
4	Absence	$<1.0x10^1$	Absence	$<1.0x10^1$
5	Absence	$<1.0x10^1$	Absence	$<1.0x10^1$

- **Counts of Sporulate bacteria** (Results expressed in cfu/g)

Over 90 days there was no growth in any control and LAE treated samples.

Samples	MIRENAT-N	Control
1	$<5.0x10^1$	$<5.0x10^1$
2	$<5.0x10^1$	$<5.0x10^1$
3	$<5.0x10^1$	$<5.0x10^1$
4	$<5.0x10^1$	$<5.0x10^1$
5	$<5.0x10^1$	$<5.0x10^1$

- **Chromatographical results:**

The stability of Lauric Arginate was maintained during the 90 days of the study.

Samples	LAE initial	LAE 90 days	LAS initial	LAS 90 days
1	78.4	85.4	0.0	0.0
2	88.6	84.2	0.0	0.0
3	87.7	85.3	0.0	0.0
4	83.9	85.5	0.0	0.0
5	84.0	99.7	0.0	0.0
χ	84.5	88.0	0.0	0.0
σ	3.6	5.9	0.0	0.0

* Units: mg/kg

- **Microbiological conditions:**

  - Total aerobic bacteria
  Culture media : Plate Count Agar (ISO 4833:1991)
  Conditions: aerobiosis, 37± 1ºC/72 hours
  Method: count in plate

  - Enteric bacteria
  Culture media : Agar V.R.B.G (ISO 7402:1985)
  Conditions: aerobiosis, 37± 1ºC / 24 hours
  Method: count in plate

  - *Staphylococcus aureus*
  Culture media : Brou baird (LMBG)
  Agar Baird Parker (ISO 6888:1983)
  Conditions: aerobiosis, 37± 1ºC / 48+48 hours
  Method: more probable number (MPN)

  - *Clostridium sp*
  Culture media : Agar SPS (CNAN)
  Agar Baird Parker (ISO 6888:1983)
  Conditions: aerobiosis, 44± 1ºC / 24+48 hours
  Method: count in tube

  - Sporulate bacteria
  Culture media : Count plate agar
  Conditions: aerobiosis, 44± 1ºC / 24+48 hours
  Method: count in tube

  - *Salmonella sp* and *Shigella sp*
  Culture media : Buffered peptone Water (ISO 6975:1993)
  Brou selenit Cistina (ISO 6975:1993)
  RVS semisolid
  Agar XLD (ISO 6975:1993)
  Agar SS (ISO 6975: 1993)
  Agar Kligler (ISO 6975:1993)
  Identification battery urea-indol and API 20E
  Conditions: aerobiosis, time-temperature depends of stage
  Method: investigation of absence/presence

- Lactobacillus

Culture media : MRS agar (according to Ministry of agriculture and Health)
Conditions: anaerobiosis, 37ºC ± 1ºC, 72 hours
Method: count in plate

TECHNICAL REPORT OF EFFICACY TESTS

LAURIC ARGINATE AS A PRESERVATIVE FOR SLICED CURED HAM

000201

I+D Department:

Sergi Figueras Study Director	Date
Carmen Codes Contributing Scientist	Date

Quality Assurance Department:

Roger Segret Principal Auditor	Date

Experimental Start Date: 25.08.04

Experimental Completion Date: 25.10.04

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000203

Terms Glossary

- **LAE :** Lauric arginate (the active ingredient is: Ethyl-N^{α}-lauroyl-*L*-arginate HCl)
- **LAS :** N^{α}-lauroyl-*L*-arginine. Hydrolysis product of LAE
- **MIRENAT-N :** is a formulation of LAE with propylene glycol and citric acid.

Introduction

The main characteristics of cooked ham are an almost neutral pH (around 6.0), a high water activity level (above 0.98) and a high concentration of proteins and sacharides. As a consequence of these characteristics, cooked ham is prone to microbiological spoilage.

Nowadays, cured ham is mainly used in slices. The manipulation process to slice cured ham increases significantly the risk of microbiological spoilage because surface of the cured ham is prone to microbiological contamination. Once the cured ham is sliced, the microbiological population is spread all over the slices resulting in a lack of safety to the product and to the consumer.

Objective of application of Lauric arginate

The aim of this study is to demonstrate how a treatment with Lauric arginate added to the mass and after treated the ham with an inmersion bath of Lauric arginate could preserve the cured ham without affecting its technological characteristics to improve the safety of the sliced cured ham.

Experimental procedure

Collaborator companies

SUBIRATS S.A
C/ Reus , number 1.
Industrial state La Florida
Sta. Perpètua de la Mogoda. 08130 Barcelona
Tlf: 93.574.31.32
NIF: A 59168112
The contact person: Mr. Miquel Jordá. (Quality responsible)

000204

Process

- The industrial process was carried out in Subirats according to the regular manufacture practises that are used in this sector.
- The ingredients employed are also the usually added to produce cured ham.
- The test was initiated by removing plastic wrapping from the product and the piece of ham was sliced in a slicing machine in hygienic conditions at Lamirsa laboratory.
- The control slices were immersed in a bath of distilled water and the treated samples were immersed in a bath at 10,000 mg LAE/L.
- An inoculum consisting in one strain of Listeria monocytogenes (ATCC 35152) was prepared the previous day in order to obtain a concentration of 1.0×10^6 cfu/g.
- Individual slices (25-35g each) were aseptically inoculated by adding 0.1 mL of the *Listeria monocytogenes* inoculum across the surface and then distributing it as a thin film over the surface. The inoculum resulted in a count of 3.0×10^3 cfu/g.
- Cooked ham slices were packed in vacuum sterile bags and stored under refrigeration (40ºF).

Treatments

Two lots of sliced cured ham were produced:

1. **Control** no preservative was added to the mass. Untreated samples were immersed in distilled water.
   Excess solution was allowed to drip from samples (30 seconds) before packaging.
2. **The dose of the active ingredient was LAE 175mg/kg** .Lauric Arginate was incorporated into the formulation by injection/tumbling.
   LAE immersion bath for the slices was prepared as at 10000 mg/L solution in deionised water for immersion for treatment of samples prior to packaging. Excess solution was allowed to drip from samples (30 seconds) before packaging.

000205

Details of the Lauric arginate used

MIRENAT-N is a formulation of LAE with propylene glycol and citric acid. The LAE used was batch No. 6420 and the MIRENAT-N was referenced as JMR-409. The analytical results were:

LAE (batch No. 6420):

Ethyl-N$^{\alpha}$-lauroyl-*L*-arginate HCl	74.9%	ID-11-1697
LAS	1.1%	ID-11-1697
Lauric acid	2.1 %	ID-11-2004
Ethyl laurate	1.4 %	ID-11-2004
Ashes (700 °C)	1.7 %	ID-11-083
Water	15.3%	ID-11-020

MIRENAT-N (ref. JMR-409)

Ethyl-N$^{\alpha}$-lauroyl-*L*-arginate HCl	19.50 %	ID-11-1697
Aspect	Clear liquid	
Density	1.046 g/cm^3	ID-11-030
Viscosity (Brookfield, spindle RV2, 50 rpm, 20°C)	88 mPa·s	ID-11-029
Index of refraction (20°C)	1.442	
pH (solution 1% H_2O deionized, 20°C)	3.26	

The methods ID-11-1697 and ID-11-2004 have been validated by an external laboratory, NOTOX.

Analysis conditions

- The analysis of each point was made over 2 samples , of the appropriated lot, that were chosen randomly.
- Frequency of analysis: initial, 1st, 2nd, 3rd, 4th, 6th and 8th weeks.
- The stored temperature for both lots was (0-5°C).
- The analysis were made at Lamirsa facilities.

- **Microbiological results**

Counts of *Listeria monocytogenes*. (Results expressed in cfu/g)

Days	Samples	Control		LAE	
		Listeria monocytogenes	**Log (cfu/g)**	***Listeria monocytogenes***	**Log (cfu/g)**
0 (Initial)	**1**	4.0×10^3	3.60	$<1.0 \times 10^2$	1.95
	2	2.0×10^3	3.30	$<1.0 \times 10^2$	1.95
	χ		3.45		1.95
1st	**1**	3.0×10^3	3.48	$<1.0 \times 10^2$	1.95
	2	2.2×10^3	3.34	$<1.0 \times 10^2$	1.95
	χ		3.41		1.95
2sd	**1**	5.8×10^3	3.76	$<1.0 \times 10^2$	1.95
	2	3.2×10^3	3.51	$<1.0 \times 10^2$	1.95
	χ		3.63		1.95
3rd	**1**	3.7×10^3	3.57	$<1.0 \times 10^2$	1.95
	2	7.2×10^3	3.86	$<1.0 \times 10^2$	1.95
	χ		3.71		1.95
4th	**1**	5.0×10^4	4.70	$<1.0 \times 10^2$	1.95
	2	2.0×10^4	4.30	$<1.0 \times 10^2$	1.95
	χ		4.50		1.95
6th	**1**	5.0×10^4	4.70	$<1.0 \times 10^2$	1.95
	2	5.0×10^4	4.70	$<1.0 \times 10^2$	1.95
	χ		4.70		1.95
8th	**1**	3.0×10^3	3.48	$<1.0 \times 10^2$	1.95
	2	3.0×10^3	3.48	$<1.0 \times 10^2$	1.95
	χ		3.48		1.95

000207

- **Microbiological conditions:**

  - *Listeria monocytogenes*
    Culture media : ALOA
    Conditions: aerobiosis, 37± 1°C / 48 hours
    Method: count in plate

Parameters analysed and frequency

Parameters analysed were:

- ✓ Organoleptical: aspect, odour and colour.
- ✓ Microbiological: *Listeria monocytogenes.*

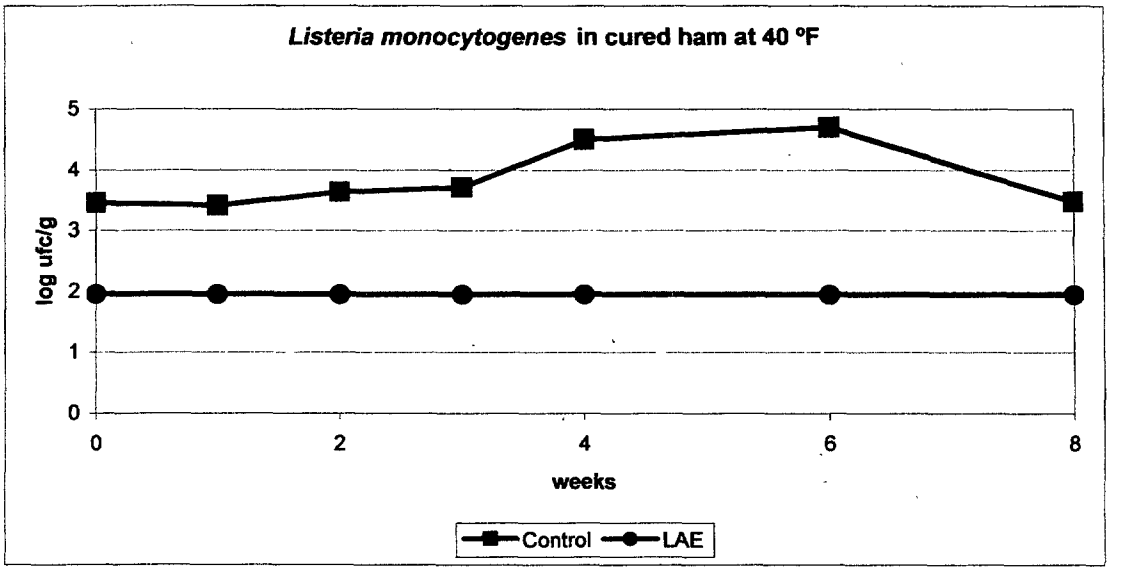
Results

Tables with the results are in ANNEXE 1.

Organoleptical results

At three weeks, the surface of the control samples was viscous and started to develop a bad odor. But treated samples had the aspect and the odor characteristic of the product during the eight weeks.

Microbiological results



Graphic 1. Evolution of *Listeria monocytogenes* in sliced cooked ham (8 weeks).

Conclusions

- Lauric arginate reduces the growth of *Listeria monocytogenes* of sliced cooked ham.
- Lauric arginate retards the degradation of slices cooked ham without affecting the organoleptical evolution of the product.

ANNEXE 1

- **Organoleptical results**

Weeks		Control	LAE 175 (active ingredient) mg/kg
Initial-2sd	Aspect	Characteristic of the product	Characteristic of the product
	Colour	Pink	Pink
	Odor	Characteristic of the product. Good odor.	Characteristic of the product. Good odor.
3rd	Aspect	Viscous surface	Characteristic of the product
	Colour	Pink	Pink
	Odor	The product has a slight bad odor.	Characteristic of the product. Good odor.
4th	Aspect	Viscous surface	Characteristic of the product
	Colour	Pink	Pink
	Odor	The product has a bad odor.	Characteristic of the product. Good odor.
6th	Aspect	Viscous surface	Characteristic of the product
	Colour	Pink	Pink
	Odor	The product has a bad odor.	Characteristic of the product. Good odor.
8th	Aspect	Viscous surface	Characteristic of the product
	Colour	Pink	Pink
	Odor	The product has a bad odor.	Characteristic of the product. Good odor.

TECHNICAL REPORT OF EFFICACY TESTS

LAURIC ARGINATE AS A PRESERVATIVE FOR DICED CURED HAM

000212

I+D Department:

Sergi Figueras Study Director	Date
Carmen Codes Contributing Scientist	Date

Quality Assurance Department:

Roger Segret Principal Auditor	Date

Experimental Start Date: 25.08.04

Experimental Completion Date: 25.10.04

000213

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000214

Terms Glossary

- **LAE :** Lauric arginate. The active ingredient is: Ethyl-N^{α}-lauroyl-*L*-arginate HCl
- **LAS :** N^{α}-lauroyl-*L*-arginine. Hydrolysis product of LAE
- **MIRENAT-N :** is a formulation of LAE with propylene glycol and citric acid.

Introduction

The main characteristics of cured ham are an almost neutral pH (around 6.0), a high water activity level (above 0.98) and a high concentration of proteins and sacharides. As a consequence of these characteristics, cured ham is prone to microbiological spoilage.

Nowadays, cured ham is mainly used in slices. The manipulation process to slice cured ham increases significantly the risk of microbiological spoilage. The surface of the cured ham is prone to microbiological contamination. Once the cured ham is diced, the microbiological population is spread all over the slices resulting in a lack of safety to the product and to the consumer.

Objective of application of Lauric arginate

The aim of this study is to demonstrate how a treatment with Lauric arginate in the mass and in a bath preserve the diced cured ham without affecting its technological characteristics to improve the safety of the diced cured ham.

Experimental procedure

Collaborator companies

SUBIRATS S.A
C/ Reus , number 1.
Industrial state La Florida
Sta. Perpètua de la Mogoda. 08130 Barcelona
Tlf: 93.574.31.32
NIF: A 59168112
The contact person: Mr. Miquel Jordá. (Quality responsible)

Process

- The industrial process was carried out in Subirats according to the regular manufacture practises that are used in this sector.
- The ingredients employed are also the usually added to produce cured ham.
- At Lamirsa facilities, the test was initiated by removing plastic wrapping from the product and the piece of ham was diced in hygienic conditions at Lamirsa laboratory.
- Ham dices were immersed in a LAE solution at 10,000 mg/L in deionised water for 30 seconds at 20ºC and allowed to drip.
- Control samples were immersed in distilled water and allowed to drip.
- An inoculum consisting in one strain of *Listeria monocytogenes* (ATCC 35152) was prepared the previous day in order to obtain a concentration of $1.0x10^7$ cfu/g.
- Individual dices were aseptically inoculated by adding 1% of the *Listeria monocytogenes* inoculum across the surface and then distributing it as a thin film over the surface. The inoculum resulted in a count of $1.0x10^5$ cfu/g.
- Cured ham dices were packed in vacuum sterile bags and stored at 40ºF and 66ºF.

Treatments

Two lots of diced cured ham were produced:

1. **Control** no preservative was added to the mass. Untreated samples were immersed in a bath of distilled water before inoculation.
   Excess solution was allowed to drip from samples (30 seconds) before packaging.
2. **The dose of active ingredient used was 175mg/kg** .Lauric Arginate was incorporated into the formulation by injection/tumbling.
   LAE immersion bath for the dices was prepared as at 10,000 mg/L solution in deionised water for the immersion of samples and before inoculation. Excess solution was allowed to drip from samples (30 seconds) before packaging.

Details of the Lauric arginate used

MIRENAT-N is a formulation of LAE with propylene glycol and citric acid. The LAE used was batch No. 6420 and the MIRENAT-N was referenced as JMR-409. The analytical results were:

LAE (batch No. 6420):

LAE (Ethyl-N^{α}-lauroyl-*L*-arginate HCl)	74.9%	ID-11-1697
LAS	1.1%	ID-11-1697
Lauric acid	2.1 %	ID-11-2004
Ethyl laurate	1.4 %	ID-11-2004
Ashes (700 °C)	1.7 %	ID-11-083
Water	15.3%	ID-11-020

MIRENAT-N (ref. JMR-409)

LAE (Ethyl-N^{α}-lauroyl-*L*-arginate HCl)	19.50 %	ID-11-1697
Aspect	Clear liquid	
Density	1.046 g/cm^3	ID-11-030
Viscosity (Brookfield, spindle RV2, 50 rpm, 20°C)	88 mPa·s	ID-11-029
Index of refraction (20°C)	1.442	
pH (solution 1% H_2O deionized, 20°C)	3.26	

The methods ID-11-1697 and ID-11-2004, have been validated by an external laboratory, NOTOX.

Analysis conditions

- The analysis of each point was made over 2 samples , of the appropriated lot, that were chosen randomly.
- The stored temperature for both lots was 40°F and 66°F
- Frequency of analysis:
  - 40°F → initial and 59th days.
  - 66°F → initial, 1st, 2nd and 3rd day.
- The analysis were made at Lamirsa facilities.

000217

Parameters analysed and frequency

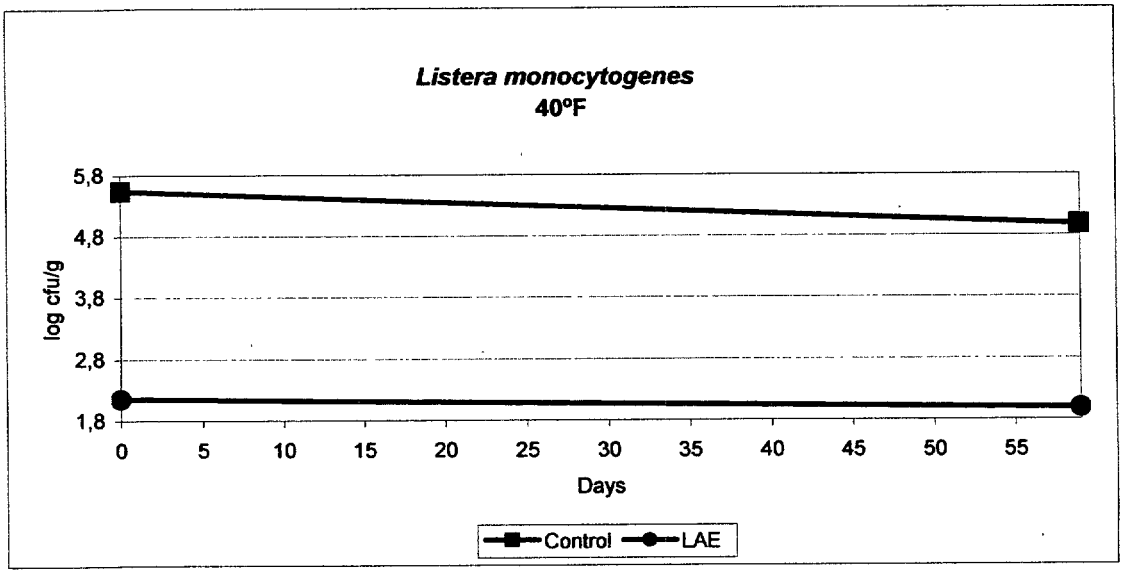
Parameters analysed were:

- ✓ Microbiological: *Listeria monocytogenes.*

Results

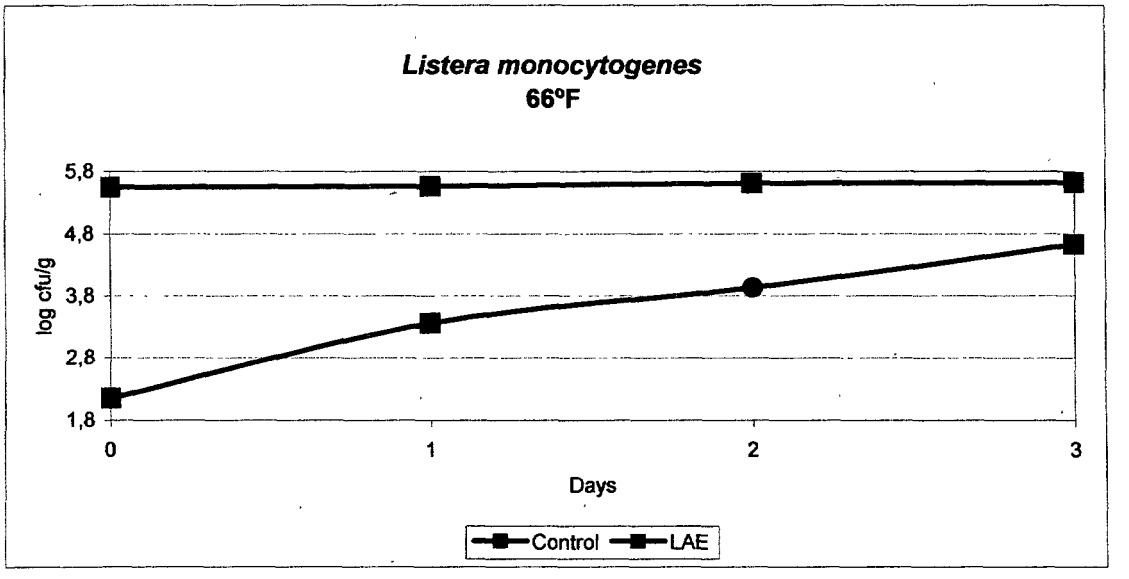
Tables with the results are in ANNEXE 1.

Microbiological results (JMR-401)



Graphic 1. Evolution of *Listeria monocytogenes* in diced cured ham stored at 40°F.

000218



Conclusions

- Lauric arginate reduces the growth of *Listeria monocytogenes* of diced cured ham.

000219

ANNEXE 1

- **Microbiological results**

Counts of *Listeria monocytogenes* at 66°F. (Results expressed in cfu/g)

Days	Samples	LAE		Control	
		Listeria monocytogenes	**Log (cfu/g)**	***Listeria monocytogenes***	**Log (cfu/g)**
0 (Initial)	**1**	1.0×10^2	2.00	3.2×10^5	5.50
	2	2.0×10^2	2.30	3.8×10^5	5.60
	χ		2.20		5.50
1st	**1**	2.0×10^3	3.30	2.4×10^5	5.40
	2	2.7×10^3	3.40	5.6×10^5	5.70
	χ		3.40		5.60
2nd	**1**	7.0×10^4	4.40	1.9×10^5	5.30
	2	7.4×10^3	3.90	8.8×10^5	5.90
	χ		3.90		5.60
3rd	**1**	3.5×10^4	4.50	3.6×10^5	5.60
	2	5.3×10^4	4.70	4.8×10^5	5.70
	χ		4.60		5.60

000220

Counts of *Listeria monocytogenes* at 40°F. (Results expressed in cfu/g)

Days	Samples	LAE		Control	
		Listeria monocytogenes	**Log (cfu/g)**	***Listeria monocytogenes***	**Log (cfu/g)**
0 (Initial)	**1**	$1.0x10^2$	2.00	$3.2x10^5$	5.50
	2	$2.0x10^2$	2.30	$3.8x10^5$	5.60
	χ		2.20		5.50
59st	**1**	$<1.0x10^2$	2.00	$3.8x10^4$	4.60
	2	$<1.0x10^2$	2.00	$2.4x10^5$	5.40
	χ		2.00		5.00

- **Microbiological conditions:**

  - *Listeria monocytogenes*

  Culture media : ALOA
  Conditions: aerobiosis, 37± 1°C / 48 hours
  Method: count in plate

000221

AM

JHeimbach LLC

2006-4158

April 28, 2006

RECD MAY 3 2006

Antonia Mattia, Ph.D.
Director
Division of Biotechnology and GRAS Notice Review (HFS-255)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Mattia:

In the spring of 2005, I submitted a notice on behalf of Laboratorios Miret, S.A., stating our basis for concluding that lauramide arginine ethyl ester (LAE) is GRAS for use as an antimicrobial agent at levels up to 200 ppm of its active ingredient (GRN 000164). The major component was described as being in the form of the hydrochloride salt. FDA had no questions concerning our determination.

We have interest in using this compound in the form of salts of GRAS acids other than HCl. In particular, lactic acid (21 CFR 184.1061), acetic acid (21 CFR 184.1005), glutamic acid (whose sodium salt is described to be GRAS in 21 CFR 182.1), citric acid (21 CFR 184.1033), and phosphoric acid (21 CFR 182.1073) are common acids that will form salts of LAE of potential interest to my client. All are considered to be GRAS with no limitations other than current good manufacturing practice[1]. We would intend to use only acids complying with food grade specifications to prepare the salts of LAE.

While we agree that reaction products of GRAS ingredients are not necessarily GRAS, we understand that it has been FDA's longstanding policy that when GRAS acids and bases are used to adjust pH, the salts they form are considered to be equally safe and lawful[2]. While this may not apply if such use would change exposure dramatically, that is not the case here where use would not have a significant effect on exposure.

---

1 The affirmation for lactic acid excludes use in infant foods and infant formula products and we would not intend to use the lactic acid salts of LAE for such foods. Indeed, our GRAS determination for the existing form of LAE does not encompass such foods.

2 The Food Additives Status List on OFAS' web site contains the following statement: "For example, in the case of boiler water additives or other minor ingredients, processing aids, or indirect additives, FDA has not objected, in certain cases, to the substitution of ammonium, calcium, magnesium, potassium, or sodium salts for each other when only one is listed in a regulation."

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tel. (+1) 202-237-8406 fax (+1) 202-478-0986 JHeimbach@aol.com

000237.001

We believe that the use described above is safe and lawful, consistent with standard regulatory interpretation, and would not justify the time and effort for FDA to review a new GRAS notification.. Because many uses of these salts would be in products under FSIS' jurisdiction, we need written assurance that FDA does not disagree with our interpretation.

We would appreciate a response confirming our understanding.

Sincerely,

(b)(6)

James T. Heimbach, Ph.D., F.A.C.N.
President

SU

JHeimbach LLC

December 14, 2006

Edmundo Garcia, Jr., Ph.D.
Consumer Safety Officer
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Edmundo:

Since you have been the Consumer Safety Officer for the GRAS notice regarding lauramide arginine ethyl ester (LAE), which was filed in 2005 as GRN 164, I wanted to bring you up to date on an expansion of the intended use of this substance. I am not submitting this change as a GRAS notice, and no reply to this letter is necessary.

My client, LAMIRSA, has determined that the addition of LAE to carbonated beverages at a maximum addition level of 100 ppm of the active ingredient, corresponding to 112.5 ppm of LAE, is also GRAS.

LAMIRSA's GRAS Expert Panel, Joseph Borzelleca, Robert Nicolosi, Michael Pariza, and John Thomas, has reviewed LAMIRSA's intended use and agrees that it is GRAS.

As noted above, I am providing this information to FDA in order to keep you informed, and am not requesting either a review of this GRAS determination or a reply to this letter.

Sincerely,

(b)(6)

James T. Heimbach, Ph.D., F.A.C.N.
President

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