GR

ORIGINAL SUBMISSION

000010

OLSSON, FRANK AND WEEDA, P.C.

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA (1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

Sender's Direct Phone (202) 518-6366
Sender's Direct Facsimile (202) 234-3550

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SUSAN P. GRYMES
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE M. BRIGGS*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

May 23, 2003

BY FEDERAL EXPRESS

03-05-279 92910-M

Dr. Linda S. Kahl
Food and Drug Administration
Office of Food Additive Safety
HFS-255
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Lactoferrin

Dear Dr. Kahl:

As counsel to aLF Ventures, LLC, we are simultaneously both providing this Notification of GRAS status to the Food and Drug Administration and filing a Request for Acceptability Determination with the United States Department of Agriculture in regard to the use of bovine milk-derived lactoferrin as an antimicrobial spray on beef carcasses. In the interest of simplification, the same submission is being provided to both FDA and USDA. We request your expedited review of this Notification because the use of lactoferrin covered by this notification will have a significant impact on food safety and decrease the incidence of food-borne illness through its antimicrobial action against the pathogen *E.coli* O157:H7.

Expeditious review is appropriate for this Notification, because lactoferrin will be used as the antimicrobial agent in a carcass wash solution, immediately after the hide has been removed in the slaughter process. This use of lactoferrin inhibits pathogenic *E.coli* O157:H7 bacteria from attaching to the carcass, and thereby substantially decreases the risk that *E.coli* O157:H7 will be present on the beef produced from carcasses which pass through a lactoferrin wash.

We anticipate that FDA and USDA will move forward with consideration of both this Notification and the request to USDA for an Acceptability Determination pursuant to the terms of the Memorandum of Understanding between the two agencies regarding the listing or approval of food ingredients used in the production of meat and poultry products. That Memorandum of Understanding provides that where "FDA receives a GRAS notice regarding the use of a substance in the production of meat or poultry products," or "when FSIS receives a request for an acceptability determination," the two agencies will consult with each other as

necessary and provide written responses to the other agency within 60 days. Because of the urgent need to the decrease food-borne illness associated with *E.coli* O157:H7, we respectfully request that both FDA and USDA complete these reviews in substantially less than this 60-day target period.

A previous GRAS Notification (GRSN 67) from aLF Ventures relating to multiple uses of lactoferrin in antimicrobial sprays for beef carcasses and cuts should help to expedite FDA review of this Notification. The previously reviewed and accepted GRAS Notification addressed all of the relevant safety factors, except for the potential allergenicity of this milk-derived select substance. Because allergenicity had not been considered by the panel conducting the GRAS review, retail product labeling was required.

The present Notification is directed exclusively to the issue of allergenicity and is specifically limited to the use of lactoferrin in carcass washes. The dietary exposure from this application is less than 10% of the potential exposure from the use of lactoferrin described in GRSN 67. This Notification is based on a GRAS determination reached by a preeminent panel of the nation's leading allergists. The panel's conclusions rely on a variety of factors including (1) the minimal levels of lactoferrin which may remain after the use of lactoferrin antimicrobial sprays, (2) the presence of equal or greater amounts of endogenous lactoferrin in the beef which we consume every day, and (3) the absence of casein and other known milk allergens in the spray.

We respectfully request the most expeditious possible response to this notification.

Sincerely,

/Philip C. Olsson

PCO.lss
Enclosures

cc: Robert C. Post, Ph.D.

OLSSON, FRANK AND WEEDA, P.C.

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA (1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

Sender's Direct Phone (202) 518-6366
Sender's Direct Facsimile (202) 234-3550

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SUSAN P. GRYMES
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE M. BRIGGS*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

May 23, 2003

BY SCHEDULED EXPRESS

Robert C. Post, Ph.D., Director
Labeling and Consumer Protection Staff
FSIS, USDA
1400 Independence Ave, S.W.
(Suite 602, Annex)
Washington, DC 20250

Re: Request for Acceptability Determination

Dear Dr. Post:

As counsel to aLF Ventures, LLC, we are simultaneously submitting both this Request for Acceptability Determination and a Notification of GRAS status to the Food and Drug Administration with regard to the use of bovine milk-derived lactoferrin as an antimicrobial spray on beef carcasses. In the interest of simplification, the same submission is being provided to both USDA and FDA. We request your expedited review of this Request because the use of lactoferrin covered by this notification will have a significant impact on food safety and decrease the incidence of food-borne illness through its antimicrobial action against the pathogen *E.coli* O157:H7.

We respectfully request the most expeditious possible response to this notification.

Sincerely,

Philip C. Olsson

PCO.lss
Enclosures

cc: Dr. Linda S. Kahl, OFAS/FDA (w/o enclosures)
Philip Derfler, Esq.

GRAS Notification for Bovine Milk-Derived Lactoferrin in Beef Carcass Spray Applications

Submitted by aLF Ventures, L.L.C.
May 23, 2003

Table of Contents

A: Section I	GRAS Claim
B: Section II	Identity of the Notified Substance
C: Section III	Information on Self-Limiting
D: Section IV	Basis for Notifier's Claim
E: Section V	Scientific Support Document
F: Section VI	GRAS Panel Report

* * *

G: Attachment A	Appendices to Scientific Support Document

A

000016

Section I

GRAS Claim

aLF Ventures, L.L.C. hereby submits this Notification regarding the use of bovine mild-derived lactoferrin as a spray on beef carcasses.

A. Name and address of Notifier:

aLF Ventures, L.L.C.
299 South Main Street
Suite 2310
Salt Lake City, Utah 84111

B. Common or Usual Name of Substance:

Bovine milk-derived lactoferrin. The published literature may also refer to this lactoferrin as red milk protein, lactosiderophilin, ekkrinosiderophilin, or lactotransferrin

C. Conditions of Use:

The bovine milk-derived lactoferrin will be used as an antimicrobial spray on beef carcasses so that the residual level of exogenous lactoferrin does not exceed 800 nanograms (ng) per gram of beef. This is equivalent to 800 parts per billion (ppb) of exogenous lactoferrin in the beef carcass. Typically, the bovine milk-derived lactoferrin will be applied as a component of an antimicrobial spray. The spray will contain up to 2.0% lactoferrin and will be applied at a rate not to exceed 0.20 mL/Kg of beef. Following application of the spray, the carcass will be rinsed with a lactic acid solution to further reduce the residual lactoferrin.

D. Basis for GRAS Determination:

aLF Ventures has convened a GRAS review panel consisting of preeminent allergenists and immunologists. These experts have concluded that bovine milk-derived lactoferrin, used as a component (at not more than two percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb), is determined to be GRAS without the requirement of labeling of food products produced from carcasses so treated.

Based on the conclusion of the expert panel, aLF Ventures has concluded that the use of bovine milk-derived lactoferrin, as described in paragraph C above is GRAS. The panel's conclusions rely on a variety of factors including the minimal levels of lactoferrin which may remain after the use of lactoferrin antimicrobial sprays, on the presence of equal or greater amounts of

endogenous lactoferrin in the beef which we consume every day, and on the absence of casein and other known milk allergens in the spray.

E. Data Availability Statement:

The data and information that are the basis for the notifier's GRAS determination are detailed in the bibliography attached to the GRAS Determination document in Section VI of this Notice. They are available for the Food and Drug Administration's (FDA) review and/or will be sent to FDA upon request during reasonable business hours at the offices of:

Claire L. Kruger, Ph.D., D.A.B.T.
Principal
Environ Health Science Institute
4350 North Fairfax Drive
Suite 300
Arlington, VA 22203
(703) 516-2309

Respectfully submitted,

Ram Nimmagudda
Vice President of Operations
aLF Ventures, L.L.C.

B

Section II

Identity of the Notified Substance

The substance that is the subject of this notification is bovine milk-derived lactoferrin. It is the same substance that was the subject of GRN 67. All information regarding the identity of bovine milk-derived lactoferrin submitted in the previous Notification is included herein by reference.

To address the issue of potential protein contamination of the bovine milk-derived lactoferrin product, aLF conducted chromatographic and other studies. No impurities of allergenic concern were identified. These studies are discussed in detail on page 4 of Section VI.

C

Section III

Information on Self-Limiting Levels of Use

As noted in Section I, this Notification is limited to those applications where the residual level of exogenous lactoferrin does not exceed 800 nanograms (ng) per gram of beef. aLF Ventures' testing has confirmed that this level is effective in reducing microbial contamination. Higher levels of the lactoferrin spray are not cost effective and are not addressed by this Notification.

D

Section IV

Basis for Notifier's Claim

Section V is a detailed report of the scientific basis for the opinion of the GRAS Review Panel. Section VI is the opinion of the GRAS review panel,"Generally Recognized As Safe (GRAS) Determination for the Use of Bovine Milk-Derived Lactoferrin as a Component of an Antimicrobial Beef Carcass Spray Without Requiring Labeling for Lactoferrin." These documents set forth in detail the basis for aLF Ventures' claim of GRAS status. This claim is based on scientific procedures as detailed in publicly available scientific literature.

E

Section V

SCIENTIFIC SUPPORT DOCUMENT FOR THE GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF BOVINE MILK-DERIVED LACTOFERRIN AS A COMPONENT OF AN ANTIMICROBIAL BEEF CARCASS SPRAY WITHOUT REQUIRING LABELING FOR LACTOFERRIN

SCIENTIFIC SUPPORT DOCUMENT FOR THE GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF BOVINE MILK-DERIVED LACTOFERRIN AS A COMPONENT OF AN ANTIMICROBIAL BEEF CARCASS SPRAY WITHOUT REQUIRING LABELING FOR LACTOFERRIN

Prepared for:

aLF Ventures, LLC
Fraser, New York

Prepared by:

ENVIRON International Corporation
Arlington, Virginia

May 2003

TABLE OF CONTENTS

		Page
I.	BACKGROUND	4
II.	RATIONALE	6
III.	LEGAL REQUIREMENTS OF FOOD LABELING	9
IV.	STRUCTURAL COMPARISON OF BOVINE MILK AND NEUTROPHIL LACTOFERRIN	11
	A. Human Milk and Neutrophil Lactoferrin	11
	B. Bovine Milk and Neutrophil Lactoferrin	12
	C. Conclusions	12
V.	ENDOGENOUS LACTOFERRIN LEVELS IN BEEF	14
	A. Neutrophil Lactoferrin Content	14
	B. Lactoferrin in Blood Plasma	15
	C. Estimating Endogenous Lactoferrin Levels in Beef	16
	1. Estimating Levels of Lactoferrin in Beef Based on a Review of Published Literature	16
	2. An Empirically Derived Estimate of Endogenous Lactoferrin Levels in Beef	18
	D. Conclusions	18
VI.	EXOGENOUS LACTOFERRIN LEVELS IN BEEF	19
VII.	ESTIMATES OF BEEF CONSUMPTION IN THE U.S.	22
VIII.	ESTIMATES OF LACTOFERRIN INTAKE	25
	A. Estimates of Endogenous Lactoferrin Intake	25
	B. Estimates of Exogenous Lactoferrin Intake	26
	C. Conclusions	27
IX.	LACTOFERRIN INTAKES FROM DAIRY AND WHEY PRODUCTS ADDED TO FOOD	29
X.	LACTOFERRIN AS A POTENTIAL HUMAN FOOD ALLERGEN	32
XI.	LITERATURE CITED	36

000028

TABLES

		Page
Table 1	EPA Beef Commodity Codes	23
Table 2	Estimates of Beef Consumption: 2-Day Average Intake and Intake per Eating Occasion	24
Table 3	Estimated Background Lactoferrin Intakes versus Estimated 50th and 90th Percentile Intakes of Bovine Milk-derived Lactoferrin from Lactoferrin-treated Beef Carcasses	28
Table 4	Estimate of per Capita Daily Intake of Bovine Lactoferrin from Milk and Whey Proteins	31

APPENDICES

Appendix I: Three Studies Conducted by National Beef to Measure Endogenous Levels of Lactoferrin in Beef

Appendix II: Four Beef Carcass Washing Studies Conducted by National Beef

Appendix III: Information Provided by National Beef Describing the Steps Involved in Meat Processing and Details Regarding the Lactoferrin Application Process

Appendix IV: Product Specification

000029

I. BACKGROUND

As described in generally recognized as safe ("GRAS") notification number 67 ("GRN 67") previously submitted to the U.S. Food and Drug Administration ("FDA") by Farmland National Beef Packaging Company, L.P. ("National Beef"), bovine milk-derived lactoferrin is to be used as a component (at not more than 2 percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses prior to a final lactic acid rinse during processing. This specific use of bovine milk-derived lactoferrin (along with two other uses) was previously determined to be GRAS by National Beef, one of the founding partners of aLF Ventures, LLC. The FDA was notified of this GRAS determination on January 9, 2001, via GRN 67. The following three specific proposed uses of an antimicrobial spray formulation containing bovine milk-derived lactoferrin were the subject of GRN 67:

Application to beef carcasses at a concentration not to exceed ("NTE") 0.20 ml of formulation per kg beef;
Application to subprimal beef parts at a concentration NTE 0.56 ml of formulation per kg beef; and
Application to finished cuts of beef at a concentration NTE 2.5 ml of formulation per kg beef.

The cumulative exposure to lactoferrin from these three proposed uses was estimated by National Beef to be 4.1 mg per person per day for the "typical" consumer of beef products and 9.1 mg per person per day for the "heavy" consumer of beef products. The FDA's response to GRN 67, dated October 23, 2001, contained the following statement:

> "... the agency has no questions at this time regarding National Beef's conclusion that milk-derived lactoferrin is GRAS under the intended conditions of use, provided that the ingredient statement of food products that contain milk-derived lactoferrin identifies the source of the protein."

Therefore, although the FDA had no questions concerning the GRAS status of bovine milk-derived lactoferrin for the proposed uses, the Agency did require that any food products containing bovine milk-derived lactoferrin resulting from the proposed uses be appropriately labeled. This labeling requirement was requested by the Agency because of potential allergenicity thought to be associated with bovine milk-derived lactoferrin exposures at the levels estimated in GRN 67. Furthermore, this labeling requirement was implemented to ensure that consumers who are allergic to milk would be aware of the potential presence of this milk-derived ingredient in beef products that they might consume.

This current document addresses only the first use of bovine milk-derived lactoferrin listed above (i.e., application to beef carcasses), and provides a legal and scientific analysis as to why the GRAS status of this specific use (which yields a much lower estimated exposure to bovine milk-derived lactoferrin than that considered under GRN 67) should not require labeling of food products produced from carcasses so treated. The labeling requirement for the two remaining proposed uses of bovine milk-derived lactoferrin (i.e., application of lactoferrin to subprimals and to finished cuts of beef) are not addressed in this document. For these two proposed uses, appropriate labeling of products to which bovine milk-derived lactoferrin has been applied remains a condition of their GRAS status.

000031
ENVIRON

II. RATIONALE

As discussed in the previous chapter, the purpose of this document is to provide a legal and scientific assessment as to why the GRAS status for the proposed use of bovine milk-derived lactoferrin to treat beef carcasses should not require the labeling of food products produced from carcasses so treated. Again, as indicated previously, this labeling requirement was implemented by the FDA as a result of GRN 67 to ensure that consumers who are allergic to milk will be aware of the potential presence of this milk-derived ingredient in beef products that they might consume. The assumption made in GRN 67 is that exposure to bovine milk-derived lactoferrin would be up to 9.1 mg per person per day in "heavy" consumers of beef.

The two key components of this assessment as to whether labeling should be required for the GRAS status of bovine milk-derived lactoferrin applied only to beef carcasses are: 1) a determination that exogenous lactoferrin exposure (resulting from its application onto beef carcasses) is estimated to be in the same range as existing background lactoferrin exposures from naturally occurring endogenous lactoferrin in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i.e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use). These two key components will be addressed in this document by demonstrating the following points:

- Legal and regulatory precedents suggest that when the addition of a processing aid does not substantively impact the total intake of that substance, and this exogenous addition will not produce a significant health or safety concern, then labeling for this substance is not required.

- Lactoferrin found naturally in uncooked beef is virtually identical (both structurally and functionally) to the bovine milk-derived lactoferrin that is to be applied to beef carcasses as an antimicrobial spray as described in GRN 67;

- The predominant source of lactoferrin in uncooked beef is from neutrophils present in residual blood, and results in lactoferrin concentrations in beef of approximately 200 ng per g or parts per billion ("ppb");

- The intended use of bovine milk-derived lactoferrin as an antimicrobial spray treatment on beef carcasses will result in concentrations of this source of lactoferrin in the edible meat of approximately 320 ng per g (ppb) if the lactoferrin is assumed to be equally distributed throughout the carcass, and approximately 82 ng per g (ppb) in boxed beef and 726 ng per g (ppb) in trim/grind if the lactoferrin is assumed to be unequally distributed throughout the carcass;

- The range of 50th percentile intakes of bovine milk-derived lactoferrin resulting from its application to beef carcasses is within the range of estimated background intakes of lactoferrin from naturally occurring endogenous levels of lactoferrin in beef;

- The estimated intakes of bovine lactoferrin from dairy and whey products added to food demonstrate the very high background dietary exposure to lactoferrin (in the mg range) from these sources relative to the estimated lactoferrin intakes (in the μg range) that could potentially result from the use of bovine milk-derived lactoferrin as an antimicrobial spray treatment on beef carcasses;

- Bovine milk-derived lactoferrin is a minor cows' milk allergen. Among individuals with documented cows' milk allergy, under 50 percent have IgE antibody binding to lactoferrin. However, the fact that: 1) milk-derived and beef-derived lactoferrin are allergenically equivalent, 2) beef allergies are uncommon and no evidence exists whether or not lactoferrin is an allergen in beef, and 3) the proposed use of bovine milk-derived lactoferrin on beef carcasses is not expected to significantly increase typical lactoferrin exposures from meat consumption above background, makes the likelihood of an allergic reaction from the use of bovine milk-derived lactoferrin as an antimicrobial spray on beef carcasses very low; and

- The lowest dose that elicits an observable allergic reaction in sensitized individuals exposed to well-recognized food allergens such as peanuts, eggs, and cows' milk (standardized on protein content) appear to be in the low milligram range or higher for most individuals with allergies to these particular foods, while estimated intakes of bovine milk-derived lactoferrin under its intended conditions of use described in this document are in the low microgram range, a number of orders of magnitude lower than the elicitation threshold for peanuts, eggs, and cows' milk in the vast majority of sensitized individuals. Therefore, the possibility that lactoferrin would elicit an allergic reaction in any individuals at the estimated levels of ingestion described in this document is unlikely.

000033

Each of these points will be further elaborated in the remaining chapters of this document. (The issue of any concern from allergenic potential of protein contamination of the bovine milk-derived lactoferrin product was addressed; several cationic exchange chromatography, gel permeation chromatography (GPC), and high performance liquid chromatography (HPLC) analyses were conducted. The four allergenic proteins present in milk that would be of concern if they were present in substantial quantity in the bovine milk-derived lactoferrin product are alpha-lactalbumin (α-La), beta-lactoglobulin (ß-Lg), caseinmacropeptide (glycomacropeptide; GMP), and bovine serum albumin (BSA). A chromatogram of a mixture of standards of these four proteins of concern and a chromatogram of a sample of bovine milk-derived lactoferrin product were compared. The figure of this overlay shows that the lactoferrin product does not have any peaks that correspond to the peaks for these proteins of concern (at a detection limit of approximately 10 mg/L or 10 ppm). Thus, there is sufficient evidence to conclude that the non-lactoferrin protein fraction does not consist of one or more of these proteins of allergenic concern (Data is included in Appendix IV). In

fact, this is expected based on the production process for the bovine milk-derived lactoferrin product. The proteins of concern (α -La, ß -Lg, BSA, GMP) are not extracted by their nature; i.e., their ionic charge is opposite that of lactoferrin. Furthermore, if residues of some of these proteins did remain on the ion exchanger column at the end of the adsorption cycle, they would be washed off in one of the washing steps before bovine milk-derived lactoferrin is recovered from the column.)

III. LEGAL REQUIREMENTS OF FOOD LABELING

The present document relates only to lactoferrin treatment of beef carcasses and not subprimals or finished cuts of beef. The carcasses are treated with bovine milk-derived lactoferrin at a concentration not to exceed 0.20 ml of formulation per kilogram of beef. The meat from these carcasses is intended for further processing prior to being packaged as a final food product for sale at the retail level. The level of ingestion of bovine milk-derived lactoferrin from this one use is therefore much lower than the cumulative exposure resulting from all three uses described in GRN 67. The FDA's response to GRN 67 reflected the higher exposure levels contained in that GRAS determination.

Bovine milk-derived lactoferrin is applied to beef carcasses simply to facilitate removal of microorganisms adhering to the carcass surface prior to further processing. The applied bovine milk-derived lactoferrin is rinsed from the carcass using a lactic acid wash, and it is not intended to have a functional effect in the finished food. Thus, bovine milk-derived lactoferrin functions as a processing aid in the preparation of beef products that may also be used as ingredients in other foods.

Section 403(i) of the Federal Food, Drug, and Cosmetic Act ("FFDCA") and 21 CFR §101.4 require the listing of all ingredients in a food fabricated from two or more ingredients, although the Secretary may establish exemptions from this requirement under appropriate circumstances. One such exemption established by the Secretary is for incidental additives present in food at insignificant levels, such as processing aids, as defined under 21 CFR §101.100(a)(3)(ii). In the current application under consideration, bovine milk-derived lactoferrin is removed to the extent practicable from the surface of the carcass with a lactic acid rinse. Furthermore, bovine milk-derived lactoferrin is not intended to have a technical or functional effect in the finished food product. As such, it is properly considered a processing aid, exempt from the requirement of inclusion in the ingredient list under 21 CFR §101.100(a)(3). 000035

The FDA's requirement that products containing bovine milk-derived lactoferrin be labeled as such arose from concerns about potential allergenicity at the levels of ingestion cited in GRN 67. The estimated levels of lactoferrin ingestion from food containing beef from carcasses treated with bovine milk-derived lactoferrin is only a small fraction of the lactoferrin intake estimates reported in GRN 67. As shown in Chapter V of this document, beef carcasses naturally contain a background level of lactoferrin that is essentially indistinguishable from the bovine milk-derived lactoferrin applied to beef carcasses under the proposed use. As shown in Chapter VIII of this document, the range of estimated intakes of endogenous lactoferrin that result from untreated beef consumption can be compared against the estimated intakes of exogenous bovine milk-derived lactoferrin that occur from consumption of beef from lactoferrin-treated carcasses. This comparison

shows that the range of 50th percentile intakes of exogenous bovine milk-derived lactoferrin is within the range of estimated background exposures to lactoferrin across all population groups of interest. For example, endogenous lactoferrin intakes are estimated to range from < 0.3 to 30 μg per occasion for adults 20 years and older, while the estimated median intake of bovine milk-derived lactoferrin from lactoferrin-treated beef carcasses in this same population group is 16 μg per eating occasion.

The FDA has previously addressed concerns about the known allergenicity of a processing aid in establishing 21 CFR §101.100(a)(4) regarding the use of sulfites in food. The FDA determined that the presence of sulfites, even if otherwise exempt as a processing aid, must be disclosed if the level of sulfites is more than "insignificant." The FDA defined an insignificant level of sulfites as 10 ppm or less. This 10 ppm upper limit was based on a threshold limit of detection, employing then-currently available analytical methodologies, rather than on some biological (i.e., allergenicity) threshold [51 Federal Register 25012, 25014 (July 9, 1986)]. But in the lactoferrin situation, where the foods already contain a naturally occurring background level of the substance, such a labeling requirement does not make sense. Consumers who eat beef are already being exposed to lactoferrin, and the estimated typical exposure from the proposed exogenous source of lactoferrin falls within this normal background range of intake. Thus, this is not a situation where a new, foreign substance is being added to food, but rather where there is only an insignificant addition of a substance already present.

Section 201(n) of the FFDCA requires that all material facts about a food be disclosed on the label of the food, including all information that may be material to a consumer regarding the safety of the food. This provision may not be interpreted, however, to require disclosure of all information that consumers merely desire to know [IDFA v. Amestoy, 92 F.3d 67, 73 (2d Cir. 1996)]. The required element of "materiality" is that it must be based on a true health or safety concern. Here, where the potential allergen is already a naturally-occurring component of food, the level of ingestion of the component in food is not increased significantly above the range of intakes derived from naturally occurring sources of the component, the component is not intended to have a technical or functional effect in the finished food, and the allergenicity of the component has not been firmly established, there can be no justification for requiring labels on foods containing beef from carcasses treated with bovine milk-derived lactoferrin to state that fact on the label.

IV. STRUCTURAL COMPARISON OF BOVINE MILK AND NEUTROPHIL LACTOFERRIN

As discussed previously, the FDA has raised the concern that the treatment of beef carcasses with lactoferrin would leave traces of bovine milk-derived lactoferrin on the carcass, and thus there is a theoretical risk that persons allergic to milk proteins might react to this residual bovine milk-derived lactoferrin. As described in the next chapter, endogenous levels of lactoferrin are present naturally in uncooked beef, and thus people are exposed daily to this source of lactoferrin. However, it could be argued that milk- and tissue-derived (i.e., neutrophil and plasma) lactoferrins are different proteins, and might therefore have different allergenic properties. This chapter discusses what is known about the structure of bovine milk versus neutrophil lactoferrins, and the impact this structure may have on allergenic properties of these proteins.

A. Human Milk and Neutrophil Lactoferrin

The first lactoferrin protein sequence to be published was for human milk lactoferrin (Metz-Boutigue et al. 1984), using amino acid sequencing. Subsequently, human neutrophil lactoferrin was cloned and sequenced from a myeloid library (Rado et al. 1987), and the deduced amino acid sequence differed by only three amino acids from that of the milk-derived protein, presumably arising from sequencing errors. It therefore appears that human milk and neutrophil lactoferrins are coded by the same gene and the protein products are identical. Although functionally different forms of lactoferrin have been proposed (Broxmeyer et al. 1980; Furmanski et al. 1989), these have not been confirmed and no supporting biochemical basis for this heterogeneity has been advanced. The only structural variation in the human lactoferrin polypeptide chain so far reported is a non-secreted splice-variant form (Δ-lactoferrin) lacking the N-terminal signal sequence (Siebert and Huang 1997). Although gene polymorphisms occur in human lactoferrin (Liu et al. 2002), it is now accepted that there is only a single functional human lactoferrin gene (Teng 2002) located on chromosome 3 (Teng et al. 1987).

000037

The one structural difference that does exist between human milk and neutrophil lactoferrins is in their glycosylation. Human milk lactoferrin glycans contain fucose, while neutrophil lactoferrin glycans do not (Derisbourg et al. 1990), and thus are more similar to the glycans of serum transferrin (Spik et al. 1994). However, apart from the lack of fucose, the composition of human neutrophil lactoferrin glycans is very similar to that of milk lactoferrin glycans (Derisbourg et al. 1990). Both forms were found to be functionally identical with regard to regulation of myelopoiesis (Broxmeyer et al. 1986) and neutralization of heparin (Wu et al. 1995). There are no reports of glycans affecting the immunogenicity of lactoferrin, and moreover, the glycans do not affect the rate of clearance of lactoferrin from the circulation by the liver (Moguilevsky et al. 1984).

B. Bovine Milk and Neutrophil Lactoferrin

Although there is less comparative structural data available for bovine milk and neutrophil lactoferrins, the situation seems very similar to that of human lactoferrin. Bovine milk lactoferrin was first cloned and sequenced from an involuting mammary gland library by Mead and Tweedie (1990). The deduced amino acid sequence of the secreted protein contained 689 amino acids, and this sequence was confirmed by Goodman and Schanbacher (1991) and by Seyfert et al. (1994), both of whom also cloned the bovine milk lactoferrin gene from a mammary gland library. An identical sequence was also deduced following cloning and sequencing from a bovine submaxillary gland library (Pierce et al. 1991). These investigators noted that the sequence showed 69% and 64% homology to human and murine lactoferrins, respectively, and contained five potential glycosylation sites. The N-terminal amino acid sequence of bovine lactoferrin isolated from tears is identical to that of the milk protein (Brown et al. 1996). As with human lactoferrin, there is only a single bovine lactoferrin gene (Teng 2002), and it has been assigned to chromosome 22 (Schwerin et al. 1994). Bovine lactoferrin gene polymorphisms have been reported (Seyfert and Kuhn 1994; Martin-Burriel et al. 1997), again paralleling the situation with human lactoferrin. Thus, although there appears to have been no attempt to directly clone bovine neutrophil lactoferrin from a myeloid library, it is highly unlikely that cloning of neutrophil lactoferrin would result in a product with a different amino acid sequence to that reported previously, using a variety of bovine tissues. No bovine analogue of the human splice-variant, Δ-lactoferrin, has so far been reported, and attempts to detect it have so far been unsuccessful (FL Schanbacher, *personal communication*, 2002).

As with human lactoferrin, there appear to be glycosylation differences between bovine milk and neutrophil lactoferrins. Hurley et al. (1993) observed multiple bands with different electrophoretic mobilities for bovine milk and neutrophil lactoferrins, which resolved into a single band with similar molecular weights for both proteins following digestion with N-glycanase. This indicates that bovine milk and neutrophil lactoferrins differ only in N-glycosylation and not in the structure of the polypeptide chain. Although the structural differences between bovine milk and neutrophil lactoferrin glycans have not been determined to the same extent as for human lactoferrin, it seems clear that the situation is entirely analogous, and that functional differences between the two proteins are unlikely to occur. Indeed, bovine milk lactoferrin is itself heterogeneous, with two major forms having been identified, lactoferrin *a* and *b*, that differ in their glycosylation, but not in their polypeptide chains (Tsuji et al. 1989; Ye et al. 1997).

C. Conclusions

000038

Thus, bovine milk and tissue (neutrophil) lactoferrins appear to be products of the same gene and possess identical polypeptide chains, any differences between them being limited to the attached glycans. Analogy to human lactoferrin suggests that these differences are unlikely to affect protein function. With regard to the question of potential allergic responses, all immunological

reactions to lactoferrin so far reported are to the polypeptide chain and not to the glycan. Therefore, there appears to be no reason to suppose that residual milk-derived lactoferrin potentially found in lactoferrin-treated beef carcasses will be any more prone to inducing allergic responses than would endogenous lactoferrin found naturally in beef carcass muscle tissue.

V. ENDOGENOUS LACTOFERRIN LEVELS IN BEEF

Lactoferrin is an iron-binding protein of the transferrin family. It is found naturally in various external secretions, especially milk and colostrum, and in the secondary (specific) granules of neutrophils. Small amounts are also present in blood plasma, and lactoferrin can bind to a variety of mammalian cells. A large number of biological effects have been attributed to lactoferrin, including antimicrobial and immunomodulatory activities. As a result, there has been some commercial interest by food and pharmaceutical companies in harnessing these properties.

Because lactoferrin is naturally present in blood neutrophils and plasma, and beef contains residual blood, there will be some endogenous lactoferrin present in beef carcasses. Levels could be further increased by lactoferrin bound to other cells in the carcass. Thus, exposure to bovine lactoferrin occurs regardless of whether or not the carcass is treated with bovine milk-derived lactoferrin. This chapter explores the quantitative aspects of endogenous lactoferrin levels in beef carcasses.

A. Neutrophil Lactoferrin Content

000040

Lactoferrin was first identified as a constituent of human neutrophil secondary granules by Masson et al. (1969), and its presence in bovine neutrophils was subsequently confirmed, although in the latter case, lactoferrin is also present in an additional type of neutrophil granule (Gennaro et al. 1983). Harmon et al. (1976) cite unpublished data in Harmon's Master's Thesis (Ohio State University, Columbus, OH, 1974) indicating that the lactoferrin content of bovine neutrophils is similar to that of humans, i.e., 3.0 μg per 10^6 cells. Subsequently, Harmon and Newbould (1980) estimated the lactoferrin content of bovine neutrophils to be 8.63 ($\pm$ 1.00) μg per 10^6 cells. The cells were isolated using EDTA as anticoagulant, a relatively mild procedure that should minimize degranulation, and hence loss of lactoferrin, so their value should be reasonably reliable. The lactoferrin was quantified by electroimmunodiffusion. Gennaro et al. (1978) reported bovine neutrophil lactoferrin content as 7.4 ($\pm$ 7.0) μg per 10^6 cells. These investigators used citrate as an anticoagulant, and quantified lactoferrin by double diffusion. Despite the differences in methodology, their figure agrees very well with that of Harmon and Newbould (1980). Sato et al. (2000) reported a somewhat lower figure of 2.9 μg per 10^6 cells (with a range of 1.5 to 4.7), using a sandwich ELISA technique. The method for obtaining neutrophils was not stated, so the possibility of some degranulation occurring cannot be excluded, and might account for the lower figure. However, all these figures are comparable to those reported for other species. Human neutrophil lactoferrin content has been variously reported as 4.3 μg per 10^6 cells (Venge et al. 1978), 3.0 μg per 10^6 cells (Masson et al. 1969), and 8.2 μg per 10^6 cells (Baynes et al. 1986), while a figure of from 2 to 4 μg per 10^6 cells has been reported for mouse neutrophils (Segars and Kinkade 1977).

Based on this review, it thus seems reasonable to take a mean figure of 8.0 μg lactoferrin per 10^6 bovine neutrophils as a basis for further calculations.

B. Lactoferrin in Blood Plasma

Lactoferrin is also present in small quantities in plasma, which is presumed to originate from neutrophils (although this has not been unequivocally established). Again, most data refer to humans, where increased plasma lactoferrin concentration has been proposed as a possible clinical marker of infection or inflammation. The true level in human plasma has been the subject of some debate, due to the fact that if neutrophils degranulate before or during plasma preparation, the levels of plasma lactoferrin will be artificially increased. Such an event is likely to occur if the blood is subjected to thermal, chemical, or physical shock prior to removal of the cells. Existing data from over 20 studies were summarized by Levay and Viljoen (1995) yielding plasma lactoferrin levels ranging from 0.02 to 3.5 μg per ml. The upper figures are almost certainly inaccurate, most likely resulting from a failure to control neutrophil degranulation. A more realistic normal range of from 0.05 to 0.2 μg per ml has been suggested by Brock (1998).

The only data on plasma lactoferrin in adult cows appears to be that of Sato et al. (2000), who reported a figure of 0.32 μg per ml (with a range of 0.16 to 0.71), using a sandwich ELISA technique. As mentioned above, it is not clear whether these authors took steps to prevent neutrophil degranulation, so this figure may be an overestimate. Two recent papers measured lactoferrin in newborn calves prior to ingestion of maternal colostrum. Talkuder et al. (2002) obtained a figure of 0.204 (± 0.019) μg per ml. Measurements were performed by double-antibody ELISA, using plasma obtained by rapid centrifugation of blood at 4°C without apparent use of an anticoagulant. This procedure should minimize degranulation, and it is significant that their figure is lower than that of Sato et al. (2000). Holloway et al. (2002) obtained a considerably higher figure of 2.5 (± 1.6) μg per ml, which is close to the highest figure reported for human plasma/serum by Levay and Viljoen (1995) in their review. However, Holloway et al. (2002) performed their assays on serum rather than plasma, and thus their figure is probably an overestimate, as neutrophil degranulation is much more likely to occur if blood is allowed to clot. Sato et al. (2000) measured plasma lactoferrin in older calves (at 3 to 4 weeks of age) and obtained a mean figure of 0.63 μg per ml, which again seems rather high.

000041

In humans, newborns were reported to have a plasma lactoferrin concentration of 0.39 μg per ml (Scott 1989), i.e., slightly above the likely normal range for adults. This figure is probably reliable as the investigator took care to separate cells from plasma rapidly. Scott (1989) also assayed adult plasma lactoferrin for which he obtained figures within the likely normal range cited above. The calf figure (0.204 ± 0.019 μg per ml) of Talkuder et al. (2002) is the lowest reported for cattle, and therefore probably the most reliable. It is also within the range found for plasma lactoferrin in human adults, and despite the fact that it refers to calves rather than adult cattle, it can

be considered to provide the best basis for calculating the likely contribution of plasma to lactoferrin levels in beef carcasses.

C. Estimating Endogenous Lactoferrin Levels in Beef

1. Estimating Levels of Lactoferrin in Beef Based on a Review of Published Literature

Bovine blood contains 7-10 x 10^6 leucocytes per ml, of which 25 to 30 percent are neutrophils (Frandson and Spurgeon 1995). This is a lower percentage than in humans, where neutrophils comprise about 60 percent of blood leucocytes. This means that bovine blood contains 1.8-3 x 10^6 neutrophils per ml. Taking the figure of 8.0 μg lactoferrin per 10^6 bovine neutrophils mentioned previously, this gives 14.4 to 24.0 μg per ml of neutrophil lactoferrin. The lower of these figures is 70 times higher than the concentration of plasma lactoferrin in calves reported by Talkuder et al. (2002), and also 70 times higher than the upper limit of the likely normal range in humans. The high end of this range is 120-fold greater than either calf or human plasma concentrations. Even the highest, and probably unreliable, figure reported by Holloway et al. (2002) still indicates that the contribution of plasma/serum lactoferrin is less than 20 percent of the lowest likely contribution from neutrophils. If one takes the adult cow figures for both neutrophil and plasma lactoferrin provided by Sato et al. (2000), the contribution of neutrophil lactoferrin is still at least 17-fold greater than that of plasma. Thus, it seems probable that the contribution of plasma lactoferrin is minor compared to that of neutrophils.

The degree to which levels of endogenous lactoferrin are comparable to residual levels of exogenous bovine milk-derived lactoferrin will depend primarily on the amount of residual blood, and specifically the number of neutrophils, remaining in the carcass. Residual blood is normally determined by estimating the amount of hemoglobin in tissue. Warriss (1977), reviewing earlier work, pointed out that this work might be unreliable due to a failure of earlier assays to distinguish between myoglobin and hemoglobin. He himself developed a gel-chromatographic assay (Warriss 1976), and in a subsequent review of more recent work, he suggested that the normal hemoglobin concentration in meat of slaughtered animals ranges from 0.2 to 1 mg per g (Warriss 1984). His own study (Warriss and Rhodes 1977), using tissues from heifers and steers, as well as randomly purchased beef samples from retail sources, gave hemoglobin values between 0.44 and 0.65 mg per gram of tissue. Samples from older animals (cows and bulls) gave higher values ranging from 1.0 to 1.1 mg per g. Although the amount of blood remaining in the whole carcass varies according to the efficiency of exsanguination at time of slaughter, the amount in muscle tissue remains relatively constant (Warriss 1984). However, delayed bleeding following slaughter can increase the hemoglobin concentration to up to 3.3 mg per g (Gregory et al. 1988).

A number of more recent reports have given hemoglobin figures consistent with the range proposed by Warriss (1984). Field et al. (1980) measured hemoglobin in the carcasses of 18- to 24-month-old steers and 4- to 6-year-old cows using a gel-chromatographic procedure. For steers, the

figures were 0.70 and 0.39 mg per g in cervical and lumbar muscle tissues, respectively, while for cows, the corresponding figures were 0.85 and 0.33 mg per g. Gregory et al. (1988), using the gel chromatographic method of Warriss (1976), reported values ranging from 0.39 to 0.61 mg per g in different muscles from cattle of unstated age.

Oellingrath et al. (1990) subsequently developed an HPLC method for separation of hemoglobin from myoglobin, as well as for hemoglobin quantification. Samples of minced beef gave hemoglobin values of 0.62 to 0.68 mg per g. Gerold and Stolle (1994) used the same method to assay hemoglobin in longissimus dorsi muscles from 16- to 18-month-old bulls, and obtained an almost identical mean figure of 0.65 mg per g (range 0.19 to 1.23). These most recent studies, using more modern analytical methodologies, give results comparable with the range suggested by Warriss (1984). Furthermore, unpublished data from R.A. Lawrie, an expert with over 50 years experience in the field of meat science, also fall within Warriss' range (R.A. Lawrie, *in litt*). Thus, it seems reasonable to accept Warriss' range of 0.2 to 1 mg per g as an accurate reflection of the hemoglobin content of meat.

Given that bovine blood has a hemoglobin content of 12 g per 100 ml (Frandson and Spurgeon 1995), the figures of Warriss (1984) imply that muscle tissue contains 1.7 to 8.3 μl of blood per gram of tissue. From the above calculations, this would indicate a neutrophil content of between 3.1-24.9 x 10^3 cells per gram of tissue, and a corresponding lactoferrin content of 25 to 199 ng per gram of muscle tissue (ppb). Therefore, a 250-g steak would contain between 6.3 and 50 μg of lactoferrin. If one takes the lower, and perhaps less reliable figure, for neutrophil lactoferrin of 0.39 μg per 10^6 cells provided by Sato et al. (2000), then the lactoferrin content of muscle would be in the range of 12 to 97 ng per gram of tissue (ppb). The above calculations assume that the neutrophil content of residual blood in muscle is the same as that in normal blood in the live animal. It could be argued that neutrophils might be more easily drained than erythrocytes from the carcass, so the tissue hemoglobin content would overestimate the number of neutrophils remaining. However, this seems highly unlikely. Most of the residual blood in muscle is in capillaries and is not depleted of neutrophils (PD Warriss, *personal communication*, 2002). Indeed, it is more likely that residual blood is enriched in neutrophils, as they may start to adhere to the capillary endothelia once the animal is slaughtered.

000043

Although lactoferrin mRNA has been detected in various tissues, neutrophils appear to be the most significant source of systemic lactoferrin. However, a large variety of cells and tissues can bind lactoferrin, either through specific receptors, or less specific interactions involving either lectin-type recognition of lactoferrin glycan chains, or charge interactions due to lactoferrin's high pI. While the less specific types of interaction may not occur *in vivo*, interactions with specific receptors are more likely to do so. There appear to be no data on lactoferrin interactions with myocytes, the major component of muscle tissue, but other cells which do contain lactoferrin receptors, such as tissue macrophages/monocytes and perhaps fibroblasts, will also be present in beef carcasses. Human macrophages/monocytes contain 2-200 x 10^6 specific binding sites per cell

(Birgens 1994), while no such data could be found for bovine macrophages. If one assumes an average of 10^7 sites per macrophage, this would mean that 10^6 macrophages can bind approximately 1 μg of lactoferrin, assuming a molecular weight of 80,000 for lactoferrin, the Avogadro constant of 6×10^{23}, and all binding sites are fully occupied.

The contribution of bound lactoferrin to total endogenous lactoferrin will depend upon the number of macrophages and other potential lactoferrin-binding cells in beef carcass tissue. The number of macrophages in muscle itself has not been examined in detail, but they are probably few in number (PD Warriss, *personal communication*, 2002), although more are likely to be present in connective tissue. However, at the very least, one can consider the potential contribution of lactoferrin bound to monocytes in the residual blood, which from the data of Frandson and Spurgeon (1995) number $0.35\text{-}0.5 \times 10^6$ per ml. Taking into account the blood content of beef, this would indicate a potential monocyte-bound lactoferrin content of 0.6 to 4.2 ng per g of tissue (ppb). This figure must be considered as very approximate, given the uncertainty as to whether all binding sites would in fact be occupied, but it does suggest that monocyte/macrophage lactoferrin could augment the lactoferrin contribution from neutrophils, possibly to a significant extent.

2. An Empirically Derived Estimate of Endogenous Lactoferrin Levels in Beef

To augment the endogenous lactoferrin estimates that were generated based on a review of the published scientific literature (as summarized above), National Beef conducted three separate studies to empirically measure the endogenous levels of lactoferrin in beef. In each study, an ELISA technique was used to measure lactoferrin levels in beef round-bottom round steak samples that were ground and pummeled prior to analysis. Four replicate samples were analyzed in each of the three studies yielding average lactoferrin levels of 202, 184, and 192 ng of lactoferrin per g tissue (ppb), respectively. Averaging these three estimates yields an overall estimate of the endogenous level of lactoferrin in beef of 193 ng per g tissue (ppb). This empirically derived estimate agrees quite favorably with the literature-derived range presented above of 25 to 199 ng per gram of tissue (ppb). Full reports of the three studies discussed above are included as Appendix I to this document.

D. Conclusions

Based on the above discussion, it is clear that endogenous lactoferrin is present in uncooked beef. Using the most reliable data in the literature, as discussed above, neutrophils in beef tissue would account for a content of between 25 to 199 ng of lactoferrin per g of muscle tissue (ppb). This is likely to be increased further by lactoferrin in plasma and lactoferrin bound to cells such as macrophages/monocytes. This range agrees quite well with empirically derived estimates from studies conducted by National Beef of around 193 ng of lactoferrin per g tissue (ppb). This concentration estimate will be employed in Chapter VIII to derive estimates of lactoferrin intake resulting from it being naturally present in beef muscle tissue.

VI. EXOGENOUS LACTOFERRIN LEVELS IN BEEF

As discussed previously, bovine milk-derived lactoferrin is the active ingredient in an antimicrobial spray product that is to be applied to raw beef carcasses prior to a final lactic acid rinse during processing. This water-based, antimicrobial spray product contains no more than two percent bovine milk-derived lactoferrin by weight. The other (inactive) components of the spray are food-grade substances approved for direct addition to foods by the FDA, and regarded as appropriate for use on meat products by the Food Safety and Inspection Service ("FSIS") of the U.S. Department of Agriculture ("USDA"). In this chapter, the estimated concentration of residual bovine milk-derived lactoferrin that could potentially be found in edible beef is calculated based on the intended conditions of use of this lactoferrin-containing spray during meat processing.

Three primary processes comprise the typical operation of a meat packing plant: 1) the slaughter process, 2) the chilling process, and 3) the fabrication process (see Appendix III). During this meat packing operation, lactoferrin-containing spray is to be employed at the end of the slaughter process, right before chilling of the carcasses begins. This spray is to be applied within an "activated lactoferrin cabinet" at a rate of 50 ml per carcass. Assuming that the spray contains two percent lactoferrin, this 50-ml application rate will yield 1 ml or 1 g of bovine milk-derived lactoferrin on each carcass, assuming a lactoferrin density of 1 g per ml. This calculation employs the worst-case assumption that the electrostatic spraying ("ESS") process used in applying the lactoferrin to beef carcasses is 100 percent efficient; that is, all 50 ml of spray containing lactoferrin adheres to the carcass. In reality, it is likely that this spray process is not 100 percent efficient. However, because no data currently exist to specifically estimate this application efficiency, the worst-case assumption of 100 percent efficiency was employed.

As indicated above, subsequent to lactoferrin application, the beef carcasses undergo a final lactic acid rinse prior to being chilled during the meat packing process. The intent of this lactic acid rinse is to remove bacteria bound to the lactoferrin, as well as any residual lactoferrin, from the carcass surface. In order to measure the amount of lactoferrin removed as a result of this lactic acid rinse, National Beef conducted four carcass washing studies employing a meat processing simulation ("MPS") system. This MPS system is a scale model of the actual twelve-step processing system used in a typical meat packing plant. For these experiments, beef round-bottom round steaks were used to simulate beef carcasses. These steaks were then processed using the MPS system and lactoferrin levels were measured on the surface of the steaks subsequent to lactoferrin application, both before and after the final lactic acid rinse. Lactoferrin levels were measured using an ELISA technique. In each study, five different lactoferrin spray times were evaluated (i.e., 2, 4, 6, 8, and 10 seconds) with four replicates at each time point. For this analysis, the two-second spray application data were selected because this is the lactoferrin spray duration that will be used in actual practice. Based on these data, the average percent lactoferrin that was estimated to have been

removed by a two-second lactic acid rinse across these four studies was 91, 86, 80, and 81 percent, respectively. Averaging these four estimates yields an overall estimate of lactoferrin removal efficiency for a two-second lactic acid rinse of 84.5 percent; thus, leaving an average of 15.5 percent of the applied lactoferrin on the carcass surface. Full reports of the four studies discussed above are included as Appendix II to this document. Multiplying the amount of lactoferrin applied to the carcass (i.e., 1 g) times 0.155 (the fraction of lactoferrin retained on the carcass after the lactic acid rinse) yields an estimate of the amount of bovine milk-derived lactoferrin remaining on the carcass surface of 0.155 grams.

Once the beef carcasses have undergone a lactic acid rinse, they enter the chilling process, whereby the carcasses are first placed in "hotboxes" and sprayed with cold water; then are transferred to the USDA grading area; and finally, are moved to the "sales cooler" where the carcasses are sorted. Next, the carcasses enter the fabrication process where they are trimmed (i.e., fat, bone, and lean trimmings are removed) and systematically broken down into "primals" (i.e., rounds, loins, ribs, chucks, and briskets) and then "subprimals." These subprimals are then bagged and boxed, and are referred to as "boxed beef." The lean trimmings and some of the fat that is removed during fabrication is designated "trim/grind," and is used for ground beef and other processed meats such as hot dogs. The bones and the remainder of the fat (primarily outside surface fat) removed during fabrication are destined for the rendering operation, and are not used in products intended for human consumption.

National Beef has estimated that about 40 percent of the lactoferrin-treated carcass surface (i.e., primarily the inner surface of the carcass body cavity) consists of bone and connective tissue destined for rendering. National Beef has also estimated that the remaining 60 percent of the lactoferrin-treated carcass surface ends up in edible beef, with 10 percent of this total going into boxed beef and 50 percent into trim/grind.

Therefore, of the estimated 0.155 grams of lactoferrin remaining on the carcass surface following the lactic acid rinse, about 40 percent of this lactoferrin residual will end up on portions of the carcass that are not used in products intended for human consumption. Of the remaining 60 percent of the lactoferrin that is retained on edible portions of the carcass, about 10 percent will end up in boxed beef and 50 percent will go into trim/grind. Thus, multiplying the estimated amount of lactoferrin remaining on each carcass (0.155 g) times 0.6 yields an estimated amount of lactoferrin adhering to the edible portions of the carcass of 0.093 g or 93 mg, with one-sixth of this total or 15.5 mg going into boxed beef and five-sixths or 77.5 mg into trim/grind.

For the purposes of this assessment, two different exposure assumptions (or scenarios) will be evaluated:

000046

1. Assume that the 93 mg of residual lactoferrin is equally distributed across all edible portions of the carcass (i.e., boxed beef and trim/grind); or
2. Assume that the 93 mg of residual lactoferrin is unequally distributed across all edible portions of the carcass, with 15.5 mg going into boxed beef and 77.5 mg into trim/grind.

The estimated concentrations of lactoferrin in edible beef that these two exposure assumptions yield can be calculated using data compiled by National Beef for fiscal year 2002 on the average weight of beef products (on a per head basis) at various points in the meat packing process (see Appendix III). These data are as follows:

- Average live animal weight: 1,245 pounds per head
- Average dressed carcass weight: 799 pounds per head
- Average boxed beef weight: 415 pounds per head
- Average trim/grind weight: 235 pounds per head
- Average weight of bone/fat to rendering: 149 pounds per head

Based on the above data, the average amount of edible meat that a beef carcass yields is about 650 pounds (295 kg), and is derived by summing the average boxed beef weight (415 pounds or 189 kg) and the average trim/grind weight (235 pounds or 107 kg) on a per head basis. Under the first exposure scenario, the estimated concentration of bovine milk-derived lactoferrin in edible beef can be derived by dividing the amount of lactoferrin adhering to edible portions of the carcass (93 mg) by the weight of the edible meat from each carcass (295 kg) to yield 0.32 mg per kg (ppm) or 320 ng per g (ppb). Under the second exposure scenario, the estimated concentration of bovine milk-derived lactoferrin in boxed beef can be generated by dividing the amount of lactoferrin potentially found in boxed beef (15.5 mg) by the total weight of boxed beef from each carcass (189 kg) to yield 0.082 mg per kg (ppm) or 82 ng per g (ppb). The estimated concentration of bovine milk-derived lactoferrin in trim/grind can be derived by dividing the amount of lactoferrin potentially found in trim/grind (77.5 mg) by the total weight of trim/grind from each carcass (107 kg) to yield 0.726 mg per kg (ppm) or 726 ng per g (ppb). These concentration estimates will be employed in Chapter VIII to derive estimates of exogenous lactoferrin intake resulting from the use of a lactoferrin-containing spray on beef carcasses during processing.

VII. ESTIMATES OF BEEF CONSUMPTION IN THE U.S.

Consumption of beef was estimated based on data from the 1994-1996 USDA Continuing Survey of Food Intakes by Individuals ("CSFII 1994-96") and its Supplemental Children's Survey ("CSFII 1998") as provided on CD-ROM (USDA 2000). The combined data from these two surveys are designated CSFII 1994-96, 1998. In the CSFII 1994-96, 1998, dietary intakes were collected through in-person interviews using 24-hour recalls on 2 nonconsecutive days. A total of 21,662 individuals of all ages provided data for the first day; of those individuals, 20,607 provided data for a second day. Each dietary recall includes detailed information on all foods consumed the previous day, including a descriptive name and the amount (gram weight) of each food consumed.

The CSFII 1994-96, 1998 survey includes more than 9,000 food codes representing the foods consumed by survey respondents. The food codes represent both single-component foods and mixed dishes that are composed of one or more ingredients. In order to estimate consumption of beef, which is consumed either as a single-component food or as one of several components in a mixed dish, it was necessary to identify the amount of beef in each of the survey food codes. This was accomplished by linking the CSFII food codes to the Food Commodity Intake Database, Version 2.1 (USEPA 2000). The Food Commodity Intake Database is the result of cooperative efforts by the USDA Agricultural Research Service ("ARS") and the U.S. Environmental Protection Agency ("EPA") Health Effects Division. It was developed for use in estimating human exposures to pesticide residues through food and beverage intake. The Food Code-to-Commodity Translation File of the database maps each food code in the CSFII 1994-96, 1998 to one or more of EPA's list of approximately 550 food commodities. Food codes that represent mixtures were assigned commodities based on their recipe ingredients, and the amount of each commodity (in grams) is identified per 100 grams of CSFII food code.

The list of EPA commodities used in the database includes a total of 10 food commodity codes for various beef fractions. These 10 commodity codes and their associated names are presented in Table 1 below. ENVIRON identified all CSFII food codes that were mapped to the beef meat, beef byproducts, and/or beef fat commodity codes, and the amounts of each commodity per 100 g food code were summed. The resulting value represents the total amount of beef per 100 g of each food code. However, the commodity codes for kidney and liver were not included in the sums because these are internal organs that are removed from the beef carcass prior to lactoferrin application. The list of beef-containing codes also includes veal-containing foods, bison-containing foods, and other beef parts (e.g., heart, brains, tongue). These food codes also were not included in the estimates of beef consumption because these types of products will not be subjected to lactoferrin treatment. Additionally, beef by-product commodities were not included in the sum of total beef values when beef by-products accounted for less than 2.5 g per 100 g of food code. These contributions of beef by-products were not included in the estimates of beef consumption because

these uses likely represent beef gelatin (e.g., in yogurt or in marshmallows) or head meat, beef by-products not subjected to lactoferrin treatment. Gelatin drinks were also excluded from this analysis for the same reason. The food codes included in the analysis and the amount of beef (by food commodity) are presented in Appendix IV.

Table 1. EPA Beef Commodity Codes

Food Commodity Code	Food Commodity Name
21000440	Beef, meat
21000441	Beef, meat- baby food
21000450	Beef, meat, dried
21000460	Beef, meat byproducts
21000461	Beef, meat byproducts- baby food
21000470	Beef, fat
21000471	Beef, fat- baby food
21000480	Beef, kidney
21000490	Beef, liver
21000491	Beef, liver- baby food

ENVIRON linked the selected beef-containing food codes to the food consumption data from CSFII 1994-96, 1998, and estimated the 10th, 50th, and 90th percentile values for beef consumption in six population groups based on reported intake of beef-containing foods and the percent of beef per food code. The six population groups were: 1) infants < 1 year old, 2) infants 1 year old, 3) children 2-5 years old, 4) children 6-12 years old, 5) teenagers 13-19 years old, and 6) adults 20+ years old.

Estimates of beef intake were calculated based on both 2-day average beef intakes and beef intake per eating occasion. All dietary intake analyses included only consumers of beef. Therefore, the 2-day average intake estimates are based upon the mean amount of beef consumed in 2 days by all individuals who reported consumption of at least one beef-containing food during the 2-day period, and the intake per eating occasion estimates are based on the amount of beef consumed on each occasion (i.e., unique time and day) an individual reported consumption of beef and/or beef-containing food(s). The 2-day average intakes provide an estimate of usual beef intake, while the intakes per eating occasion provide estimates of the amount of beef consumed at a single meal or snack occasion.

000049

Results of the dietary analyses are presented in Table 2. Children and teenagers are the subpopulations most likely to consume beef, with nearly 90 percent of each of these groups reporting consumption of one or more beef-containing foods over the 2 days of dietary recall. In the other subpopulations evaluated, approximately 80 percent of adults aged 20 years and older, as well as one-year-old infants, consumed beef over the two-day period, while beef consumption over this same period was reported in only 28 percent of infants under one year of age. Across all population groups, the total number of eating occasions on which beef consumption was reported is

approximately 2 to 2 ½ times the number of people who reported beef consumption at least once during the 2 days of dietary recall, indicating that the typical beef consumer eats a beef-containing food on one or more occasions each day.

Estimates of 2-day average intakes indicate that beef consumption is highest in populations of teenagers and adults. The 2-day average intakes are lower in populations of infants and children, with intakes increasing with age. Infants under 1 year of age consume the least amount of beef per day.

Estimates of beef intake per beef eating occasion are comparable to beef intake estimates based on 2-day average dietary intakes. In addition, both estimates exhibit a trend of increasing intake with age. Adults reported the highest intake per eating occasion, while infants under 1 year of age consumed the least amount of beef per occasion.

Table 2. Estimates of Beef Consumption: 2-Day Average Intake and Intake per Eating Occasion

Population Group	Survey Population[1]	%	2-Day Average Intake				Intake per Eating Occasion			
			N[2]	Percentile (g per day)			N[3]	Percentile (g per eating occasion)		
				10	50	90		10	50	90
Infants, < 1 y	1065	28	276	1	13	41	478	< 0.5	14	43
Infants, 1 y	972	80	782	2	19	55	1632	< 0.5	16	61
Children, 2-5 y	5437	87	4704	2	25	74	10,205	< 0.5	21	75
Children, 6-12 y	2089	89	1867	5	38	99	4289	1	29	96
Teenagers, 13-19 y	1222	88	1088	6	52	147	2617	1	35	137
Adults, 20+ y	9323	81	7556	5	52	152	15,423	1	49	156

DATA SOURCE: 1994-96, 1998 Continuing Survey of Food Intakes by Individuals (CSFII) and Food Commodity Intake Database (USDA 2000, USEPA 2000).
NOTES: Breastfeeding infants and children were excluded from the analyses. Analyses were limited to individuals who provided 2-days of recall data. Estimates were generated with USDA sample weighting factors to adjust for differences in representation of subpopulations.
[1] Number of survey respondents who provided 2-day dietary recalls.
[2] Number of respondents who reported consumption of one or more beef-containing foods during the 2 days of recall.
[3] Number of occasions on both survey days combined on which consumption of a beef-containing food (or foods) was reported.

000050

VIII. ESTIMATES OF LACTOFERRIN INTAKE

In order to assess the impact spraying lactoferrin on beef carcasses could potentially have on total lactoferrin exposure from beef consumption, ENVIRON estimated the potential intake of lactoferrin from exogenously applied lactoferrin employing several worst-case assumptions. This estimated intake of exogenous lactoferrin is compared to lactoferrin intake resulting from endogenous levels found naturally in beef. This comparison shows that the estimated intake of bovine milk-derived lactoferrin from its intended use as an anitmicrobial spray on beef carcasses (employing several worst-case assumptions) is comparable to an individual's estimated background exposure to lactoferrin found endogenously in beef. Thus, the intended use of bovine milk-derived lactoferrin would not be expected to result in any substantive increase in total lactoferrin exposure from consumption of beef products. This chapter presents the calculated estimated intakes of lactoferrin from both endogenous and exogenous sources.

A. Estimates of Endogenous Lactoferrin Intake

In Chapter V of this document, based on a review of the published scientific literature, it was estimated that beef contains approximately 25 to 199 ng of lactoferrin per gram of muscle tissue (ppb) from neutrophils contained in the residual blood that remains in this tissue after slaughter. This is by no means the only source of lactoferrin in blood, although it is the primary source. Lactoferrin is also found in blood plasma and bound to macrophages, but at much lower levels than in neutrophils. It was also demonstrated in a series of three studies conducted by National Beef that empirical measurements of endogenous levels of lactoferrin in beef round-bottom round steak averaged 193 ng lactoferrin per g of muscle tissue (ppb). This empirical value is consistent with the range estimated from a review of the published literature in Chapter V, and thus corroborates the literature-derived value.

For this step of the analysis, the objective is to derive an estimate of the range of background exposures consumers of beef may have to lactoferrin from endogenous levels in beef. It will be assumed that the concentration of endogenous lactoferrin in beef is 193 ng per gram of tissue (ppb), the empirically derived value described previously. The next step in estimating the range of background intakes of endogenous lactoferrin is to determine the amount of beef typically consumed in the diet. Because the endpoint of concern with lactoferrin is allergenicity, ENVIRON believes that the amount of beef consumed per eating occasion is the most relevant measure of beef consumption to use. It should be noted however that for beef consumption, the "per occasion" values are almost identical to the "per day" values, as shown previously in Table 2.[1] From the dietary analysis presented in Chapter VII, ENVIRON opted to use the 10th and 90th percentile

000051

[1] In this same table, it should also be noted that beef consumption by adults is approximately two to three times greater than beef consumption by children.

consumption of beef per eating occasion to represent the lower and upper limits, respectively, of the range of beef consumption in the U.S. population.

For example, for adults aged 20 years and older, the 10th percentile of beef consumption is approximately1 g per eating occasion and the 90th percentile value is 156 g per eating occasion. Multiplying both the 10th and 90th percentile consumption values for beef (i.e., 1 and 156 g per eating occasion, respectively) by an endogenous lactoferrin concentration of 193 ng per g (ppb) yields an estimated range of intakes of endogenous lactoferrin from beef consumption of 0.3 to up to 30 μg per eating occasion. Similarly, ranges of endogenous lactoferrin intake estimates can also be generated for the other population groups of interest listed in Table 2. These endogenous or background lactoferrin intake ranges are presented in Table 3 for all six population groups of interest.

B. Estimates of Exogenous Lactoferrin Intake

In Chapter VI of this document, the concentration of exogenous lactoferrin in edible beef from lactoferrin-treated carcasses was estimated to be 320 ng per g of tissue (ppb) assuming equal distribution of the applied lactoferrin throughout the meat, or 82 ng per g of tissue (ppb) of boxed beef and 726 ng per g of tissue (ppb) in trim/grind if we assume that the lactoferrin is unequally distributed within the edible beef. To derive an exogenous lactoferrin intake estimate under each of these two scenarios, the exogenous lactoferrin concentration is multiplied by the amount (and type) of beef consumed.

If the exogenous lactoferrin is assumed to be equally distributed throughout the beef carcass (scenario 1), the intake calculation is quite simple. For example, assuming an estimated concentration of bovine milk-derived lactoferrin in edible beef of 320 ng per g (ppb), the product of this lactoferrin concentration and beef intake at both the 50th and 90th percentile of beef consumption per eating occasion for adults 20+ years of age (Note: this population group has the highest consumption of beef) yields estimated intakes of exogenous bovine milk-derived lactoferrin of 16 and 50 μg per eating occasion, respectively. The 16 μg per eating occasion estimate represents lactoferrin exposure for the median or likely "typical" consumer of lactoferrin-treated beef products, while the 50 μg per eating occasion estimate represents lactoferrin exposure for the "heavy" consumer of lactoferrin-treated beef products. In this scenario, the intake of exogenous lactoferrin is directly proportional to intake of beef.

When assuming that exogenous lactoferrin concentrations in boxed beef versus trim/grind are different (scenario 2), the calculation is completed via a similar approach, though an additional step of identifying boxed versus trim/grind cuts of beef is needed. ENVIRON reviewed each of the beef-containing food codes used in the estimates of beef consumption and classified each as derived from a boxed or trim/grind cut of beef. All food codes for subprimals, foods typically prepared from subprimals (e.g., beef fajitas), and beef baby foods were classified as boxed beef. All other beef-containing food codes were classified as trim/grind. ENVIRON then generated the total

lactoferrin intake per person per beef-eating occasion by adding the amount of exogenous lactoferrin consumed from boxed beef to the amount of exogenous lactoferrin consumed from trim/grind. ENVIRON subsequently calculated the 50th and 90th percentiles of these values, which represent the typical and heavy consumers of exogenous lactoferrin per occasion, respectively, based on the assumption that the lactoferrin is not distributed evenly throughout the carcass. In this scenario, the estimated intake of exogenous lactoferrin reflects intake by product type (boxed versus trim/grind), in the U.S. population.

The range of lactoferrin intakes derived from these two scenarios for each population group (i.e., infants < 1 year old, infants 1 year old, children 2-5 years old, children 6-12 years old, teenagers 13-19 years old, and adults 20 years and older) are presented in Table 3. As seen in Table 3, both approaches yield estimated 50th percentile lactoferrin intakes that are comparable. The estimated median intake of bovine milk-derived lactoferrin ranges from 2 to 5 μg per beef-eating occasion for infants under the age of 1 year, and is estimated to be 16 μg per beef-eating occasion for adults.

At the 90th percentile of estimated exogenous lactoferrin intake, the 2 scenarios result in slightly broader ranges. For example, in the population of beef-eating adults 20 years and older, estimates of the 90th percentile intake of exogenous lactoferrin from lactoferrin-treated beef products range from 50 to 76 μg per beef-eating occasion. Estimates of 90th percentile bovine milk-derived lactoferrin intakes were consistently higher under scenario 2 as compared to estimates derived under scenario 1. Recall that in the second scenario, the concentration of exogenous lactoferrin in trim/grind beef products is assumed to be nearly 9-fold higher as compared to the concentration in boxed beef (726 versus 82 ng per g (ppb)). Results from this scenario therefore reflect both the population's intake of beef and the relative proportion of boxed beef and trim/grind beef products consumed.

In both scenarios, the values in Table 3 are likely to be overestimates of bovine milk-derived lactoferrin intakes because these calculations employ the following worst-case assumptions: 1) the ESS process employed in applying lactoferrin to beef carcasses is 100 percent efficient, and 2) all beef an individual consumes has been treated with lactoferrin.

000053

C. Conclusions

In order to evaluate the potential impact of treating beef carcasses with lactoferrin, the range of estimated intakes of endogenous lactoferrin that result from untreated beef consumption is compared against the estimated intakes of exogenous bovine milk-derived lactoferrin that could potentially occur from consumption of lactoferrin-treated beef. In Table 3, the range of 50th percentile intakes of exogenous bovine milk-derived lactoferrin are all within the range of estimated background exposures to naturally occurring endogenous lactoferrin across all population groups of interest. There are, of course, uncertainties in both the endogenous and exogenous estimates because of the assumptions that must be used to derive these values; in particular, the estimated

intakes of exogenous lactoferrin are expected to be high. Therefore, given the inherent uncertainties in both the endogenous and exogenous lactoferrin intake estimates, the intended use of bovine milk-derived lactoferrin as an antimicrobial spray treatment on beef carcasses will not result in a significant, measurable increase in lactoferrin intake relative to current background exposures to lactoferrin from beef consumption.

Table 3. Estimated Background Lactoferrin Intakes versus Estimated 50th and 90th Percentile Intakes of Bovine Milk-Derived Lactoferrin from Lactoferrin-Treated Beef Carcasses

Population Group	Estimated Range of Background Lactoferrin Intake (µg/occasion)[1]	Estimated Intake of Bovine Milk-Derived Lactoferrin (µg/occasion)[3]			
		Range of 50th Percentile Lactoferrin Intakes		Range of 90th Percentile Lactoferrin Intakes	
		Scenario 1[2]: Equal Lactoferrin Distribution	Scenario 2[3]: Unequal Lactoferrin Distribution	Scenario 1: Equal Lactoferrin Distribution	Scenario 2: Unequal Lactoferrin Distribution
Infants, < 1 y	< 0.1 to 8	5	2	14	20
Infants, 1 y	< 0.1 to 12	5	6	20	35
Children, 2-5 y	0.1 to 14	7	10	24	44
Children, 6-12 y	0.2 to 19	9	12	31	62
Teenagers, 13-19 y	0.2 to 26	11	15	44	76
Adults, 20+ y	0.3 to 30	16	16	50	76

DATA SOURCE: 1994-96, 1998 Continuing Survey of Food Intakes by Individuals (CSFII) and Food Commodity Intake Database (USDA 2000, USEPA 2000).

NOTES: Breastfeeding infants and children were excluded from the analyses. Analyses were limited to individuals who provided 2-days of recall data. Estimates were generated with USDA sample weighting factors to adjust for differences in representation of subpopulations.

[1] The range of background lactoferrin intakes is represented by the 10th and 90th percentiles of endogenous lactoferrin consumption from beef; values correspond to the 10th and 90th percentiles of beef consumption (g per occasion) multiplied by 193 ng bovine neutrophil lactoferrin per g beef (ppb), respectively. See text for further discussion of lactoferrin concentration derivation.

[2] In Scenario 1, the concentration of bovine milk-derived lactoferrin is assumed to be equally distributed throughout the treated beef carcass; this exogenous lactoferrin concentration is 320 ng per g of beef (ppb). Values correspond to the 50th or 90th percentile of beef consumption (g per occasion) multiplied by 320 ng bovine milk-derived lactoferrin per g of beef (ppb). See text for further discussion of lactoferrin concentration derivation.

[3] In Scenario 2, the concentration of bovine milk-derived lactoferrin is assumed to be unequally distributed throughout the treated beef carcass; the exogenous lactoferrin concentration in boxed beef is 82 ng per g of beef (ppb), while the exogenous lactoferrin concentration in trim/grind is 726 ng per g of beef (ppb). Values correspond to the 50th or 90th percentile of exogenous lactoferrin intake (g per occasion). For each beef-eating occasion, the amount of exogenous lactoferrin consumed was calculated by taking the sum of exogenous lactoferrin intake by type of beef (i.e., 82 ng bovine milk-derived lactoferrin per g of boxed beef (ppb) added to 726 ng bovine milk-derived lactoferrin per g of trim/grind (ppb)). See text for further discussion of lactoferrin concentration derivation.

000054

IX. LACTOFERRIN INTAKES FROM DAIRY AND WHEY PRODUCTS ADDED TO FOOD

Bovine lactoferrin has been consumed by humans for thousands of years as a naturally occurring protein found in milk. Current sources of lactoferrin in the U.S. diet include milk proteins naturally occurring in milk and milk products, and whey proteins added to foods throughout the food supply in the form of whey products. Milk proteins and whey proteins contain approximately 0.03 g and 1.4 g lactoferrin per 100 g, respectively (Barth and Behnke 1997). Using food intake data reported in the CSFII 1996, 98, ENVIRON determined the average per capita intake of milk proteins from milk and milk products (Table 4).

The estimates of milk protein intake were based on reported consumption of all milk and milk products including fluid and flavored milk, dry and reconstituted milk, milk-based beverage mixes, milk and milk products in beverage mixtures, yogurt, ice cream, cream, sour cream, and puddings. The analyses did not include cheese, infant formulas, meal replacements, whey-based beverages and beverage mixes, or butter. Additionally, the milk and milk products that were ingredients in food mixtures coded as a single item (i.e., milk used in the preparation of canned soup) were not included in the estimates. Given that whey proteins may be added to some milk products such as yogurt, ice cream, and beverage mixes, we assumed only 75 percent of the protein content of milk products to be milk protein; this allows for the addition of approximately one gram of whey protein per 100 grams of each milk product. The food codes included in the estimates of milk protein intake are listed in Appendix V.

The estimated mean intake of milk proteins by individuals in the U.S. ages 2 years and older is approximately 7.7 g per day. This is equivalent to an intake of approximately 23 mg lactoferrin per day. The 90th percentile intake of milk protein in the U.S. is approximately 18.6 g per day, or 56 mg lactoferrin per day. The 95th percentile intake of milk protein is 23.5 g per day, or approximately 70 mg lactoferrin. It is important to note that these estimates do not reflect the lactoferrin contained in milk and milk products used as an ingredient and a considerable allowance was made for the potential presence of whey proteins in milk products. Therefore, these estimates may tend to underestimate current consumption of milk proteins and consequently, intake of lactoferrin from milk proteins.

000055

Estimates of whey protein intake were derived from estimates of whey products generated by the FDA (Rulis 2000). The FDA estimated that the current consumption of whey products on a per capita basis is approximately 4.0 grams per person per day. ENVIRON has assumed that the FDA estimate of "whey products" represents the usual intake of whey from all uses in the U.S. food supply. Whey and whey products may be used in food in accordance with good manufacturing practice and are added to foods for a wide variety of functional uses. Some of the uses of whey and whey products include: nutrient, solvent/carrier/encapsulating agent, material handling aid,

appearance control agent for substances other than colors and color modifiers, leavening agent, stabilizer and thickener, flavor ingredient or flavor modifier, color control agent, texturizer, and malting/fermenting aid (Burdock 1997).

The protein content of whey products varies depending on the specific type and concentration. From current regulatory approvals and specifications for whey and whey products (21 CFR §184.1979 a,b,c), protein concentration can range from 10 percent to not less than 25 percent of these products. Whey protein isolate (based on GRN 37) contains not less than 90 percent protein. Therefore, in order to calculate protein exposures from the whey product consumption estimate, a realistic estimate of 25 percent protein was used. Using this assumption, we estimate that the per capita intake of whey protein is approximately 1.0 g per day. This is equivalent to 14 mg lactoferrin per day. We estimate that the 90th percentile intake of whey protein is approximately twice the mean intake, or 2.0 g per day, which in turn is equivalent to approximately 84 mg lactoferrin per day.

Estimates of the per capita lactoferrin intake from milk proteins in dairy products were added to the estimated per capita lactoferrin intake from whey products. These estimates of intake of lactoferrin from both dairy sources and whey protein added to food indicate that background exposure to lactoferrin can be as high as 100 mg per person per day.

This estimate of lactoferrin exposure is supported by calculations of potential lactoferrin intakes based on current recommendations in the U.S. for consumption of milk and milk products. In its Food Guide Pyramid, the USDA recommends daily intake of 2 to 3 servings of milk products (USDA 1996). Individuals consuming 3 cups of milk per day, or 3 servings, would have lactoferrin intakes of approximately 71 mg per day. Added to lactoferrin intake from whey protein sources, total lactoferrin intake is again approximately 100 mg per day.

Estimates of lactoferrin intake from dairy and other food sources presented here were submitted to the FDA as an addendum to GRN 42. This GRAS Notification was for the use of bovine lactoferrin as an ingredient in sports and functional foods. The FDA had no questions concerning the GRAS status of bovine lactoferrin for the proposed uses that were the subject of that GRAS Notification, indicating that the Agency accepted the validity of the dietary assessment presented above.

These estimated intakes of bovine lactoferrin from dairy products and whey products added to food demonstrate the very high background dietary exposure to lactoferrin (in the mg range) relative to the estimated lactoferrin intakes (in the μg range) that could potentially result from the use of lactoferrin as an antimicrobial spray treatment on beef carcasses. These dietary exposure estimates presented here provide some context in evaluating the very small intakes that would result from the proposed use of lactoferrin on beef carcasses.

TABLE 4
Estimate of Per Capita Daily Intake of Bovine Lactoferrin from Milk and Whey Proteins

	Milk Proteins		Whey Proteins		Milk and Whey Proteins
	Milk Proteins in Dairy Products [a] (g)	Lactoferrin from Milk Proteins in Dairy Products [a, b] (mg)	Whey Proteins in the Food Supply [c] (g)	Lactoferrin from Whey Proteins in the Food Supply [c, d] (mg)	Lactoferrin from Milk and Whey Proteins [e] (mg)
Mean Intake	7.7	23	1.0	14	37
90th Percentile of Intake	18.6	56	2.0	28	84
95h Percentile of Intake	23.5	70	2.0 [f]	28 [f]	98

[a] Data source: USDA 1994-96, 1998 CSFII; estimates reflect intake of protein from milk and milk products including fluid and flavored milk, dry and reconstituted milk, milk-based beverage mixes, yogurt, ice cream, cream, sour cream, and puddings. Estimates include all individuals 2 years and older who provided two 24-hour diet recalls. Estimates generated with USDA sampling weights and WesVar Complex Samples Version 3.0.
[b] Calculation of lactoferrin based on 0. 3% lactoferrin in milk protein (Barth and Behnke 1997).
[c] Data source: FDA estimate of per capita whey products consumption (Rulis 2000); assumed 25% whey protein in whey products; assumed 90th percentile of intake is twice the mean intake.
[d] Calculation of lactoferrin based on 1.4% lactoferrin in whey protein (Barth and Behnke 1997).
[e] Represents sum of estimates of lactoferrin from milk proteins in dairy products and lactoferrin from whey proteins in the food supply.
[f] Unable to estimate 95th percentile; values represent estimate of 90th percentile.

000057

X. LACTOFERRIN AS A POTENTIAL HUMAN FOOD ALLERGEN

Rather little scientific and clinical information exist to document whether bovine milk-derived lactoferrin is a cows' milk allergen. While there is some evidence to suggest that bovine milk-derived lactoferrin is a minor cows' milk allergen, the existing information is difficult to interpret because of methodological problems in the manner in which much of the data were collected.

Certainly, no argument exists that the major cows' milk allergens in IgE-mediated cows' milk allergy are casein, β-lactoglobulin, and α-lactalbumin (Besler et al. 2002; Sharma et al. 2001; Wal 1998; Wal et al. 2001). Several clinical investigators have demonstrated that some cows' milk-allergic individuals have IgE antibodies directed against lactoferrin (Baldo 1984; Businco et al. 2000; Host et al. 1992; Wal et al. 1995; Wal 1998; Wal et al. 2001). However, the vast majority of these milk-allergic infants displayed IgE antibodies to one or more of the major cows' milk allergens in addition to lactoferrin. Wal (1998) appears to have identified two milk-allergic infants out of 92 studied (2.2%) who were mono-sensitized to lactoferrin only. However, the clinical significance of this observation is unclear because the study does not indicate if these or other infants in the study had received blinded oral challenges to document their milk allergies. Without confirmation by oral challenge, the evaluation of specific IgE antibodies in patient serum can be clinically meaningless and misleading. Furthermore, in studies where quantitative estimates were obtained of the levels of specific IgE antibodies to lactoferrin (Wal et al. 1995; Wal 1998), the levels of anti-lactoferrin IgE antibodies were far lower than the levels of specific IgE antibodies to the major milk allergens in the vast majority of the patients.

Milk-allergic patients seem to occasionally have IgE antibodies directed at other minor milk proteins (Baldo 1984); the clinical significance of the presence of these antibodies is unproven. To demonstrate that bovine milk-derived lactoferrin is a clinically significant milk allergen, patients who were challenge-positive to milk ingestion would have to demonstrate specific IgE antibodies to bovine milk-derived lactoferrin (and possibly other milk proteins), positive skin prick tests to bovine milk-derived lactoferrin, and positive oral challenges to bovine milk-derived lactoferrin. Such information does not exist to unequivocally demonstrate that bovine milk-derived lactoferrin is a human allergen.

000058

Could lactoferrin be an allergen in beef? In Chapter IV, the presence of lactoferrin in beef primarily arising from its presence in bovine neutrophils has been clearly documented. Furthermore, bovine neutrophil lactoferrin is very similar, structurally and functionally, to bovine milk-derived lactoferrin (see Chapter IV for a more thorough discussion). However, even given the presence of lactoferrin in beef, allergic reactions to beef are comparatively rare and are usually relatively mild, with bovine serum albumin ("BSA") most often associated with these reactions

(Fiocchi et al. 1995; Werfel et al. 1997). Thus, the evidence suggests that bovine neutrophil lactoferrin is not an allergen in beef.

Would lactoferrin become an allergen for those consuming beef at the levels of exposure likely to occur with the use of bovine milk-derived lactoferrin described in this document? The current document addresses the spray application of bovine milk-derived lactoferrin to beef carcasses as an antimicrobial treatment. As noted in Chapter IV of this document, beef carcasses (and meat products derived from them) already contain naturally occurring lactoferrin, primarily from neutrophils, and as described in Chapter V, no significant increase in exposure to lactoferrin would occur from this use relative to current background exposures from consumption of beef. Therefore, it is unlikely that lactoferrin would become an allergen in those individuals consuming lactoferrin-treated beef.

Is bovine neutrophil lactoferrin similar to bovine milk-derived lactoferrin? Chapter IV contains a discussion of the structural similarities and differences between these two different sources of lactoferrin. From this discussion, only one structural difference emerged; that involving a difference in glycosylation (Hurley et al. 1993). With regard to the question of potential allergic responses, all immunological reactions to lactoferrin so far reported are to the polypeptide chain and not to the glycan. Differences in glycosylation are unlikely to have any effect on lactoferrin in terms of its potential allergenicity.

Furthermore, glycosylation has been investigated for possible cross-reactivity that may exist in some patients between pollen and food allergens and rather thoroughly for its impact on the IgE-binding to pollen allergens. Cross-reacting carbohydrate determinants ("CCDs") have been found associated with certain pollen and food allergens, particularly allergens in fresh fruits and vegetables (Aalberse et al. 2001; Vieths et al. 1994; Vieths 1997). Certain proteins in fruits and vegetables and certain pollen allergens possess similar glycan structures that bind to IgE antibodies in the serum of some human subjects. However, the cross-reactive IgEs directed to CCDs do not appear to have any clinical significance because they have been identified in both symptomatic and asymptomatic individuals (van der Veen et al. 1997; Vieths 1997). Thus, if these results can be extrapolated to lactoferrin, differences in glycosylation are unlikely to result in differences in the potential of these proteins to elicit allergic reactions.

Does bovine milk-derived lactoferrin have the ability to induce allergic sensitization? Studies in Brown Norway rats conducted by the British Industrial Biological Research Association ("BIBRA") in the U.K. indicate that lactoferrin can induce IgE responses in these rats when injected intraperitoneally (Atkinson and Miller 1994; Miller et al. 1998). However, this particular Brown Norway rat model is not appropriate for the prediction of human responses to ingestion of lactoferrin or other proteins. First, this model has not been validated. It does not discriminate between allergenic and non-allergenic proteins. No protein ever tested in this model with intraperitoneal injection has been shown to be negative. Therefore, the predictive value of these studies is essentially zero. In further studies with the Brown Norway rat using oral exposures, the

ability of lactoferrin to induce an IgE response was much weaker and similar to BSA, another milk protein that is also not a particularly significant milk allergen (Meredith and Atkinson 2000; Meredith and Atkinson 2001). This result raises even further questions regarding the initial observations obtained with intraperitoneal injections.

In addition, based on what is currently known regarding the lowest dose that elicits an allergic response in sensitized individuals exposed to several common (and fairly potent) food allergens (see the discussion below regarding peanuts, eggs, and cows' milk), the estimated intakes of bovine milk-derived lactoferrin resulting from its intended use as an antimicrobial spray on beef carcasses, as described in this document, are below any of these doses. Furthermore, the likelihood of an allergic response due to exposure to bovine milk-derived lactoferrin at these low levels is even more remote when one considers that there are likely to be few (if any) lactoferrin-sensitized individuals in the population. Thus, the possibility that lactoferrin would elicit an allergic reaction in any individuals at the estimated levels of ingestion described in this document is unlikely.

The most recent work related to identifying elicitation thresholds for several well-recognized food allergens (i.e., peanuts, eggs, and cows' milk) comes primarily from Hourihane et al. (1997) and Taylor et al. (2002). In Hourihane et al. (1997), investigators used a randomized, double-blind, placebo-controlled study design to challenge 14 human subjects allergic to peanuts (i.e., sensitized) with peanut doses ranging from 10 μg to 50 mg, administered in the form of a commercially available peanut flour. What these investigators found was that even in this group of well-characterized, highly sensitive subjects with established peanut allergies, the "threshold dose" of peanut protein varies. More importantly, they observed that as little as 100 μg of peanut protein could elicit symptoms in some subjects with peanut allergy.

Taylor et al. (2002) reported the results of a roundtable conference between twelve clinical allergists who were invited to share existing data on threshold doses for food allergens in human subjects already sensitized to the allergen. In this paper, the investigators stated:

> "...sufficient results are available to conclude that the threshold doses for commonly allergenic foods are finite, measurable, and above zero. However, attempting to reach consensus on the threshold doses for peanut, egg, cows' milk, fish, and mustard on the basis of the existing data would probably be premature for a number of reasons."

000060

According to the authors, the primary reason for not estimating a threshold dose on the basis of the available data is that the no-observed-adverse-effect level ("NOAEL") was not established for the vast majority of the human subjects involved in these trials. Instead, the "lowest provoking dose" for each individual patient was determined. This dose corresponds to the lowest-observed-adverse-effect level ("LOAEL"), rather than the NOAEL. However, even though threshold doses were not specifically established in this paper due to uncertainties regarding what (if any) safety factors to apply, the tabulated results suggest that the lowest dose that elicits an allergic response in sensitized

individuals exposed to peanuts, eggs, and cows' milk (standardized on protein content) appear to be in the low milligram range or higher for most individuals with allergies to these particular foods.

The estimated intakes of bovine milk-derived lactoferrin resulting from its intended use on beef carcasses, as shown in Table 3, are 76 μg or less, lower than the lowest dose (i.e., 130 μg) that elicited an observable allergic reaction in any sensitized individual exposed to peanuts, eggs, or cows' milk in the study reported by Taylor et al. (2002), and even less than the lowest dose observed in Hourihane et al. (1997) that elicited an allergic reaction in subjects with known peanut allergies (i.e., 100 μg). Moreover, the likelihood of an allergic response due to exposure to bovine milk-derived lactoferrin at these low levels is even more remote when one considers that there are likely to be few (if any) lactoferrin-sensitized individuals in the population.

Therefore, bovine milk-derived lactoferrin has not been established as a cows' milk or beef allergen, and there is no reason to believe that it will become an allergen under the conditions of use described in this document.

XI. LITERATURE CITED

Aalberse, R.C., J.H. Akkerdaas, and R. van Ree. 2001. Cross-reactivity of IgE antibodies to allergens. *Allergy* 56:478-490.

Atkinson, H.A.C. and K. Miller. 1994. Assessment of the Brown Norway rat as a suitable model for the investigation of food allergy. *Toxicol.* 91:281-288.

Baldo, B.A. 1984. Milk allergies. *Aust. J. Dairy Technol.* 39:120-128.

Barth, C.A. and U. Behnke. 1997. Nutritional significance of whey and whey components. *Nahrung* 41:S2-S12.

Baynes, R.D., W.R. Bezwoda, Q. Khan, and N. Mansoor. 1986. Plasma lactoferrin content: Differential effect of steroid administration and infective illnesses: Lack of effect of ambient temperature at which specimens are collected. *Scand. J. Haematol.* 37:353-359.

Besler, M., P. Eigenmann, and R.H. Schwartz. 2002. Cows' milk (*Bos domesticus*). Internet Symp. *Food Allergens* 4:19-106.

Birgens, H.S. 1994. The monocytic receptor for lactoferrin and its involvement in lactoferrin-mediated iron transport. *Adv. Exp. Med. Biol.* 357:99-109.

Brock, J.H. 1980. Lactoferrin in human milk: Its role in iron absorption and protection against enteric infection in the newborn infant. *Arch. Dis. Child.* 55:417-421.

Brock, J.H. 1998. Lactoferrin. In: *Human Cytokines*, Vol III, ed B.B. Aggarwal, Blackwell, M.A. Malden, 92-123.

Brown, M.H., A.H. Brightman, B.W. Fenwick, and M.A. Rider. 1996. Identification of lactoferrin in bovine tears. *Am. J. Vet. Res.* 57:1369-1372.

Broxmeyer, H.E., P. Ralph, J. Bognacki, P.W. Kincade, and M. De Sousa. 1980. A subpopulation of human polymorphonuclear neutrophils contains an active form of lactoferrin capable of inhibiting production of granulocyte-macrophage colony stimulating activities by human monocytes. *J. Immunol.* 125:903-909.

Broxmeyer, H.E., D.C. Bicknell, S. Gillis, E.L. Harris, L.M. Pelus, and G.W. Sledge. 1986. Lactoferrin: Affinity purification from human milk and polymorphonuclear neutrophils using monoclonal antibody (II2C) to human lactoferrin, development of an immunoradiometric assay using II2C, and myelopoietic regulation and receptor-binding characteristics. *Blood Cells* 11:429-446.

Burdock, G.A. 1997. *Encyclopedia of Food and Color Additives*. Boca Raton, FL: CRC Press Inc.

000062

Businco, L., P.G. Giampietro, P. Lucenti, F. Lucaroni, C. Pini, G. Di Felice, P. Iacovacci, C. Curadi, and M. Orlandi. 2000. Allergenicity of mare's milk in children with cows' milk allergy. *J. Allergy Clin. Immunol.* 105:1031-1034.

Derisbourg, P., J.M. Wieruszeski, J. Montreuil, and G. Spik. 1990. Primary structure of glycans isolated from human leucocyte lactotransferrin. Absence of fucose residues questions the proposed mechanism of hyposideraemia. *Biochem. J.* 269:821-825.

Field, R.A., L.R. Sanchez, J.E. Kunsman, and W.G. Kruggel. 1980. Heme pigment content of bovine hemopoietic marrow and muscle. *J. Food Sci.* 45:1109-1112.

Fiocchi, A., P. Restani, E. Riva, R. Qualizza, P. Bruni, A.R. Restelli, and C.L. Galli. 1995. Meat allergy. I. Specific IgE to BSA and OSA in atopic, beef sensitive children. *J. Am. Coll. Nutr.* 14:239-244.

Fiocchi, A., P. Restani, E. Riva, G.P. Mirri, I. Santini, L. Bernardo, and C.L. Galli. 1998. Heat treatment modifies the allergnicity of beef and beef serum albumin. *Allergy* 53:798-802.

Frandson, R.D. and T.L. Spurgeon. 1995. *Anatomía y fisiología de los animales domésticos*, 5th ed. Interamericana McGraw-Hill, Mexico DF, p227.

Furmanski, P., Z-P. Li, M.B. Fortuna, C.V.B. Swamy, and M.R. Das. 1989. Multiple molecular forms of human lactoferrin. *J. Exp. Med.* 170:415-429.

Gennaro, R., C. Schneider, G. de Nicola, F. Cian, and D. Romeo. 1978. Biochemical properties of bovine granulocytes. *Proc. Soc. Exp. Biol. Med.* 157:342-347.

Gennaro, R., B. Dewald, U. Horisberger, H.U. Gubler, and M. Baggiolini. 1983. A novel type of cytoplasmic granule in bovine neutrophils. *J. Cell Biol.* 96:1651-1661.

Gerold, M. and A. Stolle. 1994. Elektrostimulation. Untersuchungen über den Einfluss auf den Ausblutungsgrad von Rinderschlachtierkorpern. *Fleischwirtschaft* 74:864-869.

Goodman, R.E. and F.L. Schanbacher. 1991. Bovine lactoferrin mRNA: Sequence, analysis, and expression in the mammary gland. *Biochem. Biophys. Res. Comm.* 180:75-84.

Gregory, N.G., L.J. Wilkins, and A.M.S. Gregory. 1988. Studies on blood engorgement in beef carcasses. *J. Sci. Food Agric.* 46:43-51,

Harmon, R.J., F.L. Schanbacher, L.C. Ferguson, and K.L. Smith. 1976. Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and α-lactalbumin during acute experimental and natural coliform mastitis in cows. *Infect. Immunity* 13:533-542.

Harmon, R.J. and F.H.S. Newbould. 1980. Neutrophil leukocyte as a source of lactoferrin in bovine milk. *Am. J. Vet. Res.* 41:1603-1606.

Holloway, N.M., J. Lakritz, J.W. Tyler, and S.L. Carlson. 2002. Serum lactoferrin concentrations in colostrum-fed calves. *Am. J. Vet. Res.* 63:476-478.

Host, A., S. Husby, B. Gjesing, J.N. Larsen, and H. Lowenstein. 1992. Prospective estimation of IgG, IgG subclass and IgE antibodies to dietary proteins in infants with cow milk allergy. *Allergy* 47:218-229.

Hourihane, J. O'B., S.A. Kilburn, J.A. Nordlee, S.L. Hefle, S.L. Taylor, and J.O. Warner. 1997. An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: A randomized, double-blind placebo-controlled food challenge study. *J. Allergy Clin. Immunol.* 100:596-600.

Hurley, W.L., R.C.J. Grieve, C.E. Magura, H.M. Hegarty, and S. Zou. 1993. Electrophoretic comparisons of lactoferrin from bovine mammary secretions, milk neutrophils, and human milk. *J. Dairy Sci.* 76:377-387

Levay, P.F. and M. Viljoen. 1995. Lactoferrin: A general review. *Haematologica* 80:252-267.

Liu, L.H.E., W. Gladwell, and C.T. Teng. 2002. Detection of exon polymorphisms in the human lactoferrin gene. *Biochem. Cell. Biol.* 80:17-22.

Martin-Burriel, I., R. Osta, W. Barendse, and P. Zaragoza. 1997. New polymorphism and linkage mapping of the bovine lactotransferrin gene. *Mamm. Genome* 8:704-705.

Masson, P.L., J.F. Heremans, and E. Schonne. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* 130:643-658.

Mead, P.E. and J.W. Tweedie. 1990. cDNA and protein sequence of bovine lactoferrin. *Nucl. Acids Res.* 18:7167.

Meredith, C. and H.A.C. Atkinson. 2000. Development of methods to predict the allergenic potential of food proteins. *Toxicologist* 54:Abstract #682.

Meredith, C. and H.A.C. Atkinson. 2001. Development of methods to predict the allergenic potential of food proteins. Report to U.K. Food Standards Agency.

Metz-Boutigue, M-H., J. Jolles, J. Mazurier, F. Schoentgen, D. Legrand, G. Spik, J. Montreuil, P. Jolles. 1984. Human lactotransferrin: Amino acid sequence and comparison with other transferrins. *Eur. J. Biochem.* 145:659-676.

Miller, K., J. Laugee, and C. Meredith. 1998. The monitoring of the effects of food components on immunoreactivity in experimental animals. *Allergy* 53:35-37.

000064

Moguilevsky, N., L.A. Retegui, P.J. Courtoy, C.E. Castracane, and P.L. Masson. 1984. Uptake of lactoferrin by the liver. III. Critical role of the protein moiety. *Lab. Invest.* 50:335-340.

Naidu, A.S, ed. 2000. Lactoferrin. In *Natural Food Antimicrobial Systems*. CRC Press, Inc., Boca Raton.

Oellingrath, I.M., A. Iversen, and G. Skrede. 1990. Quantitative determination of myoglobin and haemoglobin in beef by high-performance liquid chromatography. *Meat Sci.* 28:313-320.

Pierce, A., D. Colavizza, M. Beniassa, P. Maes, A. Tartar, J. Montreuil, and G. Spik. 1991. Molecular cloning and sequence analysis of bovine lactotransferrin. *Eur. J. Biochem.* 196:177-184.

Rado, T.A., X. Wei, and E.J. Benz. 1987. Isolation of lactoferrin cDNA from a human myeloid library and expression of mRNA during normal and leukemic myelopoiesis. *Blood* 70:989-993.

Rulis, A.M. 2000. *Agency Response Letter GRAS Notice No. GRN 000037.* Accessed at http://vm.cfsan.fda.gov/~rdb/opa-g037.html.

Sato, R., K. Ohki, B. Syuto, J. Sato, and Y. Naito. 2000. Plasma lactoferrin concentration measured by ELISA in healthy and diseased cows. In *Lactoferrin: Structure, function and applications* (Ed. K. Shimazaki, et al.). Elsevier, Amsterdam, 111-116.

Schwerin, M., S. Solinas Toldo, A. Eggen, R. Brunner, H.M. Seyfert, and R. Fries. 1994. The bovine lactoferrin gene (LTF) maps to chromosome 2 and syntenic group U12. *Mamm. Genome* 5:486-489.

Scott, P.H. 1989. Enzyme immunoassay of lactoferrin in newborn term infants: Reference values and influence of diet. *Ann. Clin. Biochem.* 26:407-411

Segars, F.M. and J.M. Kinkade. 1977. Radioimmunoassay for murine lactoferrin, a protein marker of myeloid and mammary epithelial secretory cell differentiation. *J. Immunol. Meth.* 14:1-14.

Seyfert, H.M. and C. Kuhn. 1994. Characterization of a first bovine lactoferrin gene variant, based on an Ecori polymorphism. *Anim. Genet.* 25:54.

Seyfert, H-M., A. Tuckoricz, H. Interthal, D. Koczan, and G. Hobom. 1994. Structure of the bovine lactoferrin-encoding gene and its promoter. *Gene.* 143:265-269.

Sharma, S., P. Kumar, C. Betzel, and T.P. Singh. 2001. Structure and function of proteins involved in milk allergies. *J. Chromatography B* 756:183-187.

Siebert, P.D. and B.C.B. Huang. 1997. Identification of an alternative form of human lactoferrin mRNA that is expressed differently in normal tissues and tumor-derived cell lines. *Proc. Nat. Acad. Sci. USA.* 94:2198-2203

Spik, G., B. Coddeville, J. Mazurier, Y. Bourne, C. Cambillaut, and J. Montreuil. 1994. Primary and three-dimensional structure of lactotransferrin (lactoferrin) glycans. *Adv. Exp. Med. Biol.* 357:21-32.

Talkuder, M.J.R., T. Takeuchi, and E. Harada. 2002. Transport of colostral macromolecules into the cerebrospinal fluid via plasma in newborn calves. *J. Dairy Sci.* 85:514-524.

Taylor, S.L., S.L. Hefle, C. Bindslev-Jensen, S.A. Bock, A.W. Burks, Jr, L. Christie, D.J. Hill, A. Host, J.O. Hourihane, G. Lack, D.D. Metcalfe, D.A. Moneret-Vautrin, P.A. Vadas, F. Rance, D.J. Skrypec, T.A. Trautman, I.M. Malmheden, and R.S. Zeiger. 2002. Factors affecting the determination of threshold doses for allergenic foods: How much is too much? *J. Allergy Clin. Immunol.* 109:24-30.

Teng, C.T. 2002. Lactoferrin gene expression and regulation: An overview. *Biochem. Cell. Biol.* 80:7-16.

Teng, C.T., B.T. Pentecost, A. Marshall, A. Solomon, B.H. Bowman, P.A. Lalley, and S.L. Naylor. 1987. Assignment of the lactotransferrin gene to human chromosome 3 and to mouse chromosome 9. *Somat. Cell. Mol. Genet.* 13:689-693.

Tsuji, S., Y. Hirata, and K. Matsuoka. 1989. Two apparent molecular forms of bovine lactoferrin. *J. Dairy Sci.* 72:1130-1136.

U.S. Department of Agriculture (USDA). 1996. *Food Guide Pyramid.* Washington, DC: U.S. Department of Agriculture, Center for Nutrition Policy and Promotion. Home and Garden Bulletin No. 252.

U.S. Department of Agriculture, Agricultural Research Service (USDA). 2000. 1994-96, 1998 Continuing Survey of Food Intakes by Individuals [CD-ROM], data and documentation. National Technical Information Service, Accession No. PB2000-500027.

U.S. Environmental Protection Agency (USEPA). 2000. Food Commodity Intake Database (FCID) [CD-ROM], data and documentation. National Technical Information Service, Accession No. PB2000-500101.

van der Veen, M.J., R. van Ree, R.C. Aalberse, J. Akkerdaas, S.J. Koppleman, H.M. Jansen, and J.S. van der Zee. 1997. Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. *J. Allergy Clin. Immunol.* 100:327-334.

Venge, P., A. Stremberg, J.H. Braconier, L.E. Roxin, and I. Olsson. 1978. Neutrophil and eosinophil granulocytes in bacterial infection: Sequential studies of cellular and serum levels of granule proteins. *Brit. J. Haematol.* 38:475-483.

Vieths, S. 1997. Allergens in fruits and vegetables. In: *Handbook of Plant and Fungal Toxicants*, ed. J.P.F. D'Mello, CRC Press, Boca Raton FL, 157-174.

Vieths, S., M. Mayer, and M. Baumgart. 1994. Food allergy: specific binding of IgE antibodies from plant food sensitized individuals to carbohydrate epitopes. *Food Agr. Immunol.* 6:453-463.

000066

Wal, J.M. 1998. Strategies for assessment and identification of allergenicity in (novel) foods. *Int. Dairy J.* 8:413-423.

Wal, J.M., H. Bernard, M. Yvon, G. Peltre, B. David, C. Creminon, Y. Frobert, and J. Grassi. 1995. Enzyme immunoassay of specific human IgE to purified cows' milk allergens. *Food Agric. Immunol.* 7:175-187.

Wal, J.M., H. Bernard, G. Clement, J.M. Chatel, and K. Adel-Patient. 2001. Characterization of allergens in milk. *Curr. Allergy Clin. Immunol.* 14:4-10.

Warriss, P.D. 1976. The quantitative determination of haemoglobin in ovine muscles. *Anal. Biochem.* 72:104-112.

Warriss, P.D. 1977. The residual blood content of meat—A review. *J. Sci. Food Agric.* 28:457-462.

Warriss, P.D. 1984. Exsanguination of animals at slaughter and the residual blood content of meat. *Vet. Rec.* 115:292-295.

Warriss, P.D. and D.N. Rhodes. 1977. Haemoglobin concentrations in beef. *J. Sci. Food Agric.* 28:931-934.

Werfel, S.J., S.K. Cooke, and H.A. Sampson. 1997. Clinical reactivity to beef in children allergic to cows' milk. *J. Allergy Clin. Immunol.* 97:293-300.

Wu, H-F., R.L. Lundblad, and F.C. Church. 1995. Neutralization of heparin activity by neutrophil lactoferrin. *Blood* 85:421-428.

Ye, X.Y., T. Nishimura, and S. Yoshida. 1997. Characterization of the protein and glycan moieties in different forms of bovine lactoferrin. *Biosci. Biotechnol. Biochem.* 61:782-786

F

Section VI
GRAS Panel Report

GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF BOVINE MILK-DERIVED LACTOFERRIN AS A COMPONENT OF AN ANTIMICROBIAL BEEF CARCASS SPRAY WITHOUT REQUIRING LABELING FOR LACTOFERRIN.

GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF BOVINE MILK-DERIVED LACTOFERRIN AS A COMPONENT OF AN ANTIMICROBIAL BEEF CARCASS SPRAY WITHOUT REQUIRING LABELING FOR LACTOFERRIN

Prepared for:

aLF Ventures, LLC
Fraser, New York

Prepared by:

ENVIRON International Corporation
Arlington, Virginia

May 2003

GRAS EXEMPTION CLAIM

aLF Ventures, LLC, a Farmland National Beef Packaging Company, L.P. ("National Beef") and DMV International joint venture, attests that the conditions of use of the substance described below (i.e., bovine milk-derived lactoferrin) are exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act ("FFDCA") because aLF Ventures has determined such use to be Generally Recognized As Safe ("GRAS") via scientific procedures. The current GRAS determination for bovine milk-derived lactoferrin described below were prepared in compliance with proposed §170.36 of Title 21 of the Code of Federal Regulations (21 CFR §170.36), as published in the Federal Register (Vol. 62, No. 74, p. 18936 et seq., April 17, 1997).

Name of GRAS Substance

Bovine milk-derived lactoferrin, the subject of this GRAS determination, is the common or trade name for an iron-binding glycoprotein isolated from cow's milk. The published literature may also refer to this lactoferrin as red milk protein, lactosiderophilin, ekkrinosiderophilin, or lactotransferrin (Brock 1980; Naidu 2000).

Intended Technical Effect

Bovine milk-derived lactoferrin is known to be an effective natural microbial blocking agent ("MBA"). Antimicrobial sprays containing lactoferrin have been shown to reduce microbial contamination by a wide variety of microbes. The intended technical effect associated with the proposed use of bovine milk-derived lactoferrin is to detach bacteria adhering to the surface of beef carcasses in order to reduce microbial contamination of beef products produced from these carcasses. The microbial blockiing effects of lactoferrin are specific to pathogenic bacteria and, when applied in an aqueous spray, lactoferrin can detach and remove a wide variety of adherent pathogens from a beef tissue surface, including enterotoxigenic E. coli and verotoxic E. coli,including serotype O157:H7. The scientific basis substantiating this antimicrobial effect was previously presented in detail in connection with GRAS Notification number 67 ("GRN 67").

Intended Use and Consumer Exposure

This GRAS determination specifically addresses a single use of bovine milk-derived lactoferrin: application as an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not

to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb).

GRN 67, previously submitted to the FDA by National Beef, described the sequential use of bovine milk-derived lactoferrin as a component (at not more than 2 percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses prior to a final lactic acid rinse during processing, with successive applications to subprimal cuts and finished cuts. The cumulative exposure to lactoferrin from these three uses was estimated by National Beef to be 4.1 mg per person per day for the "typical" consumer of beef products and 9.1 mg per person per day for the "heavy" consumer of beef products. The cumulative exposure from these successive applications of bovine milk-derived lactoferrin was previously determined to be GRAS by National Beef, subject to the inclusion of labeling to alert consumers who are allergic to milk of the potential presence of this milk-derived ingredient in beef products so labeled. The FDA was notified of this GRAS determination on January 9, 2001 (GRN 67).

The single application of bovine milk-derived lactoferrin to beef carcasses, which is the subject of the present GRAS determination, yields a much lower exposure to bovine milk-derived lactoferrin from beef consumption than the cumulative exposure from successive applications considered under GRN 67 (i.e., intakes in the μg range versus the mg range), and therefore has been determined to be GRAS without the requirement of labeling retail food products ultimately produced from carcasses so treated.

Basis for GRAS Determination

This document addresses only the application of bovine milk-derived lactoferrin to beef carcasses to yield a final residual amount of bovine milk-derived lactoferrin on the carcass of less than 800 ng per g of beef (800 ppb) and provides an analysis through scientific procedures as to why the GRAS status of this specific use should not require labeling of retail food products ultimately produced from carcasses so treated.

Based on a review of pertinent and publicly available information (provided in Section V of the GRAS Notification: Scientific Support Document), the following conclusions were reached by an Expert Panel convened to evaluate the GRAS status of bovine milk-derived lactoferrin for use on beef carcasses as an antimicrobial spray without a labeling requirement:

- Legal and regulatory precedents suggest that when the addition of a processing aid does not substantively impact the total intake of that substance,

and this exogenous addition will not produce a significant health or safety concern, then labeling for this substance is not required.

- To address the issue of potential protein contamination of the bovine milk-derived lactoferrin product, several cationic exchange chromatography, gel permeation chromatography (GPC), and high performance liquid chromatography (HPLC) analyses were conducted. The four allergenic proteins present in milk that would be of concern if they were present in substantial quantity in the bovine milk-derived lactoferrin product are alpha-lactalbumin (α-La), beta-lactoglobulin (β-Lg), caseinmacropeptide (glycomacropeptide; GMP), and bovine serum albumin (BSA). A chromatogram of a mixture of standards of these four proteins of concern and a chromatogram of a sample of bovine milk-derived lactoferrin product were compared. The figure of this overlay shows that the lactoferrin product does not have any peaks that correspond to the peaks for these proteins of concern (at a detection limit of approximately 10 mg/L or 10 ppm). Thus, there is sufficient evidence to conclude that the non-lactoferrin protein fraction does not consist of one or more of these proteins of allergenic concern. In fact, this is expected based on the production process for the bovine milk-derived lactoferrin product. The proteins of concern (α-La, β-Lg, BSA, GMP) are not extracted by their nature; i.e., their ionic charge is opposite that of lactoferrin. Furthermore, if residues of some of these proteins did remain on the ion exchanger column at the end of the adsorption cycle, they would be washed off in one of the washing steps before bovine milk-derived lactoferrin is recovered from the column.

- The major source of the lactoferrin found naturally in beef is the neutrophils present in residual blood. The source of the lactoferrin used in the antimicrobial spray formulation that is the subject of this GRAS determination is bovine milk. Bovine milk and tissue (neutrophil) lactoferrins appear to be products of the same gene and possess identical polypeptide chains, any differences between them being limited to the attached glycans. Analogy to human lactoferrin suggests that these differences are unlikely to affect protein function and/or allergic response because all immunological reactions to lactoferrin so far reported are to the polypeptide chain and not to the glycan. In addition, it is expected that endogenous lactoferrin derived from neutrophils should be released through degranulation within a few hours, at most, of the death of the animal. Thermal or mechanical shock will hasten this process still further. Thus no appreciable difference in the availability of endogenous and exogenous lactoferrin can be expected. Therefore, there appears to be no reason to believe that residual bovine milk-derived lactoferrin potentially found in treated beef carcasses will be any more prone to inducing allergic responses than would endogenous lactoferrin found naturally in beef carcass muscle tissue.

- Lactoferrin is an iron-binding protein of the transferrin family. It is found naturally in various external secretions, especially milk and colostrum, and in the secondary (specific) granules of neutrophils. Small amounts are also present in blood plasma, and lactoferrin can bind to a variety of mammalian cells. Because lactoferrin is naturally present in blood neutrophils and plasma, and meat contains residual blood, there will be some endogenous lactoferrin present in beef carcasses. Levels could be further increased by lactoferrin bound to other cells in the carcass. Thus, exposure to bovine lactoferrin occurs regardless of whether or not the carcass is treated with bovine milk-derived lactoferrin. Using the most reliable data in the published scientific literature, neutrophils in the residual blood of beef provide an estimated 25 to 199 ng of lactoferrin per g of beef muscle tissue (ppb). This is likely to be increased further by lactoferrin in plasma and lactoferrin bound to cells such as macrophages/monocytes. This estimated range derived from the published literature agrees quite well with empirically derived estimates from studies conducted by National Beef of around 193 ng of lactoferrin per g beef muscle tissue (ppb).

- The intended use of bovine milk-derived lactoferrin as an antimicrobial spray treatment on beef carcasses will result in concentrations of this source of lactoferrin in the edible meat of approximately 320 ng per g (ppb) if the lactoferrin is assumed to be equally distributed throughout the carcass, and approximately 82 ng per g (ppb) in boxed beef and 726 ng per g (ppb) in trim/grind if the lactoferrin is assumed to be unequally distributed throughout the carcass. These estimated concentrations were derived using information on the application rate of bovine milk-derived lactoferrin, the amount of lactoferrin removed following the lactic acid rinse, and the amount of lactoferrin retained on various edible portions of the carcass versus those portions destined for rendering operations and thus not consumed.

The range of estimated intakes of endogenous lactoferrin that result from untreated beef consumption can be compared against the range of estimated intakes of exogenous bovine milk-derived lactoferrin that occur from consumption of lactoferrin-treated beef. In the following table, the range of 50th percentile intakes of exogenous bovine milk-derived lactoferrin are all within the range of estimated background exposures to naturally occurring endogenous lactoferrin across all population groups of interest. There are, of course, uncertainties in both the endogenous and exogenous estimates because of the assumptions that must be used to derive these values; in particular, the estimated intake of exogenous lactoferrin is expected to be conservatively high. Therefore, given the inherent uncertainties in both endogenous and exogenous lactoferrin intake estimates, the intended use of bovine milk-derived lactoferrin as an antimicrobial treatment on beef carcasses will not result in a significant, measurable increase in lactoferrin intake relative to current background exposures from consumption of untreated beef.

Estimated Background Lactoferrin Intakes versus Estimated 50th and 90th Percentile Intakes of Bovine Milk-Derived Lactoferrin from Lactoferrin-Treated Beef Carcasses					
Population Group	**Estimated Range of Background Lactoferrin Intake (μg/occasion)[1]**	**Estimated Intake of Bovine Milk-Derived Lactoferrin (μg/occasion)[3]**			
		Range of 50th Percentile Lactoferrin Intakes		**Range of 90th Percentile Lactoferrin Intakes**	
		Scenario 1[2]: Equal Lactoferrin Distribution	**Scenario 2[3]: Unequal Lactoferrin Distribution**	**Scenario 1: Equal Lactoferrin Distribution**	**Scenario 2: Unequal Lactoferrin Distribution**
Infants, < 1 y	< 0.1 to 8	5	2	14	20
Infants, 1 y	< 0.1 to 12	5	6	20	35
Children, 2-5 y	0.1 to 14	7	10	24	44
Children, 6-12 y	0.2 to 19	9	12	31	62
Teenagers, 13-19 y	0.2 to 26	11	15	44	76
Adults, 20+ y	0.3 to 30	16	16	50	76

DATA SOURCE: 1994-96, 1998 Continuing Survey of Food Intakes by Individuals (CSFII) and Food Commodity Intake Database (USDA 2000, USEPA 2000)

NOTES: Breastfeeding infants and children were excluded from the analyses. Analyses were limited to individuals who provided 2-days of recall data. Estimates were generated with USDA sample weighting factors to adjust for differences in representation of subpopulations.

[1] The range of background lactoferrin intakes is represented by the 10th and 90th percentiles of endogenous lactoferrin consumption from beef; values correspond to the 10th and 90th percentiles of beef consumption (g per occasion) multiplied by 193 ng bovine neutrophil lactoferrin per g beef (ppb), respectively. See text for further discussion of lactoferrin concentration derivation.

[2] In Scenario 1, the concentration of bovine milk-derived lactoferrin is assumed to be equally distributed throughout the treated beef carcass; this exogenous lactoferrin concentration is 320 ng per g (ppb) beef. Values correspond to the 50th or 90th percentile of beef consumption (g per occasion) multiplied by 320 ng bovine milk-derived lactoferrin per g (ppb) beef See text for further discussion of lactoferrin concentration derivation.

[3] In Scenario 2, the concentration of bovine milk-derived lactoferrin is assumed to be unequally distributed throughout the treated beef carcass; the exogenous lactoferrin concentration in boxed beef is 82 ng per g (ppb) beef, while the exogenous lactoferrin concentration in trim/grind is 726 ng per g (ppb) beef. Values correspond to the 50th or 90th percentile of exogenous lactoferrin intake (g per occasion). For each beef-eating occasion, the amount of exogenous lactoferrin consumed was calculated by taking the sum of exogenous lactoferrin intake by type of beef (i.e., 82 ng bovine milk-derived lactoferrin per g (ppb) boxed beef added to 726 ng bovine milk-derived lactoferrin per g (ppb) trim/grind). See text for further discussion of lactoferrin concentration derivation.

- Bovine milk-derived lactoferrin has been consumed by humans for thousands of years as a naturally occurring protein found in milk. Current sources of lactoferrin in the U.S. diet include milk proteins naturally occurring in milk and milk products, and whey proteins added to foods throughout the food supply in the form of whey products. Milk proteins and whey proteins contain approximately 0.03 g and 1.4 g lactoferrin per 100 g, respectively. Estimates of the per capita lactoferrin intake from milk proteins in dairy products were added to the estimated per capita lactoferrin intake from whey products. These estimates of intake of lactoferrin from both dairy sources and whey protein added to food indicate that background lactoferrin exposure can be as high as 100 mg per person per day. These estimated intakes of bovine

lactoferrin from dairy products and whey products added to food demonstrate the very high background dietary exposure to lactoferrin relative to the typical lactoferrin intake that could potentially result from the use of lactoferrin as an antimicrobial treatment on beef carcasses. These dietary exposure estimates from dairy products and whey proteins provide some context in evaluating the very small intakes that would result from the proposed use of lactoferrin on beef carcasses.

- Bovine milk-derived lactoferrin is a minor cows' milk allergen. Among individuals with documented cows' milk allergy, under 50 percent have IgE antibody binding to lactoferrin. However, the fact that: 1) milk-derived and beef-derived lactoferrin are allergenically equivalent, 2) beef allergies are uncommon and no evidence exists whether or not lactoferrin is an allergen in beef, and 3) the proposed use of bovine milk-derived lactoferrin on beef carcasses is not expected to significantly increase typical lactoferrin exposures from meat consumption above background, makes the likelihood of an allergic reaction from the use of bovine milk-derived lactoferrin as an antimicrobial spray on beef carcasses very low.

- The lowest dose that elicits an observable allergic reaction in sensitized individuals exposed to well-recognized food allergens such as peanuts, eggs, and cows' milk (standardized on protein content) appear to be in the low milligram range or higher for most individuals with allergies to these particular foods, while estimated intakes of bovine milk-derived lactoferrin under its intended conditions of use described in this document are in the low microgram range, a number of orders of magnitude lower than the elicitation threshold for peanuts, eggs, and cows' milk in the vast majority of sensitized individuals. Therefore, the possibility that lactoferrin would elicit an allergic reaction in any individuals at the estimated levels of ingestion described in this document is unlikely.

General Recognition of the Scientific Basis for Determination of Safety of Bovine Milk-Derived Lactoferrin

The information reviewed and employed to determine the GRAS status of bovine milk-derived lactoferrin for use as an antimicrobial spray to treat beef carcasses, as described in this document, is based on pertinent studies and data provided by aLF Ventures, or identified in literature searches conducted through online bibliographic retrieval systems, including Medline® and Dialog®. The scientific data and information on which the safety determination is based are available in the published literature or are otherwise publicly available to experts qualified by training and experience to evaluate the safety of food and food additives. Thus, the data reviewed meet the general recognition element required for all GRAS determinations.

The key scientific components of the assessment as to whether labeling should be required for the GRAS status of bovine milk-derived lactoferrin applied only to beef carcasses are: 1) a determination that exogenous lactoferrin exposure (resulting from its application onto beef carcasses) is estimated to be in the same range as existing background lactoferrin exposures from naturally occurring endogenous lactoferrin in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i..e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use).

Publicly available, peer-reviewed literature indicate that bovine milk and tissue (neutrophil) lactoferrins appear to be products of the same gene and possess identical polypeptide chains, any differences between them being limited to the attached glycans. Analogy to human lactoferrin suggests that these differences are unlikely to affect protein function. With regard to the question of potential allergic responses, all immunological reactions to lactoferrin so far reported are to the polypeptide chain and not to the glycan.

Furthermore, glycosylation has been investigated for possible cross-reactivity that may exist in some patients between pollen and food allergens and rather thoroughly for its impact on the IgE-binding to pollen allergens. The cross-reactive carbohydrate determinants do not appear to have any clinical significance because in the case of patients with pollen and food allergies, such IgE reactive to these determinants has been found in both symptomatic and asymptomatic individuals. Thus, if these results can be extrapolated to lactoferrin, differences in glycosylation are unlikely to result in differences in the potential of these proteins to elicit allergic reactions. Therefore, there appears to be no reason to conclude that residual milk-derived lactoferrin potentially found in lactoferrin-treated beef carcasses will be any more prone to inducing allergic responses than would endogenous lactoferrin found naturally in beef carcass muscle tissue.

In order to assess the impact that the spraying of lactoferrin on beef carcasses could potentially have on total lactoferrin exposure from beef consumption, the potential intake of lactoferrin from exogenously applied lactoferrin was calculated employing several worst-case assumptions. This estimated intake of exogenous lactoferrin was compared to lactoferrin intake resulting from endogenous levels found naturally in beef. This comparison shows that the estimated intake of bovine milk-derived lactoferrin from its intended use as an antimicrobial spray on beef carcasses (employing several worst-case assumptions) is comparable to an individual's estimated background exposure to lactoferrin found endogenously in beef. Thus, the intended use of bovine milk-derived lactoferrin would not be expected to result in any substantive increase in total lactoferrin exposure from consumption of beef products

Thus, for this specific use of bovine milk-derived lactoferrin as a carcass spray, the beef carcass itself already contains a naturally occurring background level of the substance. Consumers who eat beef are already being exposed to lactoferrin, and the estimated typical exposure from the proposed exogenous source of lactoferrin falls within this normal background range of intake. Thus, this is not a situation where a new, foreign

substance is being added to food, but rather where there is only an insignificant addition of a substance already present.

Rather little scientific and clinical information exist to document whether bovine milk-derived lactoferrin is a cows' milk allergen. While there is some evidence to suggest that bovine milk-derived lactoferrin is a minor cows' milk allergen, the existing information is difficult to interpret because of methodological problems in the manner in which much of the data were collected.

Certainly, no argument exists that the major cows' milk allergens in IgE-mediated cows' milk allergy are casein, β-lactoglobulin, and α-lactalbumin (Besler et al. 2002; Sharma et al. 2001; Wal 1998; Wal et al. 2001). Several clinical investigators have demonstrated that some cows' milk-allergic individuals have IgE antibodies directed against lactoferrin (Baldo 1984; Businco et al. 2000; Host et al. 1992; Wal et al. 1995; Wal 1998; Wal et al. 2001). However, the vast majority of these milk-allergic infants displayed IgE antibodies to one or more of the major cows' milk allergens in addition to lactoferrin. Wal (1998) appears to have identified two milk-allergic infants out of 92 studied (2.2%) who were mono-sensitized to lactoferrin only. However, the clinical significance of this observation is unclear because the study does not indicate if these or other infants in the study had received blinded oral challenges to document their milk allergies. Without confirmation by oral challenge, the evaluation of specific IgE antibodies in patient serum can be clinically meaningless and misleading. Furthermore, in studies where quantitative estimates were obtained of the levels of specific IgE antibodies to lactoferrin (Wal et al. 1995; Wal 1998), the levels of anti-lactoferrin IgE antibodies were far lower than the levels of specific IgE antibodies to the major milk allergens in the vast majority of the patients.

Milk-allergic patients seem to occasionally have IgE antibodies directed at other minor milk proteins (Baldo 1984); the clinical significance of the presence of these antibodies is unknown. To demonstrate that bovine milk-derived lactoferrin is a clinically significant milk allergen, patients who were challenge-positive to milk ingestion would have to demonstrate specific IgE antibodies to bovine milk-derived lactoferrin (and possibly other milk proteins), positive skin prick tests to bovine milk-derived lactoferrin, and positive oral challenges to bovine milk-derived lactoferrin. Such information does not exist to unequivocally demonstrate whether or not bovine milk-derived lactoferrin is a human allergen.

The presence of lactoferrin in beef primarily arising from its presence in bovine neutrophils has been clearly documented. Furthermore, bovine neutrophil lactoferrin is very similar, structurally and functionally, to bovine milk-derived lactoferrin. However, even given the presence of lactoferrin in beef, allergic reactions to beef are comparatively

rare and are usually relatively mild, with bovine serum albumin ("BSA") most often associated with these reactions (Fiocchi et al. 1995; Werfel et al. 1997). Thus, the evidence suggests that bovine neutrophil lactoferrin is not an allergen in beef.

Studies in Brown Norway rats conducted by the British Industrial Biological Research Association ("BIBRA") in the U.K. indicate that lactoferrin can induce IgE responses in these rats when injected intraperitoneally (Atkinson and Miller 1994; Miller et al. 1998). However, this particular Brown Norway rat model is not appropriate for the prediction of human responses to ingestion of lactoferrin or other proteins. First, this model has not been validated. It does not discriminate between allergenic and non-allergenic proteins. No protein ever tested in this model with intraperitoneal injection has been shown to be negative. Therefore, the predictive value of these studies is essentially zero. In further studies with the Brown Norway rat using oral exposures, the ability of lactoferrin to induce an IgE response was much weaker and similar to BSA, another milk protein that is also not a particularly significant milk allergen (Meredith and Atkinson 2000; Meredith and Atkinson 2001). This result raises even further questions regarding the initial observations obtained with intraperitoneal injections.

In addition, based on what is currently known regarding the lowest dose that elicits an allergic response in sensitized individuals exposed to several common (and fairly potent) allergenic foods , the estimated intakes of bovine milk-derived lactoferrin resulting from its intended use as an antimicrobial spray on beef carcasses, as described in this document, are below any of these doses. Furthermore, the likelihood of an allergic response due to exposure to bovine milk-derived lactoferrin at these low levels is even more remote when one considers that there are likely to be few (if any) lactoferrin-sensitized individuals in the population. Thus, the possibility that lactoferrin would elicit an allergic reaction in any individuals at the estimated levels of ingestion described in this document is unlikely.

Taylor et al. (2002) reported the results of a roundtable conference between twelve clinical allergists who were invited to share existing data on threshold doses for allergenic foods in human subjects already sensitized to the food. In this paper, the investigators stated:

> "...sufficient results are available to conclude that the threshold doses for commonly allergenic foods are finite, measurable, and above zero. However, attempting to reach consensus on the threshold doses for peanut, egg, cows' milk, fish, and mustard on the basis of the existing data would probably be premature for a number of reasons."

According to the authors, the primary reason for not estimating a threshold dose on the basis of the available data is that the no-observed-adverse-effect level ("NOAEL") was not established for the vast majority of the human subjects involved in these trials. Instead, the "lowest provoking dose" for each individual patient was determined. This dose corresponds to the lowest-observed-adverse-effect level ("LOAEL"), rather than the NOAEL. However, even though threshold doses were not specifically established in this paper due to uncertainties regarding what (if any) safety factors to apply, the tabulated results suggest that the lowest dose that elicits an allergic response in sensitized individuals exposed to peanuts, eggs, and cows' milk (standardized on protein content) appear to be in the low milligram range or higher for most individuals with allergies to these particular foods. Of course, allergenic foods like peanuts, eggs, and milk contain mixtures of proteins while bovine milk-derived lactoferrin is an isolated protein. Threshold doses for an individual, isolated allergenic protein from a food (e.g. casein from milk) could be lower than the threshold dose for the whole food. However, lactoferrin is not an acknowledged milk allergen so this concern is probably hypothetical in this case.

The estimated intakes of bovine milk-derived lactoferrin resulting from its intended use on beef carcasses, are 76 μg or less, lower than the lowest dose (i.e., 1 mg) that elicited an objective allergic reaction in any sensitized individual exposed to peanuts, eggs, or cows' milk, as the whole food, in the study reported by Taylor et al. (2002) Moreover, the likelihood of an allergic response due to exposure to bovine milk-derived lactoferrin at these low levels is even more remote when one considers that there are likely to be few (if any) lactoferrin-sensitized individuals in the population.

Therefore, bovine milk-derived lactoferrin is not a well-established cows' milk or beef allergen, and there is no reason to believe that it will become an allergen under the conditions of use described in this document.

Conclusions

Bovine milk-derived lactoferrin has not been established as a cows' milk or beef allergen, and there is no reason to believe that it will become an allergen under the conditions of use described in this document. Scientific consensus exists for this determination since bovine milk-derived lactoferrin is already a naturally-occurring component of food, the level of ingestion of the component in food is not increased significantly above the range of intakes derived from naturally occurring sources of the component, the allergenicity of the component has not been firmly established, and there is consensus that thresholds for allergens do exist. Therefore, there can be no justification for requiring labels on foods

containing beef from carcasses treated with bovine milk-derived lactoferrin to state that fact on the label.

LITERATURE REVIEWED

Aalberse, R.C., J.H. Akkerdaas, and R. van Ree. 2001. Cross-reactivity of IgE antibodies to allergens. Allergy 56:478-490.

Atkinson, H.A.C. and K. Miller. 1994. Assessment of the Brown Norway rat as a suitable model for the investigation of food allergy. Toxicol. 91:281-288.

Baldo, B.A. 1984. Milk allergies. Aust. J. Dairy Technol. 39:120-128.

Barth, C.A. and U. Behnke. 1997. Nutritional significance of whey and whey components. Nahrung 41:S2-S12.

Baynes, R.D., W.R. Bezwoda, Q. Khan, and N. Mansoor. 1986. Plasma lactoferrin content: Differential effect of steroid administration and infective illnesses: Lack of effect of ambient temperature at which specimens are collected. Scand. J. Haematol. 37:353-359.

Besler, M., P. Eigenmann, and R.H. Schwartz. 2002. Cows' milk (Bos domesticus). Internet Symp. Food Allergens 4:19-106.

Birgens, H.S. 1994. The monocytic receptor for lactoferrin and its involvement in lactoferrin-mediated iron transport. Adv. Exp. Med. Biol. 357:99-109.

Brock, J.H. 1980. Lactoferrin in human milk: Its role in iron absorption and protection against enteric infection in the newborn infant. Arch. Dis. Child. 55:417-421.

Brock, J.H. 1998. Lactoferrin. In: Human Cytokines, Vol III, ed B.B. Aggarwal, Blackwell, M.A. Malden, 92-123.

Brown, M.H., A.H. Brightman, B.W. Fenwick, and M.A. Rider. 1996. Identification of lactoferrin in bovine tears. Am. J. Vet. Res. 57:1369-1372.

Broxmeyer, H.E., P. Ralph, J. Bognacki, P.W. Kincade, and M. De Sousa. 1980. A subpopulation of human polymorphonuclear neutrophils contains an active form of lactoferrin capable of inhibiting production of granulocyte-macrophage colony stimulating activities by human monocytes. J. Immunol. 125:903-909.

Broxmeyer, H.E., D.C. Bicknell, S. Gillis, E.L. Harris, L.M. Pelus, and G.W. Sledge. 1986. Lactoferrin: Affinity purification from human milk and polymorphonuclear neutrophils using monoclonal antibody (II2C) to human lactoferrin, development of an immunoradiometric assay using II2C, and myelopoietic regulation and receptor-binding characteristics. Blood Cells 11:429-446.

Burdock, G.A. 1997. Encyclopedia of Food and Color Additives. Boca Raton, FL: CRC Press Inc.

Businco, L., P.G. Giampietro, P. Lucenti, F. Lucaroni, C. Pini, G. Di Felice, P. Iacovacci, C. Curadi, and M. Orlandi. 2000. Allergenicity of mare's milk in children with cows' milk allergy. J. Allergy Clin. Immunol. 105:1031-1034.

Derisbourg, P., J.M. Wieruszeski, J. Montreuil, and G. Spik. 1990. Primary structure of glycans isolated from human leucocyte lactotransferrin. Absence of fucose residues questions the proposed mechanism of hyposideraemia. Biochem. J. 269:821-825.

Field, R.A., L.R. Sanchez, J.E. Kunsman, and W.G. Kruggel. 1980. Heme pigment content of bovine hemopoietic marrow and muscle. J. Food Sci. 45:1109-1112.

Fiocchi, A., P. Restani, E. Riva, R. Qualizza, P. Bruni, A.R. Restelli, and C.L. Galli. 1995. Meat allergy. I. Specific IgE to BSA and OSA in atopic, beef sensitive children. J. Am. Coll. Nutr. 14:239-244.

Fiocchi, A., P. Restani, E. Riva, G.P. Mirri, I. Santini, L. Bernardo, and C.L. Galli. 1998. Heat treatment modifies the allergnicity of beef and beef serum albumin. Allergy 53:798-802.

Frandson, R.D. and T.L. Spurgeon. 1995. Anatomía y fisiología de los animales domésticos, 5th ed. Interamericana McGraw-Hill, Mexico DF, p227.

Furmanski, P., Z-P. Li, M.B. Fortuna, C.V.B. Swamy, and M.R. Das. 1989. Multiple molecular forms of human lactoferrin. J. Exp. Med. 170:415-429.

Gennaro, R., C. Schneider, G. de Nicola, F. Cian, and D. Romeo. 1978. Biochemical properties of bovine granulocytes. Proc. Soc. Exp. Biol. Med. 157:342-347.

Gennaro, R., B. Dewald, U. Horisberger, H.U. Gubler, and M. Baggiolini. 1983. A novel type of cytoplasmic granule in bovine neutrophils. J. Cell Biol. 96:1651-1661.

Gerold, M. and A. Stolle. 1994. Elektrostimulation. Untersuchungen über den Einfluss auf den Ausblutungsgrad von Rinderschlachtierkorpern. Fleischwirtschaft 74:864-869.

Goodman, R.E. and F.L. Schanbacher. 1991. Bovine lactoferrin mRNA: Sequence, analysis, and expression in the mammary gland. Biochem. Biophys. Res. Comm. 180:75-84.

Gregory, N.G., L.J. Wilkins, and A.M.S. Gregory. 1988. Studies on blood engorgement in beef carcasses. J. Sci. Food Agric. 46:43-51,

Harmon, R.J., F.L. Schanbacher, L.C. Ferguson, and K.L. Smith. 1976. Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and α-lactalbumin during acute experimental and natural coliform mastitis in cows. Infect. Immunity 13:533-542.

Harmon, R.J. and F.H.S. Newbould. 1980. Neutrophil leukocyte as a source of lactoferrin in bovine milk. Am. J. Vet. Res. 41:1603-1606.

Holloway, N.M., J. Lakritz, J.W. Tyler, and S.L. Carlson. 2002. Serum lactoferrin concentrations in colostrum-fed calves. Am. J. Vet. Res. 63:476-478.

Host, A., S. Husby, B. Gjesing, J.N. Larsen, and H. Lowenstein. 1992. Prospective estimation of IgG, IgG subclass and IgE antibodies to dietary proteins in infants with cow milk allergy. Allergy 47:218-229.

Hourihane, J. O'B., S.A. Kilburn, J.A. Nordlee, S.L. Hefle, S.L. Taylor, and J.O. Warner. 1997. An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: A randomized, double-blind placebo-controlled food challenge study. J. Allergy Clin. Immunol. 100:596-600.

Hurley, W.L., R.C.J. Grieve, C.E. Magura, H.M. Hegarty, and S. Zou. 1993. Electrophoretic comparisons of lactoferrin from bovine mammary secretions, milk neutrophils, and human milk. J. Dairy Sci. 76:377-387

Levay, P.F. and M. Viljoen. 1995. Lactoferrin: A general review. Haematologica 80:252-267.

Liu, L.H.E., W. Gladwell, and C.T. Teng. 2002. Detection of exon polymorphisms in the human lactoferrin gene. Biochem. Cell. Biol. 80:17-22.

Martin-Burriel, I., R. Osta, W. Barendse, and P. Zaragoza. 1997. New polymorphism and linkage mapping of the bovine lactotransferrin gene. Mamm. Genome 8:704-705.

Masson, P.L., J.F. Heremans, and E. Schonne. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. J. Exp. Med. 130:643-658.

Mead, P.E. and J.W. Tweedie. 1990. cDNA and protein sequence of bovine lactoferrin. Nucl. Acids Res. 18:7167.

Meredith, C. and H.A.C. Atkinson. 2000. Development of methods to predict the allergenic potential of food proteins. Toxicologist 54:Abstract #682.

Meredith, C. and H.A.C. Atkinson. 2001. Development of methods to predict the allergenic potential of food proteins. Report to U.K. Food Standards Agency.

Metz-Boutigue, M-H., J. Jolles, J. Mazurier, F. Schoentgen, D. Legrand, G. Spik, J. Montreuil, P. Jolles. 1984. Human lactotransferrin: Amino acid sequence and comparison with other transferrins. Eur. J. Biochem. 145:659-676.

Miller, K., J. Laugee, and C. Meredith. 1998. The monitoring of the effects of food components on immunoreactivity in experimental animals. Allergy 53:35-37.

Moguilevsky, N., L.A. Retegui, P.J. Courtoy, C.E. Castracane, and P.L. Masson. 1984. Uptake of lactoferrin by the liver. III. Critical role of the protein moiety. Lab. Invest. 50:335-340.

Naidu, A.S, ed. 2000. Lactoferrin. In Natural Food Antimicrobial Systems. CRC Press, Inc., Boca Raton.

Oellingrath, I.M., A. Iversen, and G. Skrede. 1990. Quantitative determination of myoglobin and haemoglobin in beef by high-performance liquid chromatography. Meat Sci. 28:313-320.

Pierce, A., D. Colavizza, M. Beniassa, P. Maes, A. Tartar, J. Montreuil, and G. Spik. 1991. Molecular cloning and sequence analysis of bovine lactotransferrin. Eur. J. Biochem. 196:177-184.

Rado, T.A., X. Wei, and E.J. Benz. 1987. Isolation of lactoferrin cDNA from a human myeloid library and expression of mRNA during normal and leukemic myelopoiesis. Blood 70:989-993.

Rulis, A.M. 2000. Agency Response Letter GRAS Notice No. GRN 000037. Accessed at http://vm.cfsan.fda.gov/~rdb/opa-g037.html.

Sato, R., K. Ohki, B. Syuto, J. Sato, and Y. Naito. 2000. Plasma lactoferrin concentration measured by ELISA in healthy and diseased cows. In Lactoferrin: Structure, function and applications (Ed. K. Shimazaki, et al.). Elsevier, Amsterdam, 111-116.

Schwerin, M., S. Solinas Toldo, A. Eggen, R. Brunner, H.M. Seyfert, and R. Fries. 1994. The bovine lactoferrin gene (LTF) maps to chromosome 2 and syntenic group U12. Mamm. Genome 5:486-489.

Scott, P.H. 1989. Enzyme immunoassay of lactoferrin in newborn term infants: Reference values and influence of diet. Ann. Clin. Biochem. 26:407-411

Segars, F.M. and J.M. Kinkade. 1977. Radioimmunoassay for murine lactoferrin, a protein marker of myeloid and mammary epithelial secretory cell differentiation. J. Immunol. Meth. 14:1-14.

Seyfert, H.M. and C. Kuhn. 1994. Characterization of a first bovine lactoferrin gene variant, based on an Ecori polymorphism. Anim. Genet. 25:54.

Seyfert, H-M., A. Tuckoricz, H. Interthal, D. Koczan, and G. Hobom. 1994. Structure of the bovine lactoferrin-encoding gene and its promoter. Gene. 143:265-269.

Sharma, S., P. Kumar, C. Betzel, and T.P. Singh. 2001. Structure and function of proteins involved in milk allergies. J. Chromatography B 756:183-187.

Siebert, P.D. and B.C.B. Huang. 1997. Identification of an alternative form of human lactoferrin mRNA that is expressed differently in normal tissues and tumor-derived cell lines. Proc. Nat. Acad. Sci. USA. 94:2198-2203

Spik, G., B. Coddeville, J. Mazurier, Y. Bourne, C. Cambillaut, and J. Montreuil. 1994. Primary and three-dimensional structure of lactotransferrin (lactoferrin) glycans. Adv. Exp. Med. Biol. 357:21-32.

Talkuder, M.J.R., T. Takeuchi, and E. Harada. 2002. Transport of colostral macromolecules into the cerebrospinal fluid via plasma in newborn calves. J. Dairy Sci. 85:514-524.

Taylor, S.L., S.L. Hefle, C. Bindslev-Jensen, S.A. Bock, A.W. Burks, Jr, L. Christie, D.J. Hill, A. Host, J.O. Hourihane, G. Lack, D.D. Metcalfe, D.A. Moneret-Vautrin, P.A. Vadas, F. Rance, D.J. Skrypec, T.A. Trautman, I.M. Malmheden, and R.S. Zeiger. 2002. Factors affecting the determination of threshold doses for allergenic foods: How much is too much? J. Allergy Clin. Immunol. 109:24-30.

Teng, C.T. 2002. Lactoferrin gene expression and regulation: An overview. Biochem. Cell. Biol. 80:7-16.

Teng, C.T., B.T. Pentecost, A. Marshall, A. Solomon, B.H. Bowman, P.A. Lalley, and S.L. Naylor. 1987. Assignment of the lactotransferrin gene to human chromosome 3 and to mouse chromosome 9. Somat. Cell. Mol. Genet. 13:689-693.

Tsuji, S., Y. Hirata, and K. Matsuoka. 1989. Two apparent molecular forms of bovine lactoferrin. J. Dairy Sci. 72:1130-1136.

U.S. Department of Agriculture (USDA). 1996. Food Guide Pyramid. Washington, DC: U.S. Department of Agriculture, Center for Nutrition Policy and Promotion. Home and Garden Bulletin No. 252.

U.S. Department of Agriculture, Agricultural Research Service (USDA). 2000. 1994-96, 1998 Continuing Survey of Food Intakes by Individuals [CD-ROM], data and documentation. National Technical Information Service, Accession No. PB2000-500027.

U.S. Environmental Protection Agency (USEPA). 2000. Food Commodity Intake Database (FCID) [CD-ROM], data and documentation. National Technical Information Service, Accession No. PB2000-500101.

van der Veen, M.J., R. van Ree, R.C. Aalberse, J. Akkerdaas, S.J. Koppleman, H.M. Jansen, and J.S. van der Zee. 1997. Poor biologic activity of cross-reactive IgE directed to carbohydrate determinants of glycoproteins. J. Allergy Clin. Immunol. 100:327-334.

Venge, P., A. Stremberg, J.H. Braconier, L.E. Roxin, and I. Olsson. 1978. Neutrophil and eosinophil granulocytes in bacterial infection: Sequential studies of cellular and serum levels of granule proteins. Brit. J. Haematol. 38:475-483.

Vieths, S. 1997. Allergens in fruits and vegetables. In: Handbook of Plant and Fungal Toxicants, ed. J.P.F. D'Mello, CRC Press, Boca Raton FL, 157-174.

Vieths, S., M. Mayer, and M. Baumgart. 1994. Food allergy: specific binding of IgE antibodies from plant food sensitized individuals to carbohydrate epitopes. Food Agr. Immunol. 6:453-463.

Wal, J.M. 1998. Strategies for assessment and identification of allergenicity in (novel) foods. Int. Dairy J. 8:413-423.

Wal, J.M., H. Bernard, M. Yvon, G. Peltre, B. David, C. Creminon, Y. Frobert, and J. Grassi. 1995. Enzyme immunoassay of specific human IgE to purified cows' milk allergens. Food Agric. Immunol. 7:175-187.

Wal, J.M., H. Bernard, G. Clement, J.M. Chatel, and K. Adel-Patient. 2001. Characterization of allergens in milk. Curr. Allergy Clin. Immunol. 14:4-10.

Warriss, P.D. 1976. The quantitative determination of haemoglobin in ovine muscles. Anal. Biochem. 72:104-112.

Warriss, P.D. 1977. The residual blood content of meat—A review. J. Sci. Food Agric. 28:457-462.

Warriss, P.D. 1984. Exsanguination of animals at slaughter and the residual blood content of meat. Vet. Rec. 115:292-295.

Warriss, P.D. and D.N. Rhodes. 1977. Haemoglobin concentrations in beef. J. Sci. Food Agric. 28:931-934.

Werfel, S.J., S.K. Cooke, and H.A. Sampson. 1997. Clinical reactivity to beef in children allergic to cows' milk. J. Allergy Clin. Immunol. 97:293-300.

Wu, H-F., R.L. Lundblad, and F.C. Church. 1995. Neutralization of heparin activity by neutrophil lactoferrin. Blood 85:421-428.

Ye, X.Y., T. Nishimura, and S. Yoshida. 1997. Characterization of the protein and glycan moieties in different forms of bovine lactoferrin. Biosci. Biotechnol. Biochem. 61:782-786

Expert Panel Consensus Statement

In conclusion, the undersigned group of scientific experts, qualified by scientific training and experience to evaluate the safety of food and food additives, agree that bovine milk-derived lactoferrin, used as a component (at not more than two percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb), is determined to be GRAS without the requirement of labeling of food products produced from carcasses so treated. The two key components of the assessment as to why labeling should not be required for the GRAS status of bovine milk-derived lactoferrin applied to beef carcasses are: 1) a determination that exogenous lactoferrin exposure from typical beef consumption (resulting from its application to beef carcasses) is in the range of existing background exposures due to endogenous lactoferrin levels found naturally in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i.e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use).

Steve Taylor, Ph.D.
Professor and Head
Department of Food Science and Technology
University of Nebraska-Lincoln
Institute of Agriculture and Natural Resources
143 H.C. Filley Hall, East Campus
Lincoln, NE 68583-0919
Phone: 402-472-5302
Sltaylor@unlnotes.unl.edu

Signature: _____

Date: _____

Expert Panel Consensus Statement

In conclusion, the undersigned group of scientific experts, qualified by scientific training and experience to evaluate the safety of food and food additives, agree that bovine milk-derived lactoferrin, used as a component (at not more than two percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb), is determined to be GRAS without the requirement of labeling of food products produced from carcasses so treated. The two key components of the assessment as to why labeling should not be required for the GRAS status of bovine milk-derived lactoferrin applied to beef carcasses are: 1) a determination that exogenous lactoferrin exposure from typical beef consumption (resulting from its application to beef carcasses) is in the range of existing background exposures due to endogenous lactoferrin levels found naturally in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i.e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use).

Steve Taylor, Ph.D.
Professor and Head
Department of Food Science and Technology
University of Nebraska-Lincoln
Institute of Agriculture and Natural Resources
143 H.C. Filley Hall, East Campus
Lincoln, NE 68583-0919
Phone: 402-472-5302
Sltaylor@unlnotes.unl.edu

Signature: _____

Date: May 16, 2003

Expert Panel Consensus Statement

In conclusion, the undersigned group of scientific experts, qualified by scientific training and experience to evaluate the safety of food and food additives, agree that bovine milk-derived lactoferrin, used as a component (at not more than two percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb), is determined to be GRAS without the requirement of labeling of food products produced from carcasses so treated. The two key components of the assessment as to why labeling should not be required for the GRAS status of bovine milk-derived lactoferrin applied to beef carcasses are: 1) a determination that exogenous lactoferrin exposure from typical beef consumption (resulting from its application to beef carcasses) is in the range of existing background exposures due to endogenous lactoferrin levels found naturally in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i.e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use).

Lanny J. Rosenwasser, M.D.
National Jewish Medical Research Center
Head of Allergy
1400 Jackson Street
Denver, CO 80206
Phone: 303-398-1656
RosenwasserL@njc.org

Signature: _____

Date: 5/13/03

Expert Panel Consensus Statement

In conclusion, the undersigned group of scientific experts, qualified by scientific training and experience to evaluate the safety of food and food additives, agree that bovine milk-derived lactoferrin, used as a component (at not more than two percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb), is determined to be GRAS without the requirement of labeling of food products produced from carcasses so treated. The two key components of the assessment as to why labeling should not be required for the GRAS status of bovine milk-derived lactoferrin applied to beef carcasses are: 1) a determination that exogenous lactoferrin exposure from typical beef consumption (resulting from its application to beef carcasses) is in the range of existing background exposures due to endogenous lactoferrin levels found naturally in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i.e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use).

Bo Lönnerdal, Ph.D.
Professor of Nutrition and Internal Medicine
University of California
Department of Nutrition
One Shields Avenue
Davis, CA 95616
Phone: 530-752-8347
Blonnerdal@ucdavis.edu

Signature: _____

Date: May 12, 2003

Expert Panel Consensus Statement

In conclusion, the undersigned group of scientific experts, qualified by scientific training and experience to evaluate the safety of food and food additives, agree that bovine milk-derived lactoferrin, used as a component (at not more than two percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb), is determined to be GRAS without the requirement of labeling of food products produced from carcasses so treated. The two key components of the assessment as to why labeling should not be required for the GRAS status of bovine milk-derived lactoferrin applied to beef carcasses are: 1) a determination that exogenous lactoferrin exposure from typical beef consumption (resulting from its application to beef carcasses) is in the range of existing background exposures due to endogenous lactoferrin levels found naturally in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i.e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use).

Jeremy Brock, Ph.D.
Honorary Senior Research Fellow
University of Glasgow
Department of Immunology
Western Infirmary
Dumbarton Road
Glasgow, UK G11 6NT
Phone: +44-1412-112251
j.h.brock@clinmed.gla.ac.uk

Signature: _____

Date: 14/5/03

Expert Panel Consensus Statement

In conclusion, the undersigned group of scientific experts, qualified by scientific training and experience to evaluate the safety of food and food additives, agree that bovine milk-derived lactoferrin, used as a component (at not more than two percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb), is determined to be GRAS without the requirement of labeling of food products produced from carcasses so treated. The two key components of the assessment as to why labeling should not be required for the GRAS status of bovine milk-derived lactoferrin applied to beef carcasses are: 1) a determination that exogenous lactoferrin exposure from typical beef consumption (resulting from its application to beef carcasses) is in the range of existing background exposures due to endogenous lactoferrin levels found naturally in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i.e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use).

Scott H. Sicherer, MD
Assistant Professor of Pediatrics
Department of Pediatrics
Mount Sinai School of Medicine
Jaffe Food Allergy Institute
Mount Sinai School of Medicine, Box 1198
One Gustave L. Levy Place
New York, NY 10029
Phone: 212-241-5548
scott.sicherer@msm.edu

Signature: _____

Date: 5-15-03

Expert Panel Consensus Statement

In conclusion, the undersigned group of scientific experts, qualified by scientific training and experience to evaluate the safety of food and food additives, agree that bovine milk-derived lactoferrin, used as a component (at not more than two percent by weight) of an antimicrobial spray electrostatically applied to raw beef carcasses (at a concentration of lactoferrin of 2% or less in the formulation and not to exceed 0.20 ml of formulation per kg beef, followed by lactic acid rinsing) to yield a final residual amount of bovine milk-derived lactoferrin of less than 800 ng per g of beef (800 ppb), is determined to be GRAS without the requirement of labeling of food products produced from carcasses so treated. The two key components of the assessment as to why labeling should not be required for the GRAS status of bovine milk-derived lactoferrin applied to beef carcasses are: 1) a determination that exogenous lactoferrin exposure from typical beef consumption (resulting from its application to beef carcasses) is in the range of existing background exposures due to endogenous lactoferrin levels found naturally in beef, and 2) a determination that this potentially small incremental increase in lactoferrin exposure is safe (i.e., there is no reasonable expectation that bovine milk-derived lactoferrin will become an allergen under the conditions of its intended use).

Hugh A. Sampson, MD
Kurt Hirschhorn Professor of Pediatrics
Director, Jaffe Food Allergy Institute
Division Chief, Pediatric Allergy and Immunology
Director, General Clinical Research Unit
Mount Sinai School of Medicine
One Gustave L. Levy Place
New York, NY 10029
Phone: 212-241-5548
Hugh.sampson@msm.edu

Signature: _____

Date: 5/19/03

H:\24107278 ALF Ventures USDA\Expert Panel Consensus Statement.doc

G

APPENDIX I

Three Studies Conducted by National Beef to Measure Endogenous Levels of Lactoferrin in Beef

N-terminus
RESEARCH LABORATORY

Determination of Endogenous Levels of Lactoferrin in Beef

Project: **NT(02)-38A**
Scientist in charge: **Joe Tulpinski**
Date Completed: **9/5/02**

PROTOCOL

LF detection by an ELISA method:

ELISA is a sensitive method for the detection of lactoferrin in biological systems (Adonogianaki, et al., 1996; Hetherington et al., 1983). An ELISA assay kit for the detection of bovine lactoferrin (LF) was purchased from the Bethyl Laboratories (Montgomery, TX) and performed according to the manufacturers instructions, briefly as follows:

- A 0.1 ml volume of capture antibody i.e. goat anti-bovine LF-IgG diluted in 0.05 M carbonate-bicarbonate buffer, pH 8.0, was added to coat each well of the 96-wells of a microtiter plate (Nunc MaxiSorp C bottom module). An overnight incubation was allowed to attain maximum binding of the capture antibody to the polystyrene wells.
- A 0.1 ml volume of dilutions of bovine LF standards or test samples (with unknown LF levels) were prepared in TBS-tween (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) containing 1% bovine serum albumin, was added to assigned wells. Plates were incubated at 37°C for 2-h to allow maximum antigen-antibody interaction.
- Wells were washed thoroughly for four times with TBS-tween.
- A 0.1 ml volume of an enzyme-linked antibody i.e. goat anti-bovine LF-IgG conjugated to horseradish peroxidase (HRP) prepared in TBS-tween was added to the wells and the reaction was allowed for 2-h at 37°C.
- The washing step was repeated as above.
- Finally, a 0.1 ml volume of TMB (3,3',5,5' tetramethyl benzidine) enzyme substrate solution (Bethyl Laboratories, Montgomery, TX) for HRP was prepared in added to the wells. The enzymatic reaction was terminated within 5 to 15 min (based on rapidity of color change) with 15 µl of 2N sulfuric acid.
- The color reaction was measured as absorbance at 450 nm wavelength using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).
- A standard curve for bovine LF was plotted using a commercial preparation of LF protein (Lot. 10100395) from the DMV International, NY.
- Two-fold serial dilutions of LF ranging from 500 ng/ml to 7.8 ng/ml are prepared in TBS-tween buffer containing 1% bovine serum albumin and the ELISA assay was performed as described above.
- A standard curve was plotted using absorbance values versus dilutions of LF protein standard. An exponential plot of the standard curve yielded a R-squared value: 0.98 (See attached chart).
- For determination of LF levels in test samples, the color intensity of test samples from ELISA were correlated against the standard curve.

Test Samples:

Beef Round- Bottom Round Steak (sample size; n=4) was used to determine the endogenous levels of LF. Beef was grounded, weighed out and placed in a stomacher bag containing TBS (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). Meat was pummeled on high for 120 seconds. The fluid obtained after stomaching was collected and used for LF analysis.

RESULTS

Endogenous Levels of Lactoferrin:

Sample #	Levels of LF (ng/g)
1	218.87
2	196.80
3	173.99
4	219.23

Average amount of lactoferrin in beef as determined by ELISA method:

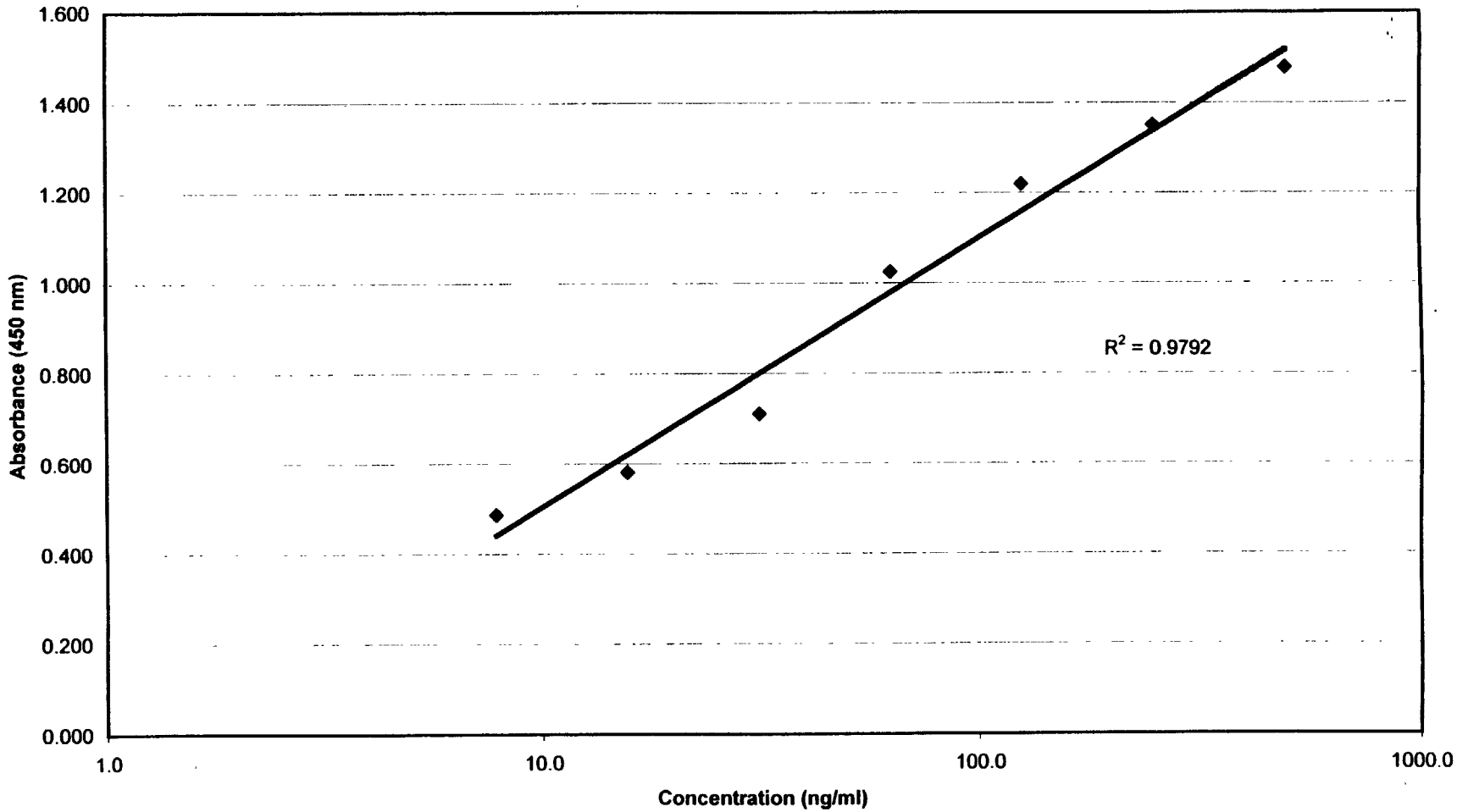
Average amount of LF (ng/g)
202.22 ± 21.55

References:

1. Adonogianaki, E., J. Mooney and D. F. Kinane (1996). "Detection of stable and active periodontitis sites by clinical assessment and gingival crevicular acute-phase protein levels." J Periodontal Res 31(2): 135-43.
2. Hetherington, S. V., J. K. Spitznagel and P. G. Quie (1983). "An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin." J Immunol Methods 65(1-2): 183-90.

M-terminus
RESEARCH LABORATORY

Bovine LF Standard Curve



N-terminus
RESEARCH LABORATORY

Determination of Endogenous Levels of Lactoferrin in Beef

Project: **NT(02)-38B**
Scientist in charge: **Joe Tulpinski**
Date Completed: **9/6/02**

PROTOCOL

LF detection by an ELISA method:

ELISA is a sensitive method for the detection of lactoferrin in biological systems (Adonogianaki, et al., 1996; Hetherington et al., 1983). An ELISA assay kit for the detection of bovine lactoferrin (LF) was purchased from the Bethyl Laboratories (Montgomery, TX) and performed according to the manufacturers instructions, briefly as follows:

- A 0.1 ml volume of capture antibody i.e. goat anti-bovine LF-IgG diluted in 0.05 M carbonate-bicarbonate buffer, pH 8.0, was added to coat each well of the 96-wells of a microtiter plate (Nunc MaxiSorp C bottom module). An overnight incubation was allowed to attain maximum binding of the capture antibody to the polystyrene wells.
- A 0.1 ml volume of dilutions of bovine LF standards or test samples (with unknown LF levels) were prepared in TBS-tween (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) containing 1% bovine serum albumin, was added to assigned wells. Plates were incubated at 37°C for 2-h to allow maximum antigen-antibody interaction.
- Wells were washed thoroughly for four times with TBS-tween.
- A 0.1 ml volume of an enzyme-linked antibody i.e. goat anti-bovine LF-IgG conjugated to horseradish peroxidase (HRP) prepared in TBS-tween was added to the wells and the reaction was allowed for 2-h at 37°C.
- The washing step was repeated as above.
- Finally, a 0.1 ml volume of TMB (3,3',5,5' tetramethyl benzidine) enzyme substrate solution (Bethyl Laboratories, Montgomery, TX) for HRP was prepared in added to the wells. The enzymatic reaction was terminated within 5 to 15 min (based on rapidity of color change) with 15 µl of 2N sulfuric acid.
- The color reaction was measured as absorbance at 450 nm wavelength using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).
- A standard curve for bovine LF was plotted using a commercial preparation of LF protein (Lot. 10100395) from the DMV International, NY.
- Two-fold serial dilutions of LF ranging from 500 ng/ml to 7.8 ng/ml are prepared in TBS-tween buffer containing 1% bovine serum albumin and the ELISA assay was performed as described above.
- A standard curve was plotted using absorbance values versus dilutions of LF protein standard. An exponential plot of the standard curve yielded a R-squared value: 0.97 (See attached chart).
- For determination of LF levels in test samples, the color intensity of test samples from ELISA were correlated against the standard curve.

Test Samples:

Beef Round- Bottom Round Steak (sample size; n=4) was used to determine the endogenous levels of LF. Beef was grounded, weighed out and placed in a stomacher bag containing TBS (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). Meat was pummeled on high for 120 seconds. The fluid obtained after stomaching was collected and used for LF analysis.

RESULTS

Endogenous Levels of Lactoferrin:

Sample #	Levels of LF (ng/g)
1	168.23
2	198.52
3	192.36
4	178.52

Average amount of lactoferrin in beef as determined by ELISA method:

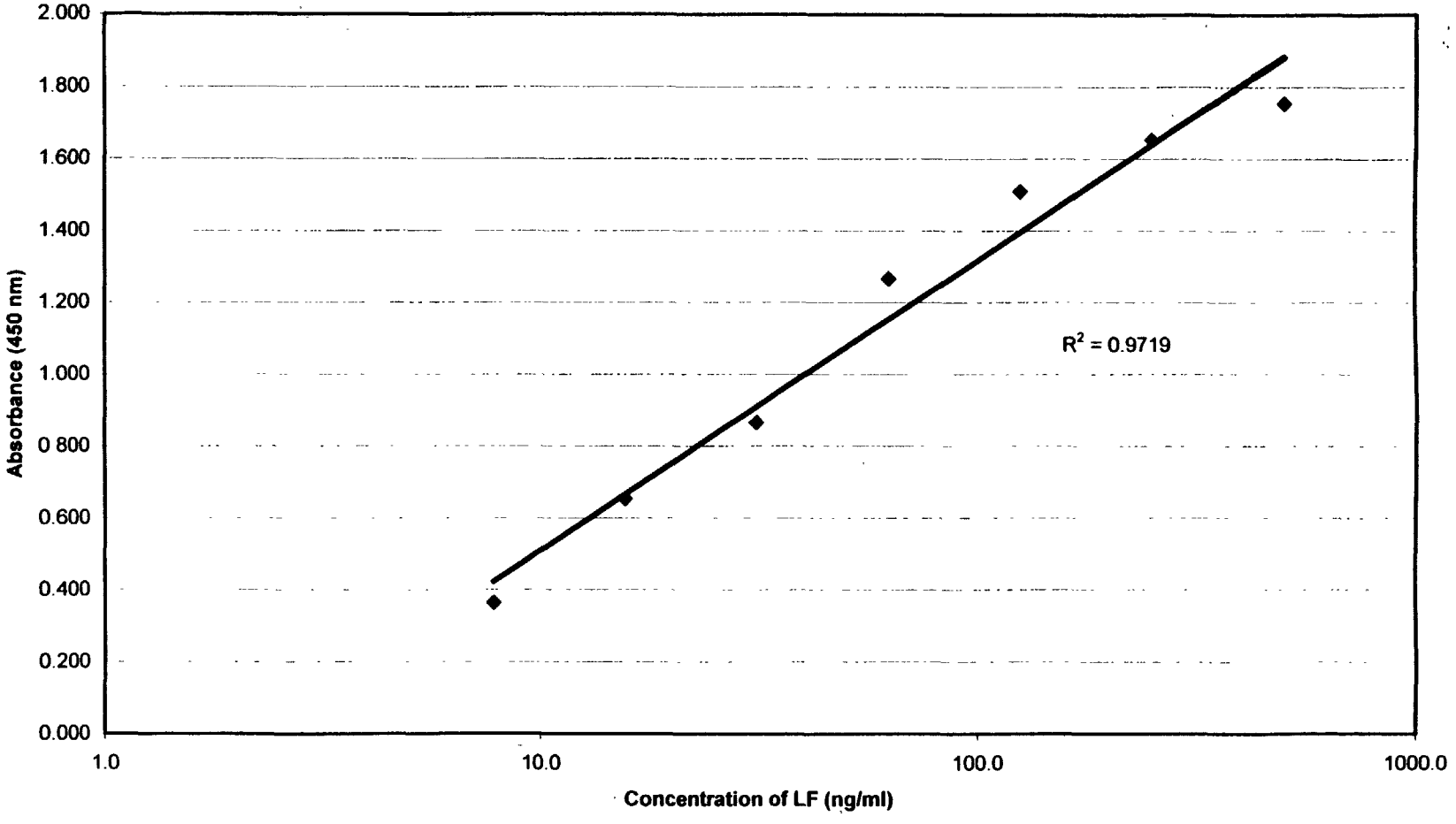
Average amount of LF (ng/g)
184.41 ± 13.65

References:

1. Adonogianaki, E., J. Mooney and D. F. Kinane (1996). "Detection of stable and active periodontitis sites by clinical assessment and gingival crevicular acute-phase protein levels." J Periodontal Res 31(2): 135-43.
2. Hetherington, S. V., J. K. Spitznagel and P. G. Quie (1983). "An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin." J Immunol Methods 65(1-2): 183-90.

N-terminus
RESEARCH LABORATORY

Bovine LF Standard Curve



000403

N-terminus
RESEARCH LABORATORY

Determination of Endogenous Levels of Lactoferrin in Beef

Project: **NT(02)-38C**
Scientist in charge: **Joe Tulpinski**
Date Completed: **9/11/02**

PROTOCOL

LF detection by an ELISA method:

ELISA is a sensitive method for the detection of lactoferrin in biological systems (Adonogianaki, et al., 1996; Hetherington et al., 1983). An ELISA assay kit for the detection of bovine lactoferrin (LF) was purchased from the Bethyl Laboratories (Montgomery, TX) and performed according to the manufacturers instructions, briefly as follows:

- A 0.1 ml volume of capture antibody i.e. goat anti-bovine LF-IgG diluted in 0.05 M carbonate-bicarbonate buffer, pH 8.0, was added to coat each well of the 96-wells of a microtiter plate (Nunc MaxiSorp C bottom module). An overnight incubation was allowed to attain maximum binding of the capture antibody to the polystyrene wells.
- A 0.1 ml volume of dilutions of bovine LF standards or test samples (with unknown LF levels) were prepared in TBS-tween (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) containing 1% bovine serum albumin, was added to assigned wells. Plates were incubated at 37°C for 2-h to allow maximum antigen-antibody interaction.
- Wells were washed thoroughly for four times with TBS-tween.
- A 0.1 ml volume of an enzyme-linked antibody i.e. goat anti-bovine LF-IgG conjugated to horseradish peroxidase (HRP) prepared in TBS-tween was added to the wells and the reaction was allowed for 2-h at 37°C.
- The washing step was repeated as above.
- Finally, a 0.1 ml volume of TMB (3,3',5,5' tetramethyl benzidine) enzyme substrate solution (Bethyl Laboratories, Montgomery, TX) for HRP was prepared in added to the wells. The enzymatic reaction was terminated within 5 to 15 min (based on rapidity of color change) with 15 µl of 2N sulfuric acid.
- The color reaction was measured as absorbance at 450 nm wavelength using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).
- A standard curve for bovine LF was plotted using a commercial preparation of LF protein (Lot. 10100395) from the DMV International, NY.
- Two-fold serial dilutions of LF ranging from 500 ng/ml to 7.8 ng/ml are prepared in TBS-tween buffer containing 1% bovine serum albumin and the ELISA assay was performed as described above.
- A standard curve was plotted using absorbance values versus dilutions of LF protein standard. An exponential plot of the standard curve yielded a R-squared value: 0.99 (See attached chart).
- For determination of LF levels in test samples, the color intensity of test samples from ELISA were correlated against the standard curve.

Test Samples:

Beef Round- Bottom Round Steak (sample size; n=4) was used to determine the endogenous levels of LF. Beef was grounded, weighed out and placed in a stomacher bag containing TBS (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). Meat was pummeled on high for 120 seconds. The fluid obtained after stomaching was collected and used for LF analysis.

RESULTS

Endogenous Levels of Lactoferrin:

Sample #	Levels of LF (ng/g)
1	178.64
2	195.23
3	205.84
4	186.54

Average amount of lactoferrin in beef as determined by ELISA method:

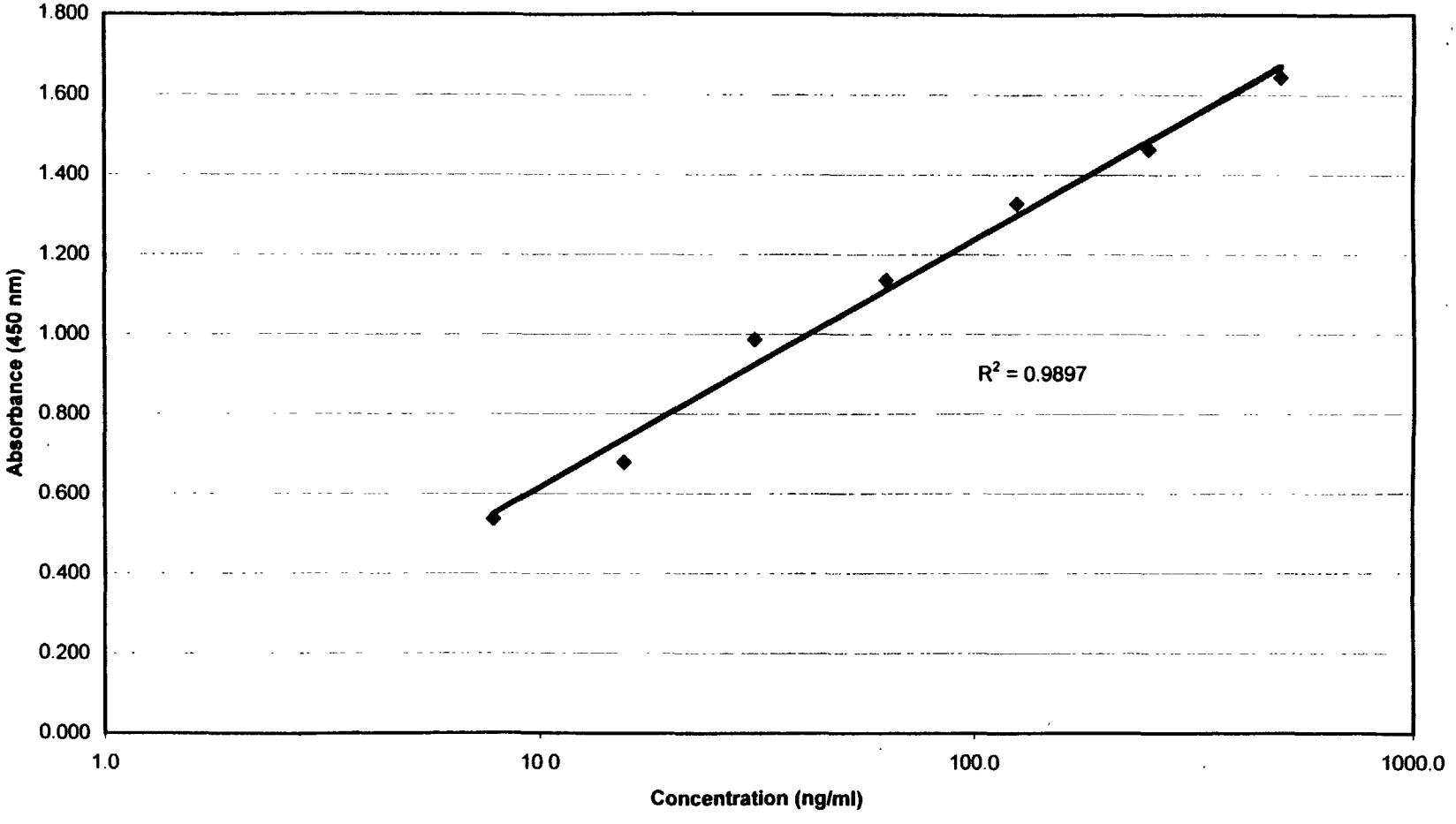
Average amount of LF (ng/g)
191.56 ± 11.68

References:

1. Adonogianaki, E., J. Mooney and D. F. Kinane (1996). "Detection of stable and active periodontitis sites by clinical assessment and gingival crevicular acute-phase protein levels." J Periodontal Res 31(2): 135-43.
2. Hetherington, S. V., J. K. Spitznagel and P. G. Quie (1983). "An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin." J Immunol Methods 65(1-2): 183-90.

N-terminus
RESEARCH LABORATORY

Bovine LF Standard Curve



APPENDIX II

Four Beef Carcass Washing Studies Conducted by National Beef

Measurement of exogenous lactoferrin levels on beef surface

Project: **NT(02)-39A**
Scientist in charge: **Joe Tulpinski**
Date Completed: **9/5/02**

PROTOCOL

The surface levels of lactoferrin was measured by an ELISA method when applied exogenously by an electrostatic spray system and washed through a meat processing simulation system according to the following protocol.

Carcass Wash using the Meat Processing Simulation (MPS) system:

MPS system. The equipment consists of a spray-, process- and drain-unit. The spray-unit has six fluid tanks of 7.6-liter capacity, connected to six separate magnetic drive (seal-less) fan cooled sanitary/chemical solution pumps (1/25 HP, 10.6 L/min, from March Manufacturing Inc., Glenview, IL). One of the fluid tanks has a built-in heating element to deliver hot water, maximum to 190°F.

The process-unit is a cabinet (72" L x 16" W x 12" H) with a safety view glass ceiling. It has a loading chamber, five pause chambers and six spray chambers (8"L x 8"W x 12"H). A drive chain passes through the center of all the twelve chambers. The chain consists of a loading trolley with a removable stainless steel cassette and spaced with six timing tabs. Inside all the spray chambers, two adjustable spray nozzles (140° angle, 7.2 L/min) were located on both sides of the drive chain to deliver a circular stream of liquid towards the middle center of the loading trolley.

The drain-unit is a secured compartment located under the process-Unit. All the six spray chambers are individually hosed to collect the wash-effluent into 500-ml borosilicate bottles. The rest of the chambers are jointly hosed to drain into an effluent basin (72" L x 16" W x 3"H).

A programmable logic controller (PLC from Allen-Bradley, Milwaukee, WI) encased in a stainless-steel box, digitally controls the MPS system. The PLC is interfaced via a message view terminal. The operator can program the spray/pause processing time, and run-cycle (test or sanitary flush), as required. The MPS system washes the meat according to specific washing steps. The following simulated steps are comparable to that of real-time sanitizing assembly of a meat processing plant:

Step	Function	Time	Spray volume	Comments
	Sample Loading			Meat (12x12 cm) loaded
1	Cold water spray	10-sec	125-mL	Pre-evicery wash step
	Process pause	10-sec		
2	Lactic acid spray	10-sec	125-mL	Pre-evicery 2% acid rinse
	Process pause	10-sec		
3	Hot water spray	20-sec	250-mL	180°F wash (DI water)
	Process pause	10-sec		
4	Cold water spray	10-sec	125-mL	Carcass rinse
5	Lactoferrin Spray	2-10 sec	2 – 10 mL	Application by ESS
6	Lactic acid spray	10-sec	125-mL	Final 2% acid rinse

Lactoferrin application by Electro-static Spray (ESS) system:

The ESS system is a pressure and charge driven instrumentation for application of lactoferrin onto a meat surface. The electrical current for the applications of the LF ranges between 5 μdcA and 15 μdcA. Pressure on the ESS from an electrical air compressor is approximately 50 psi. The ESS was setup to deliver LF at an application rate of 1 mL/sec. This application prototype was manufactured by the National Beef Packing Company (Dodge City, KS). ESS is operated as follows:

- Meat sample after passing through ***Chamber-8 of the MPS system*** described above is unhooked together with the sample loading cassette and place inside the positioning hooks of the ESS system.
- Air compressor pressure is set to 120 psi and the tank pressure is adjusted to 100 psi.
- Air hose is attached to the air compressor and to the electrostatic spray box. Air pressure is set on the electrostatic spray control box regulator to 45-55 psi.
- LF is siphoned into the syringe which is attached to the ESS system for delivery.
- Time of LF application is adjusted for each trial segment i.e. 2-, 4-, 6-, 8-, and 10-sec.
- LF is applied on meat surface with the above settings. Fluid level and consistency of liquid sprayed from ESS is monitored during the application process.
- Meat samples were either directly measured for the LF deposition or further processed subjecting to an additional acid wash (Step-6) in the MPS system to measure residual (post-wash) LF. An ELISA method, described below is used for the detection of LF on meat surface.

LF detection by an ELISA method:

ELISA is a sensitive method for the detection of lactoferrin in biological systems (Adonogianaki, et al., 1996; Hetherington et al., 1983). An ELISA assay kit for the detection of bovine lactoferrin (LF) was purchased from the Bethyl Laboratories (Montgomery, TX) and performed according to the manufacturers instructions, briefly as follows:

- A 0.1 ml volume of capture antibody i.e. goat anti-bovine LF-IgG diluted in 0.05 M carbonate-bicarbonate buffer, pH 8.0, was added to coat each well of the 96-wells of a microtiter plate (Nunc MaxiSorp C bottom module). An overnight incubation was allowed to attain maximum binding of the capture antibody to the polystyrene wells.
- A 0.1 ml volume of dilutions of bovine LF standards or test samples (with unknown LF levels) were prepared in TBS-tween (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) containing 1% bovine serum albumin, was added to assigned wells. Plates were incubated at 37°C for 2-h to allow maximum antigen-antibody interaction.
- Wells were washed thoroughly for four times with TBS-tween.
- A 0.1 ml volume of an enzyme-linked antibody i.e. goat anti-bovine LF-IgG conjugated to horseradish peroxidase (HRP) prepared in TBS-tween was added to the wells and the reaction was allowed for 2-h at 37°C.
- The washing step was repeated as above.
- Finally, a 0.1 ml volume of TMB (3,3',5,5' tetramethyl benzidine) enzyme substrate solution (Bethyl Laboratories, Montgomery, TX) for HRP was prepared in added to the wells. The enzymatic reaction was terminated within 5 to 15 min (based on rapidity of color change) with 15 μl of 2N sulfuric acid.

- The color reaction was measured as absorbance at 450 nm wavelength using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).
- A standard curve for bovine LF was plotted using a commercial preparation of LF protein (Lot. 10100395) from the DMV International, NY.
- Two-fold serial dilutions of LF ranging from 500 ng/ml to 7.8 ng/ml are prepared in TBS-tween buffer containing 1% bovine serum albumin and the ELISA assay was performed as described above.
- A standard curve was plotted using absorbance values versus dilutions of LF protein standard. An exponential plot of the standard curve yielded a R-squared value: 0.98 (See attached chart).
- For determination of LF levels in test samples, the color intensity of test samples from ELISA were correlated against the standard curve.

Test Samples:

Beef Round- Bottom Round Steak (n=4 per each time point) was used for exogenous application of LF on meat surface. Beef samples after LF application by ESS system either directly measured for LF or subjected to a further processing and the residual (post-wash after acid rinse) was measured by an ELISA. Briefly, A 50-cc sterile conical centrifuge tube (Corning) containing 5 ml of TSB was pressed against the surface of the meat and agitated for 10-sec by hand. Each tube covered an area of approximately 4.9 cm^2. This wash liquid was tested for LF levels.

Exogenous lactoferrrin levels on beef after application by ESS system

Time of Spray (sec)	Exogenous LF (ng/ cm^2)
2	1562 ± 43
4	1233 ± 51
6	1310 ± 47
8	2784 ± 132
10	2827 ± 62

Residual (post-wash) levels of exogenous lactoferrin after carcass wash

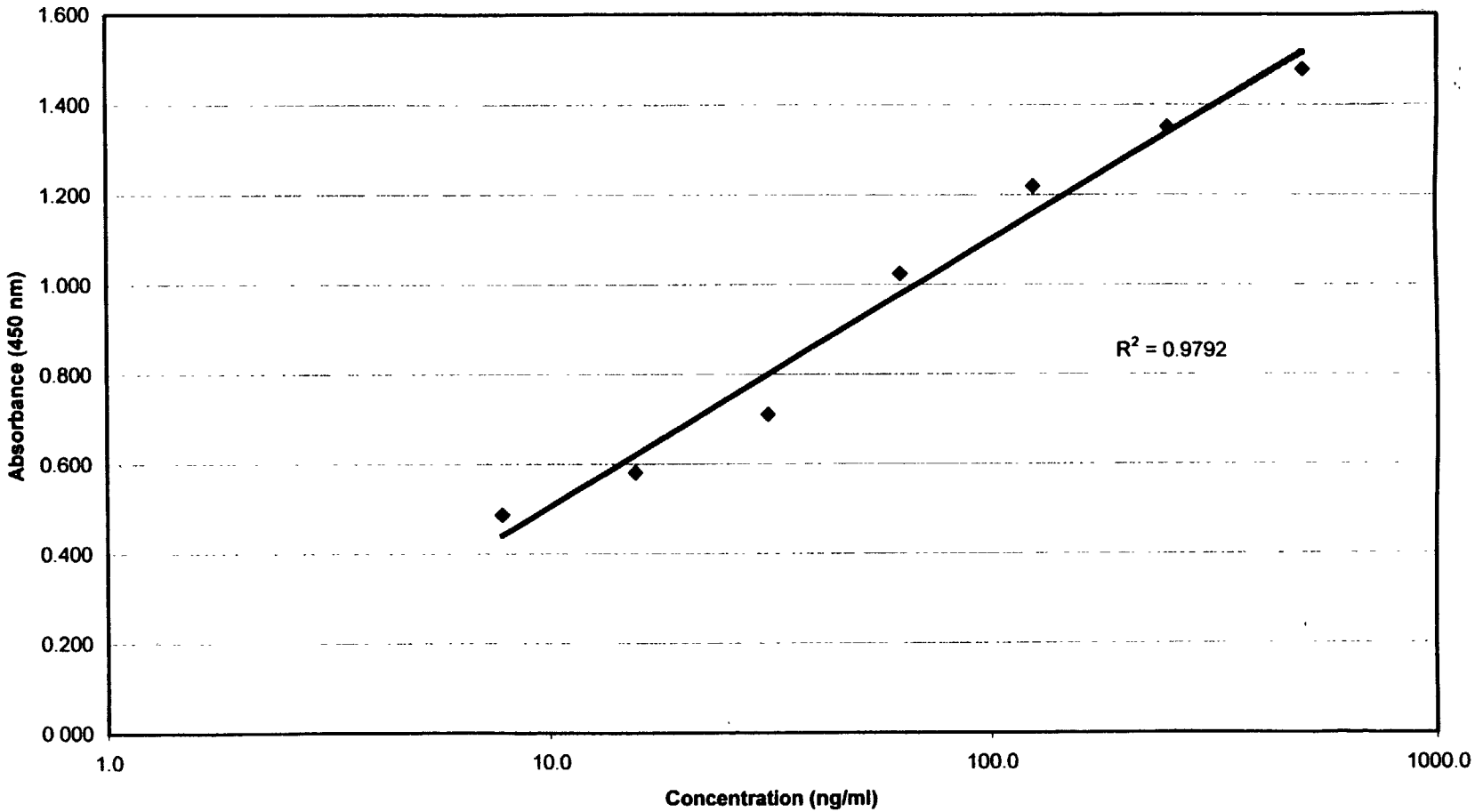
Time of Spray (sec)	Levels of LF detected (ng/ cm^2)	% LF washed-off the meat
2	145 ± 12	91
4	157 ± 11	87
6	225 ± 20	83
8	663 ± 44	76
10	926 ± 75	67

References:

1. Adonogianaki, E., J. Mooney and D. F. Kinane (1996). "Detection of stable and active periodontitis sites by clinical assessment and gingival crevicular acute-phase protein levels." J Periodontal Res 31(2): 135-43.
2. Hetherington, S. V., J. K. Spitznagel and P. G. Quie (1983). "An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin." J Immunol Methods 65(1-2): 183-90.

N-terminus
RESEARCH LABORATORY

ELISA LF Standard Curve



000111

Measurement of exogenous lactoferrin levels on beef surface

Project: **NT(02)-39B**
Scientist in charge: **Joe Tulpinski**
Date Completed: **9/6/02**

PROTOCOL

The surface levels of lactoferrin was measured by an ELISA method when applied exogenously by an electrostatic spray system and washed through a meat processing simulation system according to the following protocol.

Carcass Wash using the Meat Processing Simulation (MPS) system:

MPS system. The equipment consists of a spray-, process- and drain-unit. The spray-unit has six fluid tanks of 7.6-liter capacity, connected to six separate magnetic drive (seal-less) fan cooled sanitary/chemical solution pumps (1/25 HP, 10.6 L/min, from March Manufacturing Inc., Glenview, IL). One of the fluid tanks has a built-in heating element to deliver hot water, maximum to 190°F.

The process-unit is a cabinet (72" L x 16" W x 12" H) with a safety view glass ceiling. It has a loading chamber, five pause chambers and six spray chambers (8"L x 8"W x 12"H). A drive chain passes through the center of all the twelve chambers. The chain consists of a loading trolley with a removable stainless steel cassette and spaced with six timing tabs. Inside all the spray chambers, two adjustable spray nozzles (140° angle, 7.2 L/min) were located on both sides of the drive chain to deliver a circular stream of liquid towards the middle center of the loading trolley.

The drain-unit is a secured compartment located under the process-Unit. All the six spray chambers are individually hosed to collect the wash-effluent into 500-ml borosilicate bottles. The rest of the chambers are jointly hosed to drain into an effluent basin (72" L x 16" W x 3"H).

A programmable logic controller (PLC from Allen-Bradley, Milwaukee, WI) encased in a stainless-steel box, digitally controls the MPS system. The PLC is interfaced via a message view terminal. The operator can program the spray/pause processing time, and run-cycle (test or sanitary flush), as required. The MPS system washes the meat according to specific washing steps. The following simulated steps are comparable to that of real-time sanitizing assembly of a meat processing plant:

Step	Function	Time	Spray volume	Comments
	Sample Loading			Meat (12x12 cm) loaded
1	Cold water spray	10-sec	125-mL	Pre-evicery wash step
	Process pause	10-sec		
2	Lactic acid spray	10-sec	125-mL	Pre-evicery 2% acid rinse
	Process pause	10-sec		
3	Hot water spray	20-sec	250-mL	180°F wash (DI water)
	Process pause	10-sec		
4	Cold water spray	10-sec	125-mL	Carcass rinse
5	Lactoferrin Spray	2-10 sec	2 – 10 mL	Application by ESS
6	Lactic acid spray	10-sec	125-mL	Final 2% acid rinse

Lactoferrin application by Electro-static Spray (ESS) system:

The ESS system is a pressure and charge driven instrumentation for application of lactoferrin onto a meat surface. The electrical current for the applications of the LF ranges between 5 μdcA and 15 μdcA. Pressure on the ESS from an electrical air compressor is approximately 50 psi. The ESS was setup to deliver LF at an application rate of 1 mL/sec. This application prototype was manufactured by the National Beef Packing Company (Dodge City, KS). ESS is operated as follows:

- Meat sample after passing through ***Chamber-8 of the MPS system*** described above is unhooked together with the sample loading cassette and place inside the positioning hooks of the ESS system.
- Air compressor pressure is set to 120 psi and the tank pressure is adjusted to 100 psi.
- Air hose is attached to the air compressor and to the electrostatic spray box. Air pressure is set on the electrostatic spray control box regulator to 45-55 psi.
- LF is siphoned into the syringe which is attached to the ESS system for delivery.
- Time of LF application is adjusted for each trial segment i.e. 2-, 4-, 6-, 8-, and 10-sec.
- LF is applied on meat surface with the above settings. Fluid level and consistency of liquid sprayed from ESS is monitored during the application process.
- Meat samples were either directly measured for the LF deposition or further processed subjecting to an additional acid wash (Chamber-9) in the MPS system to measure residual (post-wash) LF. An ELISA method, described below is used for the detection of LF on meat surface.

LF detection by an ELISA method:

ELISA is a sensitive method for the detection of lactoferrin in biological systems (Adonogianaki, et al., 1996; Hetherington et al., 1983). An ELISA assay kit for the detection of bovine lactoferrin (LF) was purchased from the Bethyl Laboratories (Montgomery, TX) and performed according to the manufacturers instructions, briefly as follows:

- A 0.1 ml volume of capture antibody i.e. goat anti-bovine LF-IgG diluted in 0.05 M carbonate-bicarbonate buffer, pH 8.0, was added to coat each well of the 96-wells of a microtiter plate (Nunc MaxiSorp C bottom module). An overnight incubation was allowed to attain maximum binding of the capture antibody to the polystyrene wells.
- A 0.1 ml volume of dilutions of bovine LF standards or test samples (with unknown LF levels) were prepared in TBS-tween (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) containing 1% bovine serum albumin, was added to assigned wells. Plates were incubated at 37°C for 2-h to allow maximum antigen-antibody interaction.
- Wells were washed thoroughly for four times with TBS-tween.
- A 0.1 ml volume of an enzyme-linked antibody i.e. goat anti-bovine LF-IgG conjugated to horseradish peroxidase (HRP) prepared in TBS-tween was added to the wells and the reaction was allowed for 2-h at 37°C.
- The washing step was repeated as above.
- Finally, a 0.1 ml volume of TMB (3,3',5,5' tetramethyl benzidine) enzyme substrate solution (Bethyl Laboratories, Montgomery, TX) for HRP was prepared in added to the wells. The enzymatic reaction was terminated within 5 to 15 min (based on rapidity of color change) with 15 μl of 2N sulfuric acid.

- The color reaction was measured as absorbance at 450 nm wavelength using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).
- A standard curve for bovine LF was plotted using a commercial preparation of LF protein (Lot. 10100395) from the DMV International, NY.
- Two-fold serial dilutions of LF ranging from 500 ng/ml to 7.8 ng/ml are prepared in TBS-tween buffer containing 1% bovine serum albumin and the ELISA assay was performed as described above.
- A standard curve was plotted using absorbance values versus dilutions of LF protein standard. An exponential plot of the standard curve yielded a R-squared value: 0.97 (See attached chart).
- For determination of LF levels in test samples, the color intensity of test samples from ELISA were correlated against the standard curve.

Test Samples:

Beef Round- Bottom Round Steak (n=4 per each time point) was used for exogenous application of LF on meat surface. Beef samples after LF application by ESS system either directly measured for LF or subjected to a further processing and the residual (post-wash after acid rinse) was measured by an ELISA. Briefly, A 50-cc sterile conical centrifuge tube (Corning) containing 5 ml of TSB was pressed against the surface of the meat and agitated for 10-sec by hand. Each tube covered an area of approximately 4.9 cm^2. This wash liquid was tested for LF levels.

Exogenous lactoferrrin levels on beef after application by ESS system

Time of Spray (sec)	Exogenous LF (ng/ cm^2)
2	1465 ± 63
4	1564 ± 82
6	1881 ± 102
8	2183 ± 121
10	2659 ± 56

Residual (post-wash) levels of exogenous lactoferrin after carcass wash

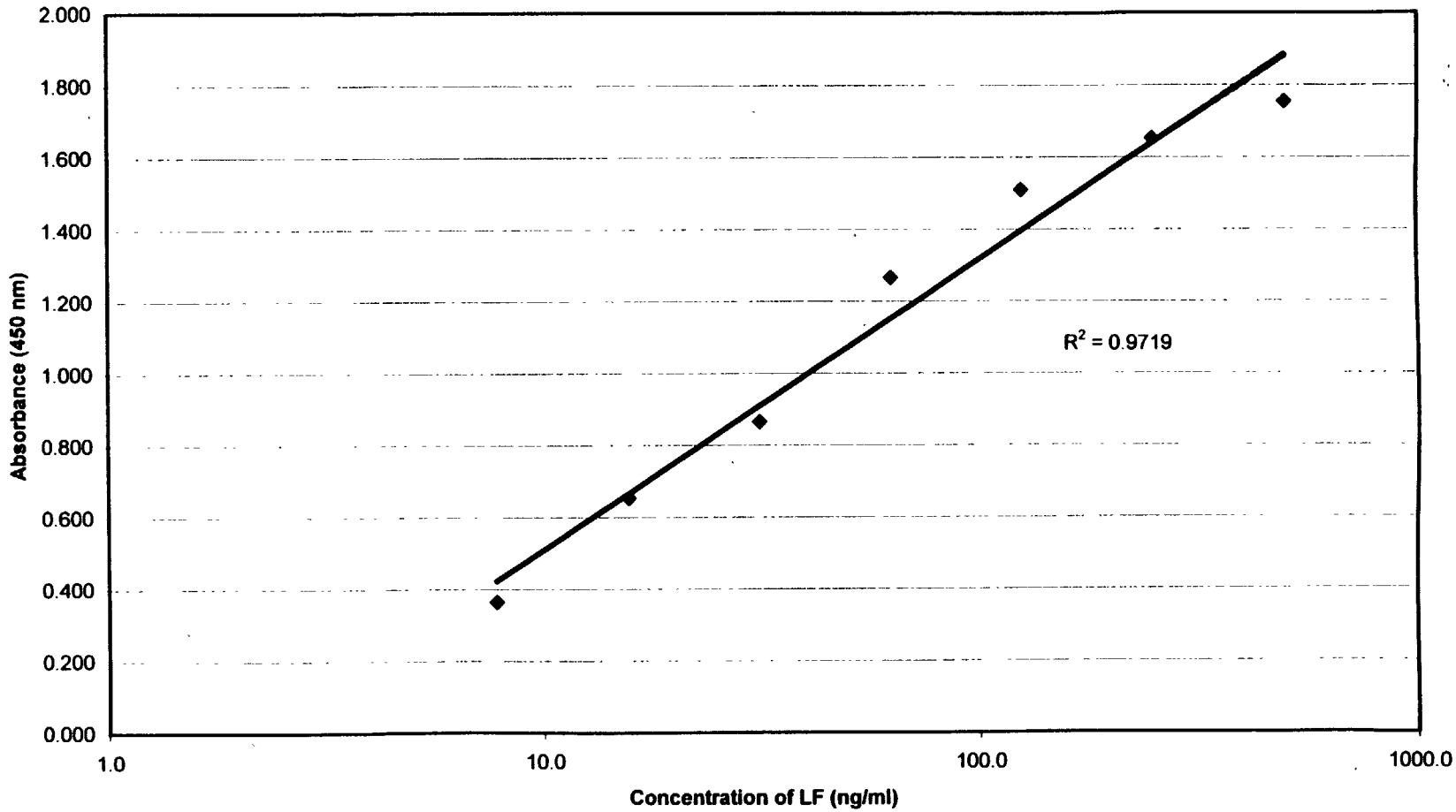
Time of Spray (sec)	Levels of LF detected (ng/ cm^2)	% LF washed-off the meat
2	211 ± 11	86
4	231 ± 15	85
6	338 ± 22	82
8	853 ± 43	61
10	1046 ± 77	61

References:

1. Adonogianaki, E., J. Mooney and D. F. Kinane (1996). "Detection of stable and active periodontitis sites by clinical assessment and gingival crevicular acute-phase protein levels." J Periodontal Res 31(2): 135-43.
2. Hetherington, S. V., J. K. Spitznagel and P. G. Quie (1983). "An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin." J Immunol Methods 65(1-2): 183-90.

M-terminus
RESEARCH LABORATORY

Bovine LF Standard Curve



Measurement of exogenous lactoferrin levels on beef surface

Project: **NT(02)-39C**
Scientist in charge: **Joe Tulpinski**
Date Completed: **9/11/02**

PROTOCOL

The surface levels of lactoferrin was measured by an ELISA method when applied exogenously by an electrostatic spray system and washed through a meat processing simulation system according to the following protocol.

Carcass Wash using the Meat Processing Simulation (MPS) system:

MPS system. The equipment consists of a spray-, process- and drain-unit. The spray-unit has six fluid tanks of 7.6-liter capacity, connected to six separate magnetic drive (seal-less) fan cooled sanitary/chemical solution pumps (1/25 HP, 10.6 L/min, from March Manufacturing Inc., Glenview, IL). One of the fluid tanks has a built-in heating element to deliver hot water, maximum to 190°F.

The process-unit is a cabinet (72" L x 16" W x 12" H) with a safety view glass ceiling. It has a loading chamber, five pause chambers and six spray chambers (8"L x 8"W x 12"H). A drive chain passes through the center of all the twelve chambers. The chain consists of a loading trolley with a removable stainless steel cassette and spaced with six timing tabs. Inside all the spray chambers, two adjustable spray nozzles (140° angle, 7.2 L/min) were located on both sides of the drive chain to deliver a circular stream of liquid towards the middle center of the loading trolley.

The drain-unit is a secured compartment located under the process-Unit. All the six spray chambers are individually hosed to collect the wash-effluent into 500-ml borosilicate bottles. The rest of the chambers are jointly hosed to drain into an effluent basin (72" L x 16" W x 3"H).

A programmable logic controller (PLC from Allen-Bradley, Milwaukee, WI) encased in a stainless-steel box, digitally controls the MPS system. The PLC is interfaced via a message view terminal. The operator can program the spray/pause processing time, and run-cycle (test or sanitary flush), as required. The MPS system washes the meat according to specific washing steps. The following simulated steps are comparable to that of real-time sanitizing assembly of a meat processing plant:

Step	Function	Time	Spray volume	Comments
	Sample Loading			Meat (12x12 cm) loaded
1	Cold water spray	10-sec	125-mL	Pre-evicery wash step
	Process pause	10-sec		
2	Lactic acid spray	10-sec	125-mL	Pre-evicery 2% acid rinse
	Process pause	10-sec		
3	Hot water spray	20-sec	250-mL	180°F wash (DI water)
	Process pause	10-sec		
4	Cold water spray	10-sec	125-mL	Carcass rinse
5	Lactoferrin Spray	2-10 sec	2 – 10 mL	Application by ESS
6	Lactic acid spray	10-sec	125-mL	Final 2% acid rinse

Lactoferrin application by Electro-static Spray (ESS) system:

The ESS system is a pressure and charge driven instrumentation for application of lactoferrin onto a meat surface. The electrical current for the applications of the LF ranges between 5 μdcA and 15 μdcA. Pressure on the ESS from an electrical air compressor is approximately 50 psi. The ESS was setup to deliver LF at an application rate of 1 mL/sec. This application prototype was manufactured by the National Beef Packing Company (Dodge City, KS). ESS is operated as follows:

- Meat sample after passing through ***Chamber-8 of the MPS system*** described above is unhooked together with the sample loading cassette and place inside the positioning hooks of the ESS system.
- Air compressor pressure is set to 120 psi and the tank pressure is adjusted to 100 psi.
- Air hose is attached to the air compressor and to the electrostatic spray box. Air pressure is set on the electrostatic spray control box regulator to 45-55 psi.
- LF is siphoned into the syringe which is attached to the ESS system for delivery.
- Time of LF application is adjusted for each trial segment i.e. 2-, 4-, 6-, 8-, and 10-sec.
- LF is applied on meat surface with the above settings. Fluid level and consistency of liquid sprayed from ESS is monitored during the application process.
- Meat samples were either directly measured for the LF deposition or further processed subjecting to an additional acid wash (Chamber-9) in the MPS system to measure residual (post-wash) LF. An ELISA method, described below is used for the detection of LF on meat surface.

LF detection by an ELISA method:

ELISA is a sensitive method for the detection of lactoferrin in biological systems (Adonogianaki, et al., 1996; Hetherington et al., 1983). An ELISA assay kit for the detection of bovine lactoferrin (LF) was purchased from the Bethyl Laboratories (Montgomery, TX) and performed according to the manufacturers instructions, briefly as follows:

- A 0.1 ml volume of capture antibody i.e. goat anti-bovine LF-IgG diluted in 0.05 M carbonate-bicarbonate buffer, pH 8.0, was added to coat each well of the 96-wells of a microtiter plate (Nunc MaxiSorp C bottom module). An overnight incubation was allowed to attain maximum binding of the capture antibody to the polystyrene wells.
- A 0.1 ml volume of dilutions of bovine LF standards or test samples (with unknown LF levels) were prepared in TBS-tween (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) containing 1% bovine serum albumin, was added to assigned wells. Plates were incubated at 37°C for 2-h to allow maximum antigen-antibody interaction.
- Wells were washed thoroughly for four times with TBS-tween.
- A 0.1 ml volume of an enzyme-linked antibody i.e. goat anti-bovine LF-IgG conjugated to horseradish peroxidase (HRP) prepared in TBS-tween was added to the wells and the reaction was allowed for 2-h at 37°C.
- The washing step was repeated as above.
- Finally, a 0.1 ml volume of TMB (3,3',5,5' tetramethyl benzidine) enzyme substrate solution (Bethyl Laboratories, Montgomery, TX) for HRP was prepared in added to the wells. The enzymatic reaction was terminated within 5 to 15 min (based on rapidity of color change) with 15 μl of 2N sulfuric acid.

- The color reaction was measured as absorbance at 450 nm wavelength using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).
- A standard curve for bovine LF was plotted using a commercial preparation of LF protein (Lot. 10100395) from the DMV International, NY.
- Two-fold serial dilutions of LF ranging from 500 ng/ml to 7.8 ng/ml are prepared in TBS-tween buffer containing 1% bovine serum albumin and the ELISA assay was performed as described above.
- A standard curve was plotted using absorbance values versus dilutions of LF protein standard. An exponential plot of the standard curve yielded a R-squared value: 0.99 (See attached chart).
- For determination of LF levels in test samples, the color intensity of test samples from ELISA were correlated against the standard curve.

Test Samples:

Beef Round- Bottom Round Steak (n=4 per each time point) was used for exogenous application of LF on meat surface. Beef samples after LF application by ESS system either directly measured for LF or subjected to a further processing and the residual (post-wash after acid rinse) was measured by an ELISA. Briefly, A 50-cc sterile conical centrifuge tube (Corning) containing 5 ml of TSB was pressed against the surface of the meat and agitated for 10-sec by hand. Each tube covered an area of approximately 4.9 cm^2. This wash liquid was tested for LF levels.

Exogenous lactoferrrin levels on beef after application by ESS system

Time of Spray (sec)	Exogenous LF (ng/ cm^2)
2	1321 ± 12
4	1453 ± 56
6	1645 ± 79
8	2284 ± 63
10	2946 ± 88

Residual (post-wash) levels of exogenous lactoferrin after carcass wash

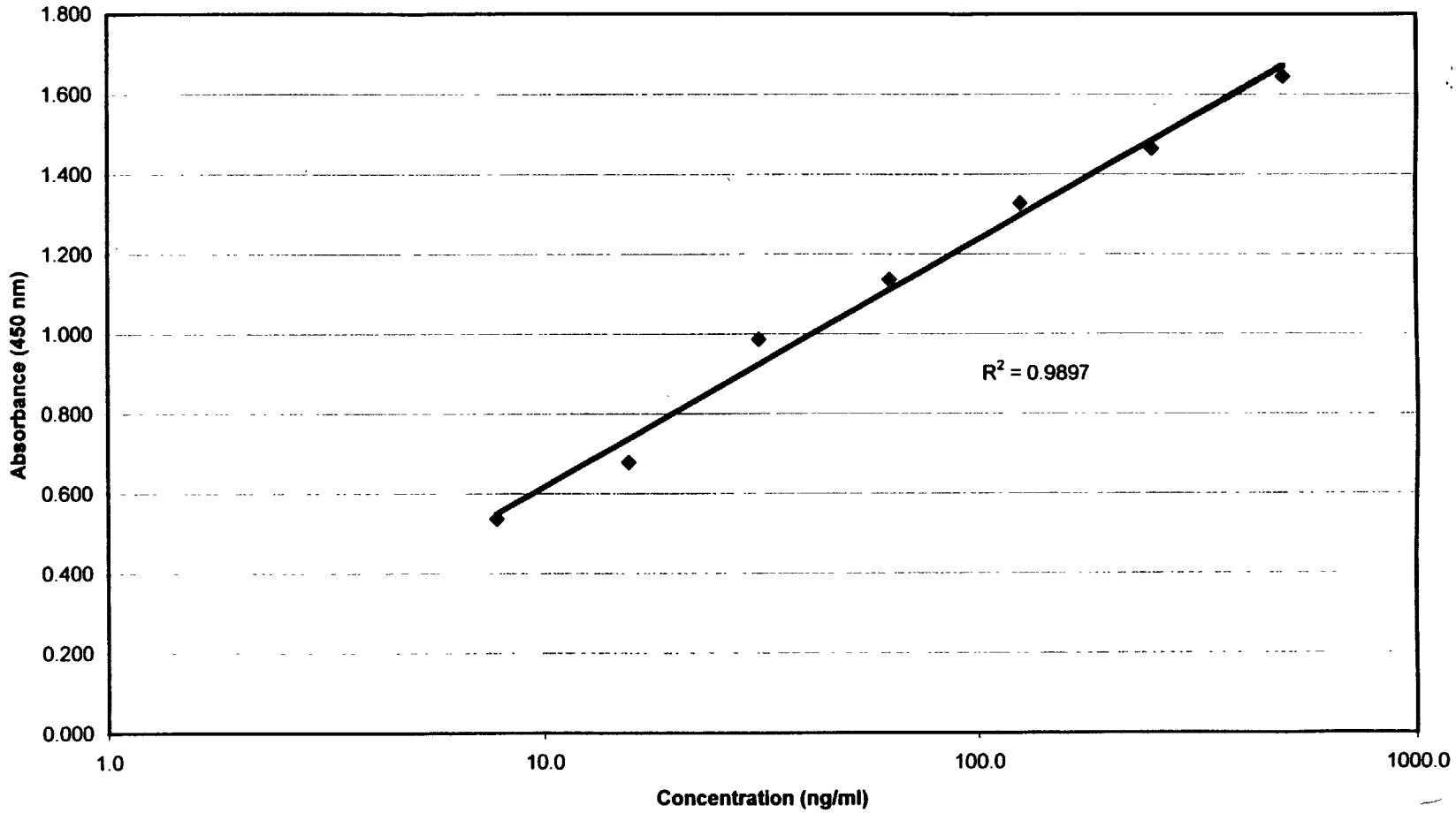
Time of Spray (sec)	Levels of LF detected (ng/ cm^2)	% LF washed-off the meat
2	263 ± 43	80
4	427 ± 57	70
6	703 ± 49	57
8	900 ± 142	61
10	1135 ± 213	61

References:

1. Adonogianaki, E., J. Mooney and D. F. Kinane (1996). "Detection of stable and active periodontitis sites by clinical assessment and gingival crevicular acute-phase protein levels." J Periodontal Res 31(2): 135-43.
2. Hetherington, S. V., J. K. Spitznagel and P. G. Quie (1983). "An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin." J Immunol Methods 65(1-2): 183-90.

N-terminus
RESEARCH LABORATORY

Bovine LF Standard Curve



-terminus
RESEARCH LABORATORY

Measurement of exogenous lactoferrin levels on beef surface

Project: **NT(02)-39D**
Scientist in charge: **Joe Tulpinski**
Date Completed: **9/12/02**

PROTOCOL

The surface levels of lactoferrin was measured by an ELISA method when applied exogenously by an electrostatic spray system and washed through a meat processing simulation system according to the following protocol.

Carcass Wash using the Meat Processing Simulation (MPS) system:

MPS system. The equipment consists of a spray-, process- and drain-unit. The spray-unit has six fluid tanks of 7.6-liter capacity, connected to six separate magnetic drive (seal-less) fan cooled sanitary/chemical solution pumps (1/25 HP, 10.6 L/min, from March Manufacturing Inc., Glenview, IL). One of the fluid tanks has a built-in heating element to deliver hot water, maximum to 190°F.

The process-unit is a cabinet (72" L x 16" W x 12" H) with a safety view glass ceiling. It has a loading chamber, five pause chambers and six spray chambers (8"L x 8"W x 12"H). A drive chain passes through the center of all the twelve chambers. The chain consists of a loading trolley with a removable stainless steel cassette and spaced with six timing tabs. Inside all the spray chambers, two adjustable spray nozzles (140° angle, 7.2 L/min) were located on both sides of the drive chain to deliver a circular stream of liquid towards the middle center of the loading trolley.

The drain-unit is a secured compartment located under the process-Unit. All the six spray chambers are individually hosed to collect the wash-effluent into 500-ml borosilicate bottles. The rest of the chambers are jointly hosed to drain into an effluent basin (72" L x 16" W x 3"H).

A programmable logic controller (PLC from Allen-Bradley, Milwaukee, WI) encased in a stainless-steel box, digitally controls the MPS system. The PLC is interfaced via a message view terminal. The operator can program the spray/pause processing time, and run-cycle (test or sanitary flush), as required. The MPS system washes the meat according to specific washing steps. The following simulated steps are comparable to that of real-time sanitizing assembly of a meat processing plant:

Step	Function	Time	Spray volume	Comments
	Sample Loading			Meat (12x12 cm) loaded
1	Cold water spray	10-sec	125-mL	Pre-evicery wash step
	Process pause	10-sec		
2	Lactic acid spray	10-sec	125-mL	Pre-evicery 2% acid rinse
	Process pause	10-sec		
3	Hot water spray	20-sec	250-mL	180°F wash (DI water)
	Process pause	10-sec		
4	Cold water spray	10-sec	125-mL	Carcass rinse
5	Lactoferrin Spray	2-10 sec	2 – 10 mL	Application by ESS
6	Lactic acid spray	10-sec	125-mL	Final 2% acid rinse

Lactoferrin application by Electro-static Spray (ESS) system:

The ESS system is a pressure and charge driven instrumentation for application of lactoferrin onto a meat surface. The electrical current for the applications of the LF ranges between 5 μdcA and 15 μdcA. Pressure on the ESS from an electrical air compressor is approximately 50 psi. The ESS was setup to deliver LF at an application rate of 1 mL/sec. This application prototype was manufactured by the National Beef Packing Company (Dodge City, KS). ESS is operated as follows:

- Meat sample after passing through ***Chamber-8 of the MPS system*** described above is unhooked together with the sample loading cassette and place inside the positioning hooks of the ESS system.
- Air compressor pressure is set to 120 psi and the tank pressure is adjusted to 100 psi.
- Air hose is attached to the air compressor and to the electrostatic spray box. Air pressure is set on the electrostatic spray control box regulator to 45-55 psi.
- LF is siphoned into the syringe which is attached to the ESS system for delivery.
- Time of LF application is adjusted for each trial segment i.e. 2-, 4-, 6-, 8-, and 10-sec.
- LF is applied on meat surface with the above settings. Fluid level and consistency of liquid sprayed from ESS is monitored during the application process.
- Meat samples were either directly measured for the LF deposition or further processed subjecting to an additional acid wash (Chamber-9) in the MPS system to measure residual (post-wash) LF. An ELISA method, described below is used for the detection of LF on meat surface.

LF detection by an ELISA method:

ELISA is a sensitive method for the detection of lactoferrin in biological systems (Adonogianaki, et al., 1996; Hetherington et al., 1983). An ELISA assay kit for the detection of bovine lactoferrin (LF) was purchased from the Bethyl Laboratories (Montgomery, TX) and performed according to the manufacturers instructions, briefly as follows:

- A 0.1 ml volume of capture antibody i.e. goat anti-bovine LF-IgG diluted in 0.05 M carbonate-bicarbonate buffer, pH 8.0, was added to coat each well of the 96-wells of a microtiter plate (Nunc MaxiSorp C bottom module). An overnight incubation was allowed to attain maximum binding of the capture antibody to the polystyrene wells.
- A 0.1 ml volume of dilutions of bovine LF standards or test samples (with unknown LF levels) were prepared in TBS-tween (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) containing 1% bovine serum albumin, was added to assigned wells. Plates were incubated at 37°C for 2-h to allow maximum antigen-antibody interaction.
- Wells were washed thoroughly for four times with TBS-tween.
- A 0.1 ml volume of an enzyme-linked antibody i.e. goat anti-bovine LF-IgG conjugated to horseradish peroxidase (HRP) prepared in TBS-tween was added to the wells and the reaction was allowed for 2-h at 37°C.
- The washing step was repeated as above.
- Finally, a 0.1 ml volume of TMB (3,3',5,5' tetramethyl benzidine) enzyme substrate solution (Bethyl Laboratories, Montgomery, TX) for HRP was prepared in added to the wells. The enzymatic reaction was terminated within 5 to 15 min (based on rapidity of color change) with 15 μl of 2N sulfuric acid.

- The color reaction was measured as absorbance at 450 nm wavelength using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA).
- A standard curve for bovine LF was plotted using a commercial preparation of LF protein (Lot. 10100395) from the DMV International, NY.
- Two-fold serial dilutions of LF ranging from 500 ng/ml to 7.8 ng/ml are prepared in TBS-tween buffer containing 1% bovine serum albumin and the ELISA assay was performed as described above.
- A standard curve was plotted using absorbance values versus dilutions of LF protein standard. An exponential plot of the standard curve yielded a R-squared value: 0.99 (See attached chart).
- For determination of LF levels in test samples, the color intensity of test samples from ELISA were correlated against the standard curve.

Test Samples:

Beef Round- Bottom Round Steak (n=4 per each time point) was used for exogenous application of LF on meat surface. Beef samples after LF application by ESS system either directly measured for LF or subjected to a further processing and the residual (post-wash after acid rinse) was measured by an ELISA. Briefly, A 50-cc sterile conical centrifuge tube (Corning) containing 5 ml of TSB was pressed against the surface of the meat and agitated for 10-sec by hand. Each tube covered an area of approximately 4.9 cm^2. This wash liquid was tested for LF levels.

Exogenous lactoferrrin levels on beef after application by ESS system

Time of Spray (sec)	Exogenous LF (ng/ cm^2)
2	1362 ± 45
4	1487 ± 86
6	1741 ± 24
8	2659 ± 153
10	2977 ± 84

Residual (post-wash) levels of exogenous lactoferrin after carcass wash

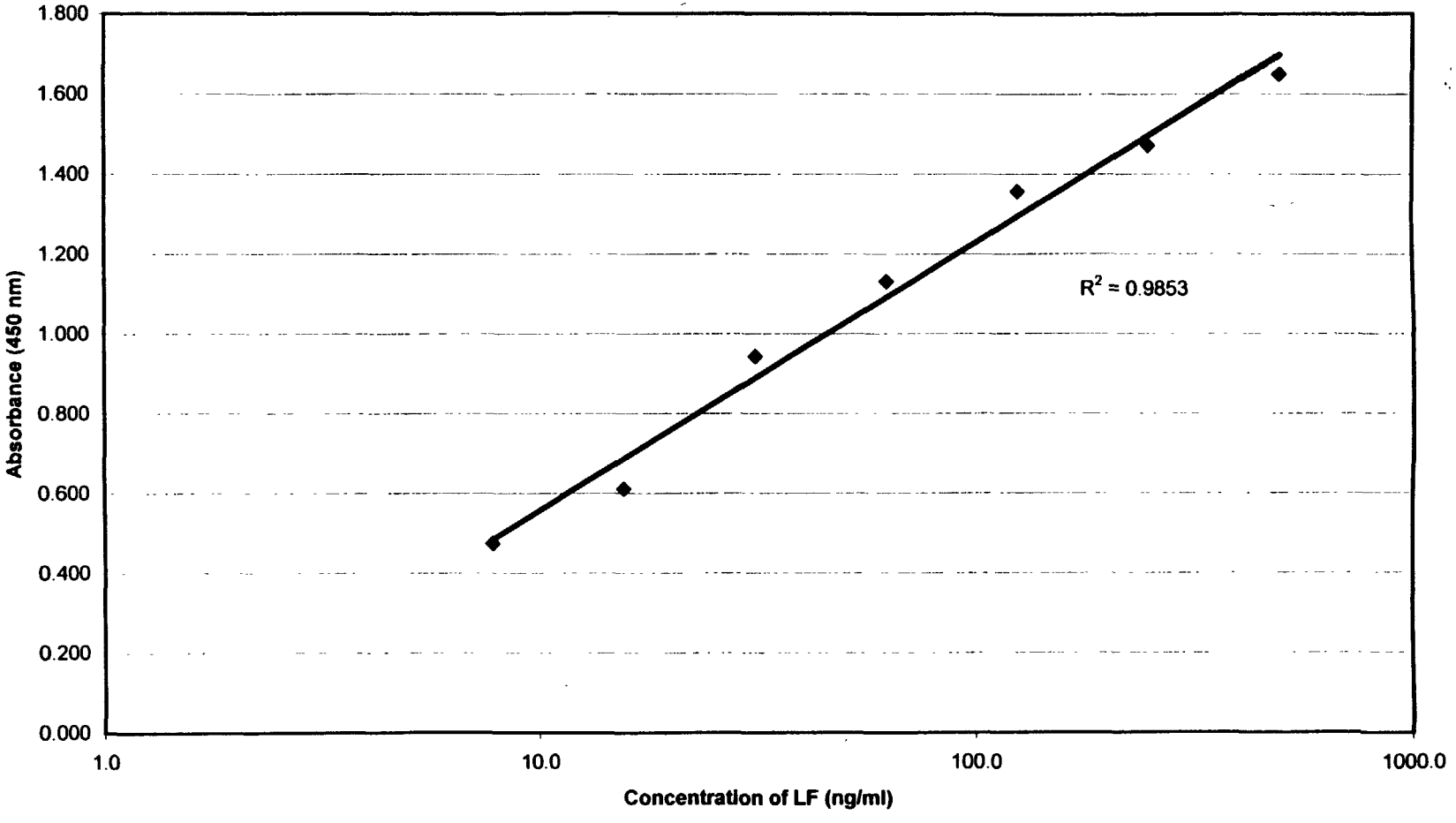
Time of Spray (sec)	Levels of LF detected (ng/ cm^2)	% LF washed-off the meat
2	263 ± 55	81
4	385 ± 41	74
6	327 ± 87	81
8	645 ± 92	76
10	865 ± 73	71

References:

1. Adonogianaki, E., J. Mooney and D. F. Kinane (1996). "Detection of stable and active periodontitis sites by clinical assessment and gingival crevicular acute-phase protein levels." J Periodontal Res 31(2): 135-43.
2. Hetherington, S. V., J. K. Spitznagel and P. G. Quie (1983). "An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin." J Immunol Methods 65(1-2): 183-90.

N-terminus
RESEARCH LABORATORY

ELISA bovine LF Standard Curve



0001123

APPENDIX III

Information Provided by
National Beef Describing the
Steps Involved in Meat Processing and
Details Regarding the Lactoferrin Application Process

Farmland
Proud to be farmer owned
National Beef
A Farmland • US Premium Beef Company

QUALITY BEEF FROM FARM TO TABLE

12200 N. Ambassador Dr.
Kansas City, MO 64163-1244
1-800-449-BEEF

September 23, 2002

Subject: Activated Lactoferrin Application at Farmland National Beef

The following is the normal process that live animals are brought in and the steps that we go through to end up in finished product which is boxed beef, trimmings destined for grind, ground beef, and bone/fat for inedible rendering. This scenario is based upon our facility in Liberal Kansas and is not meant to be an all-inclusive description of the process but rather a brief description. The plant runs 2 eight-hour shifts normally 6-days per week on both slaughter and fabrication floors.

Slaughter Process:

- Live animals are brought into the facility at a rate of approximately 35,000 head per week.
- The animals are humanely stunned and bled.
- The carcasses then are started through the skinning process. The carcasses go through multiple steps where the hide is removed leaving a de-hided carcass. The carcass goes through a series of steam vacuums to remove incidental contamination.
- Once the hide is removed, the carcass goes through a "Pre-evisceration" cabinet where a 2% lactic acid rinse is applied for microbial reduction.
- After the cabinet, the head is removed and the carcass is eviscerated.
- After evisceration, the carcass is split into two equal halves (sides) along the backbone.
- The carcass sides travel along the chain to the final rail inspection where Government FSIS Inspectors visually evaluate the carcass for defects and conduct a "post-mortem" inspection.
- During this whole process knife trimming occurs removing defects.
- After the final rail inspection the carcass sides travel through a Thermal Pasteurization Cabinet (Hot Water).
- They then enter the Activated Lactoferrin Cabinet.
- Next the carcasses enter a house tempered carcass wash.
- The final step on the slaughter floor is a "Final Lactic Acid" rinse before the carcasses enter the HotBoxes for chilling.

Chilling Process:

- Carcasses enter the Hotboxes where they are sprayed with chilled water to start their chilling process. They stay in the HotBoxes for 24 to 36 hours.
- They then are transferred to the USDA Grading area where they are evaluated for Yield, Quality and individual program classifications.
- The carcasses then are transferred to the Sales Cooler where the are sorted by grade and program designation. They normally are transferred to the fabrication floor when the are 3 – 4 days of age (from kill date).

Fabrication Process:

- Carcasses are transferred into the Fabrication floor be grade.
- As the carcasses enter the fabrication floor they are systematically broken down by primal (rounds, loins, ribs, chucks, briskets).

- These large primals are put onto main product belts and as they travel down the line are broken into smaller portions. During this process we remove fat, bone, and lean trimmings to meet our finished product specifications.
- The finished product at the end of these tables are then bagged into cryovac bags, sealed, boxed, and sent to our material handling building. This is what we are calling boxed beef.
- The lean and some of the fat that is removed during this process is diverted to side belts and is destined for either in-house grinding for ground beef or to 2000 pound combos for outside customer use (normally grinding or further processing into products like hot dogs).
- The bones and some of the fat (outside surface fat with no lean attached) are diverted to belts destined for our rendering operation.

The following is a summary of a yearly average of individual weights. A yearly average (fiscal year 2002) was used to account for the seasonal variations of carcass weights).

Average Live Animal Weight:	1,245 Lbs.
Average Dressed Carcass Weight:	799 lbs. (average side weight 400 lbs.).
Average Trim/Grind Weight	235 lbs./head
Average Boxed Beef Weight	415 lbs./head
Average Bone/Fat Weight to Rendering:	149 lbs./head

Projected Amount of Surface Coverage Activated Lactoferrin (where the treated surface ends up – the exogenous Lactoferrin surface distribution):

- When Activated Lactoferrin is applied on the carcass side in slaughter, the surface that is treated consists of the internal cavity, and the external fat surface.
- The internal cavity consists of bone and tissue that normally go to rendering.
- The remaining surface area is trimmed down on the fabrication floor to meet our customer specifications and the pure fat with no lean attached is also sent to rendering.
- Trim that goes to our in-house grind or that goes to out-side grinders consists of all the trimmings removed from the carcasses (both internal and external fat/lean).
- Most of the finished box beef produced is trimmed to a maximum ¼" fat cover or less.
- With this in mind, we have determined that approximately 40% of the original treated surface goes to rendering, 10% remains on boxed beef, and 50% goes to the in-house grind/out side grind sales.

APPENDIX IV

Product Specifications

Finished Product Specifications

The manufacturing specifications for bovine lactoferrin appear in Table 1. The product consists of 93.0% protein, 6% moisture and 1% ash. Lactoferrin makes up 95% of the protein content. Typical pH range of the finished product is between 5.5 to 6.5 with a water activity (a_w) of 0.2. Complete solubility is achieved when 2 g of lactoferrin is added to 100 ml of 20°C water. The iron binding capacity of the final product is ≥70%.

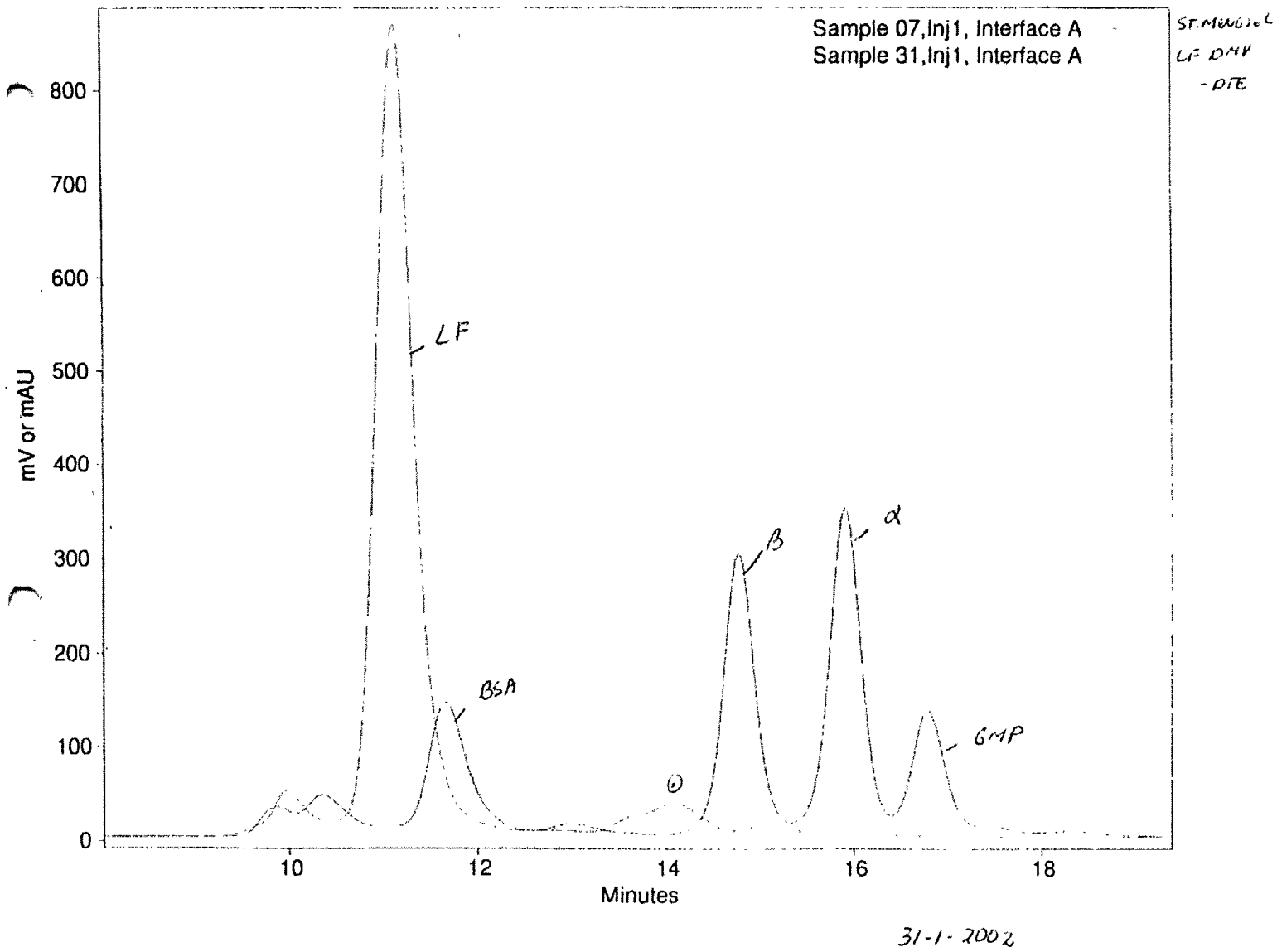
Table 1. Manufacturing Specifications for Bovine Lactoferrin

Chemical/Physical/Microbial Characteristics	Specifications
Protein	≥ 93.0%
• Lactoferrin	≥ 95.0%
Moisture	≤ 6.0%
Ash	≤ 1.0%
pH (2%, 20°C)	5.5-6.5
a_w – not measured on a routine basis	.2
Solubility	
• In water (2%, 20°C)	≥ 100%
• Transmittance, 2% sol., 600nm	≥ 80%
Iron Binding	
Spectrophotometric method at 465 nm on solids	≥ 70%
Foreign matter (10g)	Absent
Heavy metals (as Pb)	≤ 1 mg/kg
Lead	<0.5 mg/kg
Arsenic	<1.0 mg/kg
Cadmium	<0.05 mg/kg
Mercury	<0.05 mg/kg
Copper	<2.0 mg/kg
PCB's [1]	<0.1 mg/kg
Pesticides [2]	<0.1 mg/kg
Antibiotics	<0.005 IU/ml
Alfatoxin MI	<0.5 mcg/kg
Radioactivity [3]	<5.0 Bq/kg
Standard plate count	≤ 1000/g
Enterobacteriaceae	≤ 10/g
Salmonella 2 x 1 g	Negative/50g
S. aureus	Negative
Total yeast and mold	≤ 10/g

[1] PCB 28, PCB 52, PCB 101, PCB 118, PCB 138, PCB 153, PCB 180
[2] Dieldrin, heptachlor epoxide, hexachlorobenzene, α-hexachlorocyclohexane, β-hexachlorocyclohexane, lindane, DDT-total
[3] Cs 134 + Cs 137

000128

Figure 1. Comparison of Bovine Milk – Derived Lactoferrin Product to Allergenic Protein Standards



0001129

Table 2
Comparison of Bovine Milk-Derived Lactoferrin Product to Allergenic Protein Standards*

Protein	Concentration (mg/L)	Retention Time (min.)
LF	1000	11.2
BSA	800	11.8
β - Lg	500	14.7
α - La	800	16.0
GMP	500	17.0
Unknown	60-70	14.0 (approx)

*Data from Figure 1
(Abbreviations : LF, lactoferrin ; BSA, bovin serum albumin ; β - Lg, beta-lactoglobulin ; α - La, alpha-lactoalbumin ; GMP, glycomacropeptide)

000130

000131

SUBMISSION END

AM

OLSSON, FRANK AND WEEDA, P.C.

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

Sender's Direct Phone (202) 518-6327
Sender's Direct Facsimile (202) 234-2686

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA (1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES.

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SUSAN P. GRYMES
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE M. BRIGGS*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

June 6, 2003

BY FACSMILE

Mr. Jeremiah Fasano
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice No. GRN 000130

Dear Mr. Fasano:

We have received your June 4, 2003 letter acknowledging receipt of the GRAS Notice filed on behalf of aLF Ventures, L.L.C. on May 23, 2003 (GRN 000130). The intent of this letter is to clarify the purpose of the GRAS Notice. Please note that GRN 00130 informs FDA that, in the view of aLF Ventures, L.L.C., the use of bovine milk-derived lactoferrin used as an antimicrobial spray on beef carcasses so that the residual level of endogenous lactoferrin does not exceed 800 nanograms per gram of beef is generally recognized as safe without any need for product labeling.

We are looking forward to working with FDA on its review of this notice. Should you have any questions, please do not hesitate to contact me.

Sincerely yours,

Mark L. Itzkoff

MLI:jdm
cc: Dr. Ram Nimmagudda
Claire L. Kruger, Ph.D.
Philip C. Olsson

AM

OLSSON, FRANK AND WEEDA, P.C.

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

Sender's Direct Phone (202) 518-6327
Sender's Direct Facsimile (202) 234-2686

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA (1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES.

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE B. SOLOMON*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

June 6, 2003

VIA FEDERAL EXPRESS
AND HAND-DELIVERY

Mr. Jeremiah Fasano
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice No. GRN 000130

Dear Dr. Fasano:

The purpose of this letter is to respond to your request for additional information regarding the aLF Ventures GRAS Notice (GRN 130). Specifically, you asked for citations in support of the following statement from page 33 of the Section V of GRN 130:

> With regard to the question of potential allergic responses, all immunological reactions to lactoferrin so far reported are to the polypeptide chain and not to the glycan.

A list of citations in support of this statement as well as copies of the cited studies are attached.

Should you have any additional questions, please do not hesitate to contact us.

Sincerely yours,

Mark L. Itzkoff

MLI:lss
cc: Dr. Ram Nimmagudda
Claire L. Kruger, Ph.D.
Philip C. Olsson

Additional Citations for GRN 130

1. van Berkel, P.H., van Veen, H.A., Geerts, M.E., de Boer, H.A., Characterization of monoclonal anitbodies against human lactoferrin, J. Immun. Methods 267 (2002) 139-150.

2. Skubitz, K.M., Christiansen, N.P., Mendiola, J.R., Preperation and Characterization of Monoclonal Antibodies to Human Neutrophil Cathepsin G, Lactoferrin, Eosinophil Peroxidase, and Eosinophil Major Basic Protein, J Leukocyte Biol. 46 (1989) 109-118.

3. van Berkel, P.H., van Veen, H.A., Geerts, M.E., Mericskay, M., de Boer, H.A., Nuijens, J.H., N-Terminal Stretch Arg^2, Arg^3, And Arg^5 of Human Lactoferrin is Essential for Binding to Heparin, Bacterial Lipopolysaccharide, Human Lysozyme and DNA, Biochem J. 328 (1997) 145-151

BEST ORIGINAL COPY

OLSSON, FRANK AND WEEDA, P.C. AM
ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220

SENDER'S PHONE: (202) 518-6366
SENDER'S FACSIMILE: (202) 234-3550
SENDER'S E-MAIL: polsson@ofwlaw.com

FACSIMILE TRANSMISSION

June 30, 2003

TO: Dr. Linda S. Kahl

COMPANY: Food and Drug Administration, CFSAN

FAX: 202-418-3179

FROM: Philip C. Olsson

RE: Supplement to Petition for Approval of Lactoferrin-based Spray

REMARKS: Attached is the letter I sent to Dr. Robert Post at USDA, along with a prior approval from USDA.

THE INFORMATION CONTAINED IN THIS COMMUNICATION IS CONFIDENTIAL, MAY BE ATTORNEY-CLIENT PRIVILEGED, AND IS INTENDED ONLY FOR THE USE OF THE ADDRESSEE. IF YOU HAVE RECEIVED THIS COMMUNICATION IN ERROR, PLEASE NOTIFY US IMMEDIATELY BY TELEPHONE AT 202.789.1212 SO THAT WE MAY ARRANGE FOR RETRIEVAL OF THE DOCUMENT AT NO COST TO YOU. THANK YOU.

THE TOTAL NUMBER OF PAGES INCLUDING THIS PAGE IS 7. IF YOU DO NOT RECEIVE ALL PAGES, PLEASE CALL MY OFFICE.

OLSSON, FRANK AND WEEDA, P.C.
ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

Sender's Direct Phone (202) 518-6366
Sender's Direct Facsimile (202) 234-3550

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA (1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE B. SOLOMON*

OF COUNSEL
JUR. T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

June 30, 2003

BY FACSIMILE

BEST ORIGINAL COPY

Robert C. Post, Ph.D.
Director
Labeling and Consumer Protection Staff
Food Safety and Inspection Service
U.S. Department of Agriculture
300 12th Street, S.W., Room 602-ANX
Washington, D.C. 20250

Re: Supplement to Petition for Approval of Lactoferrin-based Spray

Dear Dr. Post:

The purpose of this letter is to supplement the November 29, 2001 "Petition for Approval of Lactoferrin-based Spray to inhibit microbial growth on beef products" submitted by aLF Ventures, L.L.C. We are hereby submitting an alternative formulation for the lactoferrin spray, described in that petition. Table 1, attached, provides the formulations of the original spray and the alternative formulation. As you can see, the only differences in the two formulations are: (1) the use of disodium EDTA in place of citric acid; and (2) the substitution of pectin for agar or carrageenan.

EDTA

Disodium ethylenediamine tetraacetate (EDTA) is cleared for use in a variety of food applications. USDA has authorized the use of EDTA both as a hog scald agent, as well to protect and preserve flavor in meat fat margarine. In this alternative lactoferrin spray formulation, EDTA would be an incidental additive because its presence as a residual would be insignificant and no longer functional.

BEST ORIGINAL COPY

Pectin

Pectin would be used to immobilize lactoferrin, in the same manner that agar was previously accepted for that purpose. [see your letter to James T. Heimbach, Ph.D. F.A.C.N., Principal, Environ International Corporation, dated December 21, 2001].

Should you have any questions, please do not hesitate to contact me.

Sincerely,

Philip C. Olsson

/lss
Attachments

cc: Dr. Linda S. Kahl, FDA

BEST ORIGINAL COPY

Table 1

Lactoferrin Spray Formulations
(per liter of spray)

Original Formulation		New Formulation	
Milk Derived Lactoferrin	10.0 – 20.0 g	Milk Derived Lactoferrin	10.0 – 20.0 g
Agar or Carrageenan	0.1 – 0.25 g	Pectin	0.15 – 0.25 g
Sodium chloride	5.0 – 6.5 g	Sodium chloride	5.0 – 6.5 g
Sodium bicarbonate	0.5 – 1.5 g	Sodium bicarbonate	0.5 – 1.5 g
Citric Acid	0.1 – 3.0 g	Disodium EDTA	0.30 – 0.50 g
Deionized water (balance)		Deionized water (balance)	

United States Department of Agriculture
Food Safety and Inspection Service
Office of Policy, Program Development and Evaluation
Washington, D.C. 20250

BEST ORIGINAL COPY

DEC 21 2001

James T. Heimbach, Ph.D., F.A.C.N., Principal
Environ International Corporation
4350 North Fairfax Drive
Arlington, VA 22203

Dear Dr. Heimbach:

I am responding to the documentation that you submitted, dated November 29, 2001, in response to the Food and Drug Administration's (FDA) Agency Response Letter (dated October 23, 2001) regarding Generally Recognized as Safe (GRAS) Notice No. GRN 000067. The subject of the GRAS Notice is milk-derived lactoferrin as a component of a spray solution that includes other constituents.

The Notice was submitted by you to FDA on behalf of Farmland National Beef Packaging Company, L.P. (National Beef) in January 2001, and informs the FDA of the view of National Beef that milk-derived lactoferrin is GRAS, through scientific procedures, for the use as a component of an antimicrobial spray that contains up to 2 percent lactoferrin that would be applied to uncooked beef (carcasses, subprimals, and cuts). The maximum concentration to be used is 3.26 milliliters spray per kilogram beef (or 65.2 milligrams lactoferrin per kilogram beef).

Based on the information provided by National Beef, as well as other information available to FDA, the agency stated in its Response Letter to you that it had no questions regarding National Beef's conclusion that milk-derived lactoferrin is GRAS under the intended conditions of use, provided that the ingredients statement of food products that contain milk-derived lactoferrin identifies the source of the protein. Furthermore, the Response Letter stated that FDA had not made its own determination regarding the GRAS status of the subject use of milk-derived lactoferrin. It is the continuing responsibility of National Beef to ensure that the food ingredient is safe and otherwise in compliance with all applicable legal and regulatory requirements.

However, with regard to suitability of use, the Response Letter also stated that, after consultation with the Food Safety and Inspection Service (FSIS), there were additional data that were needed. Such data were needed to adequately address issues related to the efficacy and suitability of the use of the lactoferrin spray as an antimicrobial agent on uncooked beef. Specifically, the data that were identified as being needed included the identification of all components of the spray; data that establish the lowest level of the blend (and the amounts of each ingredient in the blend) necessary to achieve the intended functional effect (i.e., inhibition of the growth of microorganisms); and data regarding the actual concentrations of each component of the spray that will remain in the beef that will be available to the consumer. National Beef was also advised that data must also address whether the appearance of the treated product would mislead

BEST ORIGINAL COPY

consumers into perceiving the product as "fresher" when compared to untreated beef and whether the use of this spray extends the shelf life beyond actual microbial shelf life.

Lastly, the Response Letter indicated that it was the decision of FDA and FSIS that all lactoferrin-treated uncooked beef products would need to be labeled to alert consumers of the presence of the spray. A prominently placed statement such as "treated with lactoferrin from milk" or "treated with lactoferrin, a milk protein" would be needed on the labeling of treated product from the point of slaughter to retail sale.

We have performed a thorough review of the data that you submitted on behalf of National Beef. The data explain that the spray consists of milk-derived lactoferrin (10-20 g), agar or carrageenan (0.1-0.25 g), sodium chloride (5.0-6.5 g), sodium bicarbonate (0.5-1.5 g), citric acid (0.1-3.0 g), and deionized water (to bring the solution volume to 1.0 liter), and that these levels are the lowest necessary to achieve the intended technical effect. The component primarily responsible for the antimicrobial effect of the spray is the lactoferrin. The data show that, under the prescribed conditions of use, the microbial blocking effect of the milk-derived lactoferrin in the spray performs the technical effect of detaching and removing a wide variety of pathogens from the uncooked beef tissue surface. The agar and carrageenan serve to immobilize a fraction of the lactoferrin. The remaining components serve to buffer and dilute the spray solution to the effective use concentration. Moreover, based on the figures you provided, with the exception of lactoferrin, the levels of the components in the spray, under the prescribed conditions of use, would become incidental additives upon application because their presence as residuals would be insignificant and no longer functional.

With regard to the effects of the spray solution on the characteristics of treated uncooked beef (strip loin subprimals and steaks), notable effects on the aesthetic characteristics of tenderness, juiciness, and flavor were not observed. However, the data showed that there is some effect on prolonging the shelf-life of treated uncooked beef and inhibiting microbial growth on treated beef as compared to untreated beef. Because these effects were attributed to the active component of the spray, i.e., lactoferrin, the need for labeling the presence of lactoferrin becomes necessary, in addition to the reason that lactoferrin is a potential allergen.

In summary, based on the data you presented, we have concluded that the data satisfactorily address all of the data points that FDA listed in their Response Letter which are described above. Thus, the lactoferrin spray solution may be applied to uncooked beef consistent with the conditions that were outlined in the FDA Response Letter.

I must add one additional element in response to your data submission. It relates to the special statement (e.g., "treated with lactoferrin from milk") that is needed on the labeling of uncooked beef products treated with the lactoferrin spray. The statement must appear on the principal display panel (PDP) on the labeling of the treated beef products in accordance with FSIS regulations. Furthermore, labeling bearing such statements must be submitted to FSIS for evaluation prior to applying the labeling to products for sale in commerce.

James T. Heimbach, Ph.D., F.A.C.N., Principal BEST ORIGINAL COPY

If you need additional information regarding this matter, please do not hesitate to contact Bill Jones, Sally [illegible] at (202) 205-0279.

Sincerely,

for

Robert C. Post, Ph.,D., Director
Labeling and Consumer Protection Staff

AM

OLSSON, FRANK AND WEEDA, P.C.

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA(1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SUSAN P. GRYMES
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE M. BRIGGS*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

Sender's Direct Phone (202) 518-6366
Sender's Direct Facsimile (202) 234-3550

July 11, 2003

BY ELECTRONIC MAIL

Dr. Jeremiah Fasano
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: Thermal Denaturation and Digestion of Lactoferrin

Dear Dr. Fasano:

During the July 10, 2003 meeting between the Center for Food Safety and Applied Nutrition and aLF Ventures, LLC, you and your colleagues requested additional citations regarding the effects of cooking on lactoferrin and information regarding the digestion of lactoferrin. Attached is a list of citations on these two subjects.

We are obtaining printed copies of these citations and will provide these to you as soon as possible.. In the meantime, if you have any further questions, please do not hesitate to contact us.

Sincerely,

Philip C. Olsson

PCO:jdm
Attachment

Thermal Denaturation of Lactoferrin

1. Paulsson, MA; Svensson U; Kishore AR; Naidu AS (1993) - Thermal behaviour of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. **J. Dairy Sci. 76, 3711-3720**
2. Sanchez, L; Peiro JM; Castillo H; Perez MD; Ena JM; Calvo M (1992b) - Kinetic parameters for denaturation of bovine milk lactoferrin. **J.Food Sci. 57 (4), 873-879.**

Intestinal Digestion of Lactoferrin

1. Brock JH; Arzabe F; Lampreave F; Pineiro A (1976) - The effect of trypsin on bovine transferrin and lactoferrin. **Biochim.Biophys.Acta 446: 214-225.**
2. Brines RD; & Brock JH (1983) - The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron binding properties of lactoferrin in human milk and bovine colostrums; unusual resistance of human apolactoferrin to proteolytic digestion. **Biochim. Biophys.Acta 759: 229-235.**
3. Spik G; Brunet B; Mazurier-Dehaine C; Fontaine G; Montrueil J (1982) - Characterization and properties of the human and bovine lactotransferrins extracted from the faeces of new born infants. **Acta Paedatr.Scand 71: 979-985.**
4. Line W; Sly D; Bezkorovainy A (1976) - Limited cleavage of human lactoferrin with pepsin. **Int. J. Biochem 7: 203 – 208**
5. Bellamy W; Takase M; Yamauchi K; Wakabayashi H; Kawase K Tomita M (1992) - Identification of bacterial domain. **Biochim . Biophys. Acta 1121: 130-136**
6. Freddy J. Troost ; Jan Steijns; Wim H. M.Saris and Robert-Jan M. Brummer (2001) - Gastric digestion of bovine lactoferrin In Vivo in adults. **J. Nutr 131: 2101-2104**

AM

OLSSON, FRANK AND WEEDA, P.C.

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA (1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE B. SOLOMON*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

Sender's Direct Phone (202) 518-6366
Sender's Direct Facsimile (202) 234-3550

July 15, 2003

03-07-18A11:26 RCV

BY FEDERAL EXPRESS

Dr. Jeremiah Fasano
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRSN 130

Dear Dr. Fasano:

On behalf of aLF Ventures, LLC, and further to my July 11, 2003 letter to you regarding scientific literature citations on the thermal denaturation and digestion of lactoferrin, we are hereby forwarding copies of the literature citations listed in that letter.

Cordially

Philip C. Olsson

PCO:lss
Attachments

Attachments

1. Paulsson, MA; Svensson U; Kishore AR; Naidu AS (1993) - Thermal behaviour of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. **J. Dairy Sci. 76 , 3711-3720**

2. Sanchez, L; Peiro JM; Castillo H; Perez MD; Ena JM; Calvo M (1992b) - Kinetic parameters for denaturation of bovine milk lactoferrin. **J.Food Sci. 57 (4), 873-879.**

3. Brock JH; Arzabe F; Lampreave F; Pineiro A (1976) - The effect of trypsin on bovine transferrin and lactoferrin. **Biochim.Biophys.Acta 446: 214-225.**

4. Brines RD; & Brock JH (1983) - The effect of trypsin and chymotrypsin on the in vitro antimicrobial and iron binding properties of lactoferrin in human milk and bovine colostrums; unusual resistance of human apolactoferrin to proteolytic digestion. **Biochim. Biophys.Acta 759: 229-235.**

5. Spik G; Brunet B; Mazurier-Dehaine C; Fontaine G; Montrueil J (1982) - Characterization and properties of the human and bovine lactotransferrins extracted from the faeces of new born infants. **Acta Paedatr.Scand 71: 979-985.**

6. Line W; Sly D; Bezkorovainy A (1976) - Limited cleavage of human lactoferrin with pepsin. **Int. J. Biochem 7: 203 – 208**

7. Bellamy W; Takase M; Yamauchi K; Wakabayashi H; Kawase K Tomita M (1992) - Identification of bacterial domain. **Biochim . Biophys. Acta** 1121: 130-136

8. Freddy J. Troost; Jan Steijns; Wim H. M.Saris and Robert-Jan M. Brummer (2001) - Gastric digestion of bovine lactoferrin In Vivo in adults. **J. Nutr 131: 2101-2104**

AM

OLSSON, FRANK AND WEEDA, P.C.

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA(1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SUSAN P. GRYMES
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE M. BRIGGS*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

July 18, 2003

BY ELECTRONIC MAIL
AND FEDERAL EXPRESS

Dr. Linda S. Kahl
Food and Drug Administration
Center for Food Safety and Applied Nutrition
1110 Vermont Avenue, N.W., Room 1119, (HFS-255)
Washington, D.C. 20201

Dr. Jeremiah Fasano
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRN 130

Dear Dr. Fasano:

The purpose of this letter is to follow-up with you on yesterday's telephone conversations regarding aLF Ventures, LLC's GRAS notification for bovine milk derived lactoferrin (GRN 130). The attached letter prepared by ENVIRON is intended to respond to your questions regarding the lactic acid rinse of lactoferrin-sprayed carcasses, the comparison of the intakes of exogenous and endogenous lactoferrin and the basis for the two scenarios used to calculate the comparative exposures.

We trust you now have sufficient information to complete your review of GRN 130. Should you have any further questions, please contact us immediately.

Cordially,

000171

Philip C. Olsson

OFW:lss
Attachment

ENVIRON
Health Sciences Institute

July 18, 2003

Phillip C. Olsson, Esq.
Olsson, Frank and Weeda, P.C.
1400 16th Street, N.W.
Suite 400
Washington, DC 20036

Dear Phil,

Please find attached the information pertaining to the three points of clarification requested by FDA during the phone conference of July 17th with Ted Berner and Phil Olsson. Please forward this information to FDA for their files pertaining to GRAS Notification GRN 130 for bovine milk-derived lactoferrin submitted on behalf of aLF Ventures.

Sincerely yours,

Claire L. Kruger, Ph.D., D.A.B.T.
Principal

Attachments

000172

The ENVIRON Health Sciences Institute
4350 North Fairfax Drive, Suite 300, Arlington, Virginia 22203 USA
Tel: +1 703 516 2309 · Fax: +1 703 516 2304 · Email: ckruger@environcorp.com

1. ***Correction of two typographical errors contained in "GRAS Notification for Bovine Milk-Derived Lactoferrin in Beef Carcass Spray Applications" (GRN 130)***

Please note that in Section V (*Scientific Support Document*) of the above-referenced GRAS Notification, there are two typographical errors that need correcting. Both errors occur near the top of page 20 of Section V. In the first line on that page, the words "two-second" should be replaced with the words "ten-second." Also, on the third line of that same page, again, the words "two-second" should be replaced with the words "ten-second."

2. *Table to facilitate the comparison between endogenous and exogenous lactoferrin intakes*

Table 3a. A Comparison of Estimated Endogenous Lactoferrin Intakes with Estimated Exogenous Lactoferrin Intakes from Lactoferrin-Treated Beef Carcasses

Population Group	Estimated Endogenous Lactoferrin Intakes Based on 50th Percentile Beef Consumption (µg/occasion)[1]	Estimated Endogenous Lactoferrin Intakes Based on 90th Percentile Beef Consumption (µg/occasion)	Estimated Exogenous Lactoferrin Intakes Based on 50th Percentile Beef Consumption (µg/occasion)		Estimated Exogenous Lactoferrin Intakes Based on 90th Percentile Beef Consumption (µg/occasion)	
			Scenario 1[2]	Scenario 2[3]	Scenario 1	Scenario 2
Infants, < 1 y	3	8	5	2	14	20
Infants, 1 y	3	12	5	6	20	35
Children, 2-5 y	4	14	7	10	24	44
Children, 6-12 y	6	19	9	12	31	62
Teenagers, 13-19 y	7	26	11	15	44	76
Adults, 20+ y	9	30	16	16	50	76

DATA SOURCE: USDA 1994-96, 1998 Continuing Survey of Food Intakes by Individuals (CSFII) and USEPA Food Commodity Intake Database (USDA 2000, USEPA 2000).
NOTES: Breastfeeding infants and children were excluded from the analyses. Analyses were limited to individuals who provided 2-days of recall data. Estimates were generated with USDA sample weighting factors to adjust for differences in representation of subpopulations.

[1] The endogenous (background) lactoferrin intakes in columns 2 and 3 of the above table were calculated by taking the 50th and 90th percentiles of beef consumption (in g per occasion) for each population group of interest and multiplying each of them by 193 ng bovine neutrophil lactoferrin per g beef (ppb). See the text for a further discussion of the derivation of the estimated endogenous lactoferrin concentration in beef of 193 ng/g.

[2] Under Scenario 1, the amount of bovine milk-derived lactoferrin adhering to the treated beef carcass is assumed to be equally distributed throughout the edible portions of the carcass; this assumption yields an estimated exogenous lactoferrin concentration in all beef products of 320 ng per g of beef (ppb). Then, intake values corresponding to the 50th and 90th percentiles of beef consumption (in g per occasion) for each population group of interest were multiplied by this concentration of 320 ng bovine milk-derived lactoferrin per g of beef (ppb) to yield estimates of exogenous lactoferrin intakes listed above under "Scenario 1." See the text for a further discussion of the derivation of the estimated exogenous lactoferrin concentration in lactoferrin-treated beef of 320 ng/g.

[3] Under Scenario 2, the amount of bovine milk-derived lactoferrin adhering to the treated beef carcass is assumed to be distributed differentially within the treated beef carcass based on the surface area of the carcass that ends up in "boxed beef" vs. "trim/grind"; this assumption yields an estimated exogenous lactoferrin concentration in "boxed beef" of 82 ng per g of beef (ppb) and an estimated exogenous lactoferrin concentration in "trim/grind" of 726 ng per g of beef (ppb). Then, for each beef-eating occasion, it was determined whether the beef consumed would have most likely been derived from "boxed beef" or "trim/grind." If from the "boxed beef," the amount of beef consumed was multiplied by the concentration of exogenous lactoferrin in "boxed beef" of 82 ng bovine milk-derived lactoferrin per g. If from the "trim/grind," the amount of beef consumed was multiplied by the concentration of exogenous lactoferrin in "trim/grind" of 726 ng bovine milk-derived lactoferrin per g. These exogenous lactoferrin intakes were then summed across all beef-eating occasions and 50th and 90th percentile intake values were generated. See the text for a further discussion of the derivation of the estimated exogenous lactoferrin concentrations in lactoferrin-treated beef.

0001174

3. Clarification regarding assumptions employed in "Scenario 1" versus "Scenario 2"

For "Scenario 1," the assumption employed was that the amount of bovine milk-derived lactoferrin adhering to the exterior of the treated beef carcass will become equally distributed throughout all parts of the edible beef within the carcass during processing, so that all beef products produced from the treated carcass will contain the same concentration of bovine milk-derived (i.e., exogenous) lactoferrin (i.e., 320 ng/g). Then, intake values corresponding to the 50th and 90th percentiles of beef consumption (in g per occasion) for each population group of interest were multiplied by this concentration of 320 ng bovine milk-derived lactoferrin per g of beef (ppb) to yield 50th and 90th percentile estimates of exogenous lactoferrin intakes for inclusion in Table 3.

For "Scenario 2," the assumption employed was that the amount of bovine milk-derived lactoferrin adhering to the exterior of the treated beef carcass will become distributed differentially into different cuts of beef based on the relative proportion of the surface area of the treated carcass that ends up in "boxed beef" versus "trim/grind," so that the concentration of bovine milk-derived (i.e., exogenous) lactoferrin in a particular beef product derived from a treated carcass depends on whether that product was derived from "boxed beef" or "trim/grind." For beef products derived from "boxed beef" (e.g., steaks, roasts, tenderloins, etc.), the concentration of bovine milk-derived lactoferrin in these products was calculated to be 82 ng/g. For beef products derived from "trim/grind" (e.g., ground beef, hot dogs, sausages, etc.), the concentration of bovine milk-derived lactoferrin in these products was calculated to be 726 ng/g. Then, for each beef-eating occasion, it was determined whether the beef consumed would have most likely been derived from "boxed beef" or "trim/grind." If from "boxed beef," the amount of beef consumed was multiplied by the concentration of exogenous lactoferrin in "boxed beef" of 82 ng bovine milk-derived lactoferrin per g beef. If from "trim/grind," the amount of beef consumed was multiplied by the concentration of exogenous lactoferrin in "trim/grind" of 726 ng bovine milk-derived lactoferrin per g beef. These exogenous lactoferrin intakes were then summed across all beef-eating occasions and 50th and 90th percentile intake values were generated for inclusion in Table 3.

AM

OLSSON, FRANK AND WEEDA, P.C.

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA(1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SUSAN P. GRYMES
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE M. BRIGGS*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

Sender's Direct Phone (202) 518-6366
Sender's Direct Facsimile (202) 234-3550

July 24, 2003

BY FACSIMILE
and ELECTRONIC MAIL

Dr. Linda S. Kahl
Food and Drug Administration
Office of Food Additive Safety
HFS-255
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Weight Variations

Dear Dr. Kahl:

You have asked whether weight variations among cattle would be likely to lead to concentrations of lactoferrin in edible beef > 800 ng per gram. USDA data regarding slaughter weights shows that this is unlikely to occur.

Estimated concentrations of lactoferrin in edible beef can be calculated using data compiled by National Beef for fiscal year 2002 on the average weight of beef products (on a per head basis) at various points in the meat packing process. These data are as follows:

- Average live animal weight: 1,245 pounds per head
- Average dressed carcass weight: 799 pounds per head
- Average boxed beef weight: 415 pounds per head
- Average trim/grind weight: 235 pounds per head
- Average weight of bone/fat to rendering: 149 pounds per head

Based on the above data, the average amount of edible meat that a beef carcass yields is about 650 pounds (295 kg), and is derived by summing the average boxed beef weight (415 pounds or 189 kg) and the average trim/grind weight (235 pounds or 107 kg) on a per head basis.

An application rate of 50 ml per carcass is targeted to result in an 800 ng per gram estimated concentration of bovine milk-derived lactoferrin for a carcass with 250 kg edible meat, and proportionately less (680 ng per gram) for a carcass with 295 kg of edible meat. In the National Beef table, the dressed weight for a carcass with 295 kg of edible meat is 799 pounds. By extrapolation the dressed weight for a carcass with 250 kg of edible meat will be 678 pounds.

USDA Market News Service reports dressed weights for cattle slaughter. Examining Market News reports for April 30, 2003 and July 23, 2003, the average dressed weights for steers and heifers range from a low of 690 pounds in the April 30 report to as much as 839 pounds in the July 23 report. Only in the July 23 report, was there a group of cattle in which some carcasses had a dressed weight of less than 677 pounds. In that group there were 4,917 cattle with a weight range of 654-813 pounds and an average weight of 744 pounds. Application of 50 ml per carcass to animals in this weight range would result in average concentrations of bovine milk-derived lactoferrin of only 729 ng per gram.

Since the external trimmings (the portion with the highest concentration) from such carcasses would always be blended with lean meat and trim from other carcasses the average concentration of bovine derived lactoferrin in any ground products would always be less than 800 ng per gram. Therefore, we believe it is extraordinarily unlikely that concentrations of bovine milk-derived lactoferrin in edible meat products would ever exceed 800 ng per gram.

Sincerely,

Philip C. Olsson

PCO.lss

BEST ORIGINAL COPY

LM_CT100
St Joseph, MO Wed Apr 30, 2003 USDA Market News Service

5 AREA DAILY WEIGHTED AVERAGE DIRECT SLAUGHTER CATTLE - NEGOTIATED
Texas/Oklahoma; Kansas; Nebraska; Colorado; Iowa/Minnesota feedlots
Recap for: Tuesday 4/29/03

Head Count:	3,910	Week Ago:	2,225	Last Year:	2,832
Week to Date	4,516	Week Ago:	2,905	Last Year:	3,722

LIVE FOB BASIS - Beef Breeds

	Head Count	Weight Range	Price Range	Avg Weight	Avg Price
STEERS					
Over 80% Choice	38	1,375-1,375	79.00-79.00	1,375	79.00
65 - 80% Choice	939	1,225-1,300	78.00-78.50	1,254	78.12
35 - 65% Choice	75	1,250-1,250	78.00-78.00	1,250	78.00
0 - 35% Choice		-	-		
Total all grades	1,052	1,225-1,375	78.00-79.00	1,258	78.15
HEIFERS					
Over 80% Choice		-	-		
65 - 80% Choice	466	1,125-1,175	77.00-78.00	1,145	77.70
35 - 65% Choice	135	1,100-1,100	78.00-78.00	1,100	78.00
0 - 35% Choice		-	-		
Total all grades	601	1,100-1,175	77.00-78.00	1,135	77.77

DRESSED DELIVERED BASIS - Beef Breeds

	Head Count	Weight Range	Price Range	Avg Weight	Avg Price
STEERS					
Over 80% Choice		-	-		
65 - 80% Choice	138	803-803	125.00-125.00	803	125.00
35 - 65% Choice	270	800-805	125.00-125.00	804	125.00
0 - 35% Choice		-	-		
Total all grades	408	800-805	125.00-125.00	804	125.00
HEIFERS					
Over 80% Choice		-	-		
65 - 80% Choice	262	690-778	125.00-126.00	716	125.71
35 - 65% Choice	557	690-690	125.00-125.00	690	125.00
0 - 35% Choice		-	-		
Total all grades	819	690-778	125.00-126.00	698	125.23

WEEKLY ACCUMULATED	Head Count	Avg Weight	Avg Price
Live Steer	1,285	1,252	77.83
Live Heifer	646	1,136	77.89
Dressed Steer	408	804	125.00
Dressed Heifer	927	701	125.13

SAME PERIOD LAST WEEK	Head Count	Avg Weight	Avg Price
Live Steer	175	1,199	79.31
Live Heifer			
Dressed Steer	561	817	125.82
Dressed Heifer			

SAME PERIOD LAST YEAR	Head Count	Avg Weight	Avg Price
Live Steer	418	1,259	64.30
Live Heifer	210	1,162	64.86
Dressed Steer	1,023	803	104.74

000178

Dressed Heifer	1,194	733	104.85

Source: USDA Market News Service, St. Joseph, MO
816-238-0678 email: stjoe.lgmn@usda.gov
www.ams.usda.gov/mnreports/lm_ct100.txt

1100C

BEST ORIGINAL COPY

000179

BEST ORIGINAL COPY

LM_CT100
St Joseph, MO Wed Jul 23, 2003 USDA Market News Service

5 AREA DAILY WEIGHTED AVERAGE DIRECT SLAUGHTER CATTLE - NEGOTIATED
Texas/Oklahoma; Kansas; Nebraska; Colorado; Iowa/Minnesota feedlots
Recap for: Tuesday 7/22/03

Head Count:	236,118	Week Ago:	188,046	Last Year:	5,265
Week to Date	242,580	Week Ago:	206,871	Last Year:	8,124

LIVE FOB BASIS - Beef Breeds

	Head Count	Weight Range	Price Range	Avg Weight	Avg Price
STEERS					
Over 80% Choice	1,179	1,275-1,325	77.00-78.00	1,304	77.86
65 - 80% Choice	6,353	1,175-1,400	75.00-78.50	1,296	77.51
35 - 65% Choice	73,042	1,100-1,375	74.50-78.25	1,262	76.51
0 - 35% Choice	8,843	1,120-1,425	75.00-77.00	1,238	76.22
Total all grades	89,417	1,100-1,425	74.50-78.50	1,263	76.57
HEIFERS					
Over 80% Choice	1,602	1,100-1,250	77.00-79.00	1,187	78.10
65 - 80% Choice	7,373	1,075-1,350	76.00-78.25	1,159	77.20
35 - 65% Choice	54,535	1,020-1,300	74.00-78.25	1,129	76.51
0 - 35% Choice	1,790	1,065-1,175	76.00-77.00	1,120	76.20
Total all grades	65,300	1,020-1,350	74.00-79.00	1,133	76.61

DRESSED DELIVERED BASIS - Beef Breeds

	Head Count	Weight Range	Price Range	Avg Weight	Avg Price
STEERS					
Over 80% Choice	1,785	725-900	122.00-125.00	832	123.89
65 - 80% Choice	8,988	725-905	121.00-124.00	817	123.19
35 - 65% Choice	16,932	725-889	120.00-124.50	819	123.13
0 - 35% Choice	519	784-861	121.00-124.00	839	123.64
Total all grades	28,224	725-905	120.00-125.00	820	123.21
HEIFERS					
Over 80% Choice	942	710-774	120.00-125.00	745	123.07
65 - 80% Choice	4,917	654-813	122.00-124.00	744	123.14
35 - 65% Choice	12,431	675-844	119.00-124.00	752	123.19
0 - 35% Choice	144	730-730	123.00-123.00	730	123.00
Total all grades	18,434	654-844	119.00-125.00	750	123.17

WEEKLY ACCUMULATED	Head Count	Avg Weight	Avg Price
Live Steer	91,260	1,263	76.57
Live Heifer	68,336	1,133	76.61
Dressed Steer	28,267	820	123.20
Dressed Heifer	18,434	750	123.17

SAME PERIOD LAST WEEK	Head Count	Avg Weight	Avg Price
Live Steer	69,442	1,254	73.11
Live Heifer	52,893	1,132	73.21
Dressed Steer	29,823	818	117.71
Dressed Heifer	19,746	750	117.82

SAME PERIOD LAST YEAR	Head Count	Avg Weight	Avg Price
Live Steer	2,027	1,262	63.35
Live Heifer	240	1,191	63.04
Dressed Steer	1,364	854	99.63

000180

Dressed Heifer	308	745	99.59

Source: USDA Market News Service, St. Joseph, MO
816-238-0678 email: stjoe.lgmn@usda.gov
www.ams.usda.gov/mnreports/lm_ct100.txt

1100C

BEST ORIGINAL COPY

000181

BEST ORIGINAL COPY

OLSSON, FRANK AND WEEDA, P.C.
ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220

SENDER'S PHONE: (202) 518-6366
SENDER'S FACSIMILE: (202) 234-3550
SENDER'S E-MAIL: polsson@ofwlaw.com

AM

FACSIMILE TRANSMISSION

July 28, 2003

TO: Dr. Jeremiah Fasano

COMPANY: OFAS/CFSAN/FDA

FAX: (202) 418-3179

FROM: Philip C. Olsson

RE: Application and Destination of Applied LF

REMARKS: The material in the last section of page 2 of this memorandum is responsive to the question raised by Dr. Kahl this am. The memorandum is included in GRN 130 at Tab G, Appendix 3.

THE INFORMATION CONTAINED IN THIS COMMUNICATION IS CONFIDENTIAL, MAY BE ATTORNEY-CLIENT PRIVILEGED, AND IS INTENDED ONLY FOR THE USE OF THE ADDRESSEE. IF YOU HAVE RECEIVED THIS COMMUNICATION IN ERROR, PLEASE NOTIFY US IMMEDIATELY BY TELEPHONE AT 202.789.1212 SO THAT WE MAY ARRANGE FOR RETRIEVAL OF THE DOCUMENT AT NO COST TO YOU. THANK YOU.

THE TOTAL NUMBER OF PAGES INCLUDING THIS PAGE IS __3____. IF YOU DO NOT RECEIVE ALL PAGES, PLEASE CALL MY OFFICE.

BEST ORIGINAL COPY

12200 N. Ambassador Dr.
Kansas City, MO 64163-1244
1-800-449-BEEF

September 23, 2002

Subject: Activated Lactoferrin Application at Farmland National Beef

The following is the normal process that live animals are brought in and the steps that we go through to end up in finished product which is boxed beef, trimmings destined for grind, ground beef, and bone/fat for inedible rendering. This scenario is based upon our facility in Liberal Kansas and is not meant to be an all-inclusive description of the process but rather a brief description. The plant runs 2 eight-hour shifts normally 6-days per week on both slaughter and fabrication floors.

Slaughter Process:

- Live animals are brought into the facility at a rate of approximately 35,000 head per week.
- The animals are humanely stunned and bled.
- The carcasses then are started through the skinning process. The carcasses go through multiple steps where the hide is removed leaving a de-hided carcass. The carcass goes through a series of steam vacuums to remove incidental contamination.
- Once the hide is removed, the carcass goes through a "Pre-evisceration" cabinet where a 2% lactic acid rinse is applied for microbial reduction.
- After the cabinet, the head is removed and the carcass is eviscerated.
- After evisceration, the carcass is split into two equal halves (sides) along the backbone.
- The carcass sides travel along the chain to the final rail inspection where Government FSIS Inspectors visually evaluate the carcass for defects and conduct a "post-mortem" inspection.
- During this whole process knife trimming occurs removing defects.
- After the final rail inspection the carcass sides travel through a Thermal Pasteurization Cabinet (Hot Water).
- They then enter the Activated Lactoferrin Cabinet.
- Next the carcasses enter a house tempered carcass wash.
- The final step on the slaughter floor is a "Final Lactic Acid" rinse before the carcasses enter the HotBoxes for chilling.

Chilling Process:

- Carcasses enter the Hotboxes where they are sprayed with chilled water to start their chilling process. They stay in the HotBoxes for 24 to 36 hours.
- They then are transferred to the USDA Grading area where they are evaluated for Yield, Quality and individual program classifications.
- The carcasses then are transferred to the Sales Cooler where the are sorted by grade and program designation. They normally are transferred to the fabrication floor when the are 3 – 4 days of age (from kill date).

Fabrication Process:

- Carcasses are transferred into the Fabrication floor be grade.
- As the carcasses enter the fabrication floor they are systematically broken down by primal (rounds, loins, ribs, chucks, briskets).

BEST ORIGINAL COPY

- These large primals are put onto main product belts and as they travel down the line are broken into smaller portions. During this process we remove fat, bone, and lean trimmings to meet our finished product specifications.
- The finished product at the end of these tables are then bagged into cryovac bags, sealed, boxed, and sent to our material handling building. This is what we are calling boxed beef.
- The lean and some of the fat that is removed during this process is diverted to side belts and is destined for either in-house grinding for ground beef or to 2000 pound combos for outside customer use (normally grinding or further processing into products like hot dogs).
- The bones and some of the fat (outside surface fat with no lean attached) are diverted to belts destined for our rendering operation.

The following is a summary of a yearly average of individual weights. A yearly average (fiscal year 2002) was used to account for the seasonal variations of carcass weights).

Average Live Animal Weight:	1,245 Lbs.
Average Dressed Carcass Weight:	799 lbs. (average side weight 400 lbs.).
Average Trim/Grind Weight	235 lbs./head
Average Boxed Beef Weight	415 lbs./head
Average Bone/Fat Weight to Rendering:	149 lbs./head

Projected Amount of Surface Coverage Activated Lactoferrin (where the treated surface ends up – the exogenous Lactoferrin surface distribution):

- When Activated Lactoferrin is applied on the carcass side in slaughter, the surface that is treated consists of the internal cavity, and the external fat surface.
- The internal cavity consists of bone and tissue that normally go to rendering.
- The remaining surface area is trimmed down on the fabrication floor to meet our customer specifications and the pure fat with no lean attached is also sent to rendering.
- Trim that goes to our in-house grind or that goes to out-side grinders consists of all the trimmings removed from the carcasses (both internal and external fat/lean).
- Most of the finished box beef produced is trimmed to a maximum ¼" fat cover or less.
- With this in mind, we have determined that approximately 40% of the original treated surface goes to rendering, 10% remains on boxed beef, and 50% goes to the in-house grind/out side grind sales.

AM

OLSSON, FRANK AND WEEDA, P.C.

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA(1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SUSAN P. GRYMES
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE M. BRIGGS*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

July 29, 2003

BY ELECTRONIC MAIL

Dr. Jeremiah Fasano
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: Slaughter Sequence and Carcass Wash

Dear Dr. Fasano:

Yesterday I supplied a September 23, 2002 National Beef memorandum to you and Dr. Kahl for the purpose of demonstrating the application and eventual destination of activated lactoferrin to beef carcasses. Upon further review of that document, I notice that it contains an additional point which I would like to bring to your attention.

The last three items under the heading "Slaughter Process" are as follows:

- They [the carcasses] then enter the Activated Lactoferrin Cabinet.
- Next the carcasses enter a house tempered carcass wash.
- The final step on the slaughter floor is a "Final Lactic Acid" rinse…

In our recent discussions of the process of application for activated lactoferrin, I had failed to focus on the tempered water (85-90°F) carcass wash, which occurs immediately after the carcasses leave the Activated Lactoferrin Cabinet and prior to the Final Lactic Acid rinse. This tempered water carcass wash should probably be referenced in the statement of conditions of use.

In connection with Dr. Kahl's suggestion that aLF Ventures consider re-wording the conditions of use for GRN 000130 as follows:

> "Used as a component of an antimicrobial spray that would deliver one gram lactoferrin (LF) to dressed beef carcasses, followed by a washing with a lactic acid solution, resulting in less than 800 micrograms LF per kilogram of edible beef."

we suggest that the conditions of use be stated as:

> Used as a component of an antimicrobial spray that would deliver one gram lactoferrin (LF) **per carcass** to dressed beef carcasses, followed by a washing with **tempered water and** a lactic acid **rinse**, resulting in less than 800 micrograms LF per kilogram of edible beef. **(Bold type added to show changes.)**

I trust that this information is helpful. If we can provide additional information, please let me know.

Sincerely,

Philip C. Olsson

PCO.lss

REPLY TO JEREMIAH FASANO, FOOD & DRUG ADMINISTRATION

I wish to reply to your email inquiry received on July 31, 2003 regarding the digestibility of lactoferrin. This issue has arisen as part of the FDA review of GRN 130.

In your message, you make references to several different notices on lactoferrin. Please know that the GRAS Panel has been comprised of the same members during all of this time so that the Panel members are aware of all of the information that has been shared with the FDA. Therefore, the deliberations on GRN 130 build upon the earlier deliberations of the Panel and include consideration of all of the scientific information that has been made available to the Panel throughout the various petition processes. The Panel has seen and reviewed all information that was presented to FDA.

In answer to your first question, the Panel was very much aware of the existing scientific information on digestibility and thermostability of bovine lactoferrin. This information was primarily discussed during our deliberations on GRN 42 but this information was also considered in the context of our deliberations on GRN 130. The scientific information on the digestibility and thermostability of bovine lactoferrin was not considered by the experts to be a serious concern with respect to GRN 130. As I have stated before, the Panel reached its conclusions primarily on a comparison of endogenous vs. exogenous levels of bovine lactoferrin from this particular application. In this context, all other considerations became secondary although they were obviously considered. Furthermore, this issue was raised and discussed in the July 11 meeting with FDA and the Panel offered their opinions at that time that this was not an important consideration for GRN 130.

In answer to your second question (part 1), infants and young children have the highest prevalence of milk allergy, while adults with milk allergy are rarely encountered, though quite well described in the medical literature. The Panel considered all age groups in its deliberations. Certainly, young children eat beef, especially ground beef and all age groups were considered in the exposure assessment for bovine lactoferrin from this particular application. The exposure of very young infants to beef is probably minimal but they were not ignored in our considerations.

The scientific evidence indicates that bovine lactoferrin does survive digestion to some limited extent in young infants. When bovine lactoferrin is administered to young infants, some residual lactoferrin is detected in the feces. The Panel was aware of the information on that issue presented to FDA in the earlier petitions. However, several facts led the Panel to conclude that this was not an important consideration. First and foremost, the digestibility of bovine lactoferrin is substantially enhanced if the lactoferrin is heated. Beef products will mostly be ingested after cooking and the lactoferrin will be subjected to heat especially since it is applied to the surface of the beef carcass. Furthermore, especially with young children, exposure to beef is primarily through very

well cooked products (baby food products). Second, the efficiency of digestion of bovine lactoferrin is greater in older children and adults even in the unheated state. So, young infants would appear to be the only group which apparently may be limited in their digestion of lactoferrin. Even in such young infants, the limited digestion of bovine lactoferrin seems to be restricted primarily to consumption of unheated lactoferrin at high levels. The limited digestion of bovine lactoferrin appears to be transitory and limited to the first 4 months of life when the infant gut and its functions are immature and ingestion of beef is likely to be extremely limited. In early considerations of DMV International to add lactoferrin to infant formula, the process would have required the aseptic addition of the lactoferrin to the infant formula after the sterilization operation because of considerations regarding the thermostability and digestibility of heated lactoferrin.

In answer to your second question (part 2), the detection of anti-lactoferrin IgE in milk-allergic individuals occurs only on a rather rare basis. The clinical significance of these anti-lactoferrin IgE antibodies is not well documented as we have already indicated previously. Presumably, anti-lactoferrin IgE antibodies are indeed formed early in life in response to the ingestion of milk during a stage when bovine lactoferrin is not as efficiently digested (even though one would presume that most milk fed to young infants has been heat-treated in some fashion; obviously, some lactoferrin must survive digestion in order to provoke formation of the IgE antibodies).

Hopefully, I have provided answers to your questions.

AM

OLSSON, FRANK AND WEEDA, P.C.

PHILIP C. OLSSON
RICHARD L. FRANK
DAVID F. WEEDA (1948-2001)
DENNIS R. JOHNSON
ARTHUR Y. TSIEN
JOHN W. BODE*
STEPHEN D. TERMAN
MARSHALL L. MATZ
MICHAEL J. O'FLAHERTY
DAVID L. DURKIN
NEIL F. O'FLAHERTY
PAMELA J. FURMAN
BRETT T. SCHWEMER

*PRACTICE WITHIN THE DISTRICT OF COLUMBIA IS LIMITED TO MATTERS AND PROCEEDINGS BEFORE FEDERAL COURTS AND AGENCIES.

ATTORNEYS AT LAW
SUITE 400
1400 SIXTEENTH STREET, N.W.
WASHINGTON, D.C. 20036-2220
(202) 789-1212
FACSIMILE (202) 234-3550

TISH E. PAHL
ROBERT A. HAHN
NAOMI J. L. HALPERN
STEPHEN L. LACEY
SHARON D. BROOKS
RYAN W. STROSCHEIN
EVAN P. PHELPS
VALERIE B. SOLOMON*

OF COUNSEL
JUR T. STROBOS
JACQUELINE H. EAGLE
KENNETH D. ACKERMAN
MARK L. ITZKOFF

03-08-13P06:10 RCVD

August 7, 2003

BY ELECTRONIC MAIL

Dr. Linda S. Kahl
Food and Drug Administration
Center for Food Safety and Applied Nutrition
1110 Vermont Avenue, N.W., Room 1119, (HFS-255)
Washington, D.C. 20201

Dr. Jeremiah Fasano
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dr. Michael J. DiNovi
Food and Drug Administration
Center for Food Safety and Applied Nutrition
1110 Vermont Avenue, N.W., Room 705 (HFS-255)
Washington, D.C. 20201

Re: GRN 130 – Non-detectable Proteins

Dear Drs. Kahl, Fasano and DiNovi:

You have requested additional information regarding the absence of four allergenic milk-derived proteins. These proteins are alpha-lactalbumin (α-La), beta-lactoglobulin (β-Lg), caseinmacropeptide (glycomacropeptide: GMP), and bovine serum albumin (BSA). For the reasons set forth below, aLF Ventures is confident that none of these proteins is present at a detectable level.

1. The process is based on selective extraction of lactoferrin from a mixture of whey proteins. The proteins of concern are not extracted by their nature; i.e. their ionic charge is opposite

to lactoferrin. Furthermore, if some of these other proteins were to remain on the ion exchanger column at the end of the adsorption cycle, they would be washed off in one of the washing steps before lactoferrin is recovered from the column.

2. High-pressure liquid chromatography (HPLC) is the accepted standard technology for evaluating the presence and quantity of bovine milk-derived proteins. An HPLC chromatographic analysis of aLF Ventures' lactoferrin product showed no detectable amounts of the four proteins of concern. For chromatographic resolution, the test was conducted using lactoferrin at a level of 1000 milligrams per liter (Appendix IV, Table 2) with a detection limit of 10 milligrams per liter. A separate test was then conducted using standards for each of the four proteins of concern. Standards for two of these proteins were prepared at concentrations of 500 milligrams per liter and for the other two at 800 milligrams per liter (Appendix IV, Table 2) and analyzed with a detection limit of 10 milligrams per liter. The resulting chromatograms are set forth in the overlay (Appendix IV, Figure 1) demonstrating that the proteins of concern were not detectable in the lactoferrin analysis. With its test sensitivity of 10 milligrams per liter, the HPLC analysis would have detected a protein of concern if that protein had represented 10 milligrams or more of the 1000 milligrams of lactoferrin in the test solution. The results of the HPLC evaluations of aLF Venture's lactoferrin product are consistent with expectations based on the nature of the lactoferrin production process. This HPLC evaluation was conducted to determine whether these proteins are present in the product in a qualitative sense and was not designed to measure concentrations of these proteins below this level of detection. This test was not conducted for the purpose of setting a specification, but for the purpose of demonstrating the absence of the proteins of concern for the benefit of the expert panel. That panel reviewed the HPLC data and was satisfied that even if the non-detectable milk-derived proteins were to be present below the HPLC detection limit, those levels would not create allergenic concerns for this product and this use.

Sincerely,

Philip C. Olsson

PCO.lss