GR

ORIGINAL SUBMISSION

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Proliant Inc.

REC'D MAY 23 2005

Memo

TO: Dr. Robert L. Martin, Center for Food Safety and Applied Nutrition

FROM: Pam Mercer — Administrative Assistant, Quality Assurance

DATE: May 16, 2005

RE: GRAS Petition for Proliant Inc.'s Spray-Dried Bovine Globulin

cc:

Enclosed you will find (4) copies of our GRAS Petition.

Although I did not use colored notebooks as recommended in petition preparation information, I did color code the covers of the notebooks using the coding system recommended and hope this assists in distribution.

Please contact me by telephone at 515/289-5133, by fax at 515/289-4363 or by email at pamela.mercer@proliantinc.com with any questions or if additional information is necessary.

Thank you for your assistance in this matter.

:pjm
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Enclosures (4)

Proliant
HEALTH INGREDIENTS
The Art of Service.
The Science of Protein.

RECD MAY 23 2005

May 16, 2005

Dr. Robert L. Martin
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740-3835
Phone: 301/436-1219

RE: GRAS Petition Submission for Proliant Inc. Spray-Dried Bovine Globulin

Dear Dr. Martin:

We are pleased to enclose Proliant Inc.'s petition for GRAS self-affirmation for our Spray-Dried Bovine Globulin for your review.

After your December 2004 meeting with Mr. Mel Vanden Berg, our Vice President of Quality Assurance, we revised the petition by offering an Executive Summary outlining the historical perspective of the development and the acceptance of the product into the feed and food industries.

We submitted our GRAS petition for peer review to Dr. Paul J. Flakoll, Ph.D. and Dr. James E. Pettigrew. Both men are experts in their field and have years of experience regarding the use of our product. Their review letters are part of the petition.

Proliant Inc.'s Spray-Dried Bovine Globulin is presently positioned as a food additive or nutraceutical/nutritional supplement and is therefore exempt from the

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pre-market approval requirements of the Food, Drug and Cosmetic Act because Proliant Inc. has determined that its use in such products is GRAS.

Please contact either Mr. Mel Vanden Berg or me at 515/289-5100 if you have any questions concerning the content of this petition.

Best regards,

(b)

Bonnie J. Humm
Director of Quality Assurance

BJH:pjm
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Enclosures (4)

REC'D MAY 23 2005

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EXECUTIVE SUMMARY

SPRAY-DRIED BOVINE GLOBULIN GRAS PETITION

The basis of our self-affirmation of GRAS for our spray-dried bovine globulin has been determined through years of supplying this protein for use in human food and animal feeds throughout the world. More recently, in the last 8 years, we have established a large acceptance and market for feeding young calves. This successful application for blood products in young animal diets resulted in the introduction of blood products into human dietary supplements and foods. Extensive studies exist on the benefits of feeding blood proteins to young animals that demonstrate both the increased growth of the young animals and provide a long history outlining the safe use of these products.

APC, Inc., the technical division of the Lauridsen Group Inc. and sister company to Proliant Inc., initially produced our blood products believing that they would simply be an alternative source of proteins for the feeding of animals. However, after only a limited number of feeding studies it became immediately evident that young swine animals remained healthy during their early growth period and showed significantly higher feed conversion and growth rates when consuming our product. More importantly, to date APC has had no documented reports of the products having a negative effect on the health of young swine or bovine animals.

Plasma Use in Porcine Animals

Since this revelation, and over the past 10 years, spray-dried plasma has been used in the diets of over 95% of all newly weaned pigs in the United States. Since 1994 over 900 million pigs have consumed plasma. The total consumption of plasma in young swine in 2004 was 20-25 million pounds. APC, Inc. produces 80-85% of the United States' spray-dried plasma supply. The average percentage of plasma in the newly weaned pigs' diet is 3-10% resulting in a total daily intake of 24 grams of plasma, at 15% IgG, per

[redacted]

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animal per day or 3.6 grams of IgG per day. The average percentage of serum globulin in this plasma product is between 15% to 20%.

Although our spray-dried plasma costs the producer as much as 10 times the cost of other ingredients (Table 1), its use is considered by many producers to be essential for the raising of swine in confinement production.

Table 1

COST COMPARISON * (5 TO 10 YEAR AVERAGES)	
Soy Meal – 48% Protein	10 – 12 cents per pound
Fish Meal – 62-65% Protein	15 –25 cents per pound
Spray-Dried Plasma – 70% Protein	$1.35 - $1.80 per pound

* Information for Table 1 from historical sales records

Throughout the 20 years that plasma has been utilized in the feeding of swine, numerous published documents have appeared regarding the value of spray-dried plasma in swine production. More important to this notification is that while many of these publications mention the positive effects of plasma, few ever mention a negative effect on an animal that was traced to this feed ingredient. We have provided several of these studies as references (Section 9.0). While many of these references do not mention morbidity or mortality, the absence of any information implies that there were no observable incidents that would suggest illness or death resulting from the feeding of plasma. None of these articles, nor any of the others we have found, reveal adverse health issues with the animals, but instead showed

[redacted]

only improvements in nutritional performance. Throughout APC, Inc.'s experience of producing plasma, there has never been an issue brought to our attention where our plasma products have jeopardized or caused an issue with the health of swine during the nursery stages.

Plasma Use in Bovine Animals

In more recent years, APC, Inc. began to explore the benefits of feeding plasma and more specifically, bovine serum globulin, as a colostrum supplement to young bovine animals. The feeding of our serum globulin to newly born bovine animals has been shown to provide an alternative to colostrum and is being suggested by industry and university experts as a possible means to break the Johnes' cycle of maternal transmission via colostrum/milk (Section 9.0, Tab A).

During the past 10 years, APC, Inc. has experienced only one documented incident in which a calf feeder claimed that the feeding of our product resulted in the death of calves. After a thorough investigation by APC, Inc. and tests results of the APC feed by recognized outside laboratory experts, it was concluded that the animals did not die as a result of consuming our feed (Section 9.0, Tab B). Poor sanitary conditions in the environment were more likely to blame.

A newborn calf weighing 75 pounds would be fed one pound of plasma in two liters of water as a colostrum supplement. Using 15% IgG plasma, this would provide 68.1 grams of Immunoglobulin (2 g/kg body weight). It should be emphasized here that newly born calves have an "open gut" that allows the serum globulin to directly enter the blood stream. This results in direct means of correcting the problem of failure of passive immunity when colostrum is insufficient in providing the necessary IgG to the calf. The data provided in this petition clearly demonstrates not only the safety of our product, but also the positive impact of improving the IgG levels in newly born calves (Section 9.0, Tab C).

Previously we have been discussing the extensive experiences and scientific data we have accumulated on the feeding of plasma and serum globulin in young animals. The consumption of blood and blood derivatives also has a

long history in man's diet. Proliant Inc., the edible division of Lauridsen Group, Inc. and sister company to APC, Inc., has manufactured spray-dried bovine plasma for use in human food for over 8 years.

Blood Plasma Use in Humans

Biblical references describe the drinking of animal blood. Blood sausage has been consumed by humans for over 2,000 years and is still a common commodity in France and other European countries. Recipes for blood sausage indicate that the sausage consists of 33 to 50% whole blood (Section 9.0, Tab D). If the blood sausage contains 33% blood, the percentage of globulin in the sausage would be 4.18%. The consumption of globulin would be the following per listed serving size:

- 4 ounces of blood sausage = 1.56 grams of globulin per 4 oz. serving
- 6 ounces of blood sausage = 2.34 grams of globulin per 6 oz. serving

Proliant Inc. has sold spray-dried plasma into the surmimi industry for 10 years with a total of 40 to 50 million pounds being consumed in the Asian and European markets. More recently, Proliant Inc. has manufactured bovine serum globulin for use in the neutraceutical industry. The recommended dose is 75mg/kg body weight with a range in application of 37.5 to 125 mg/kg body weight. Daily dosage for a child would be 1-2 grams; daily dosage for an adult (150 pounds) would be 2-5 grams.

Regulatory Status

The USDA/FSIS permits animal blood as an ingredient in meat food products as found in Title 9 CFR 318.6(b)(7). Further, USDA/FSIS in their *Food Standards and Labeling Policy* book lists blood, blood and tongue sausage, blood pudding, and blood sausage specifying the restrictions and conditions for the product and product labeling (Section 9.0, Tab E). There are no restrictions as to the quantity of blood used in these products other than they would have to contain some of the other ingredients which are listed in the definition.

Human Studies

Additionally, several adult studies and child feeding trials have been conducted to research the potential benefits of consuming bovine blood derivatives. In the Lembcke study, up to 50% of the control sample milk

protein was replaced with bovine serum concentrate (BSC) (Section 9.0, Tab F). Conclusion of the Lembcke study: "Each of the diets was well accepted by the study children and there was no evidence of any adverse effects of bovine serum concentrate." In another study, children were fed 3.1 grams of IgG per day for up to 8 months, or up to 8% IgG in their milk and rice diet (Section 9.0, Tab G). This unpublished study provides significant data to support the premise that spray-dried serum consumption by young children is similarly safe as that found in our animal studies.

We are also providing additional unpublished data on human studies supporting the previously mentioned conclusions with regards to human safety (Section 9.0, Tabs H and I).

Allergens

Neither meat nor blood products are listed in the U.S. nor EU as one the top 8 major food or food groups considered to be allergens: milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans (Section 9.0, Tab J). Therefore Proliant Inc. does not consider this bovine product to be allergenic or problematic. Since the term bovine or beef is carried through on all labeling, the consumer would have proper information for decision making, whether cultural or dietary in nature.

Bovine Spongiform Encephalopathy (BSE)

Proliant Inc. is not concerned about the transmission of BSE from our blood products because all of the blood is derived from animals slaughtered in U.S. slaughter facilities under the continuous inspection of USDA/FSIS. All animals have been passed as fit for human consumption. Both ante- and post-mortem inspections are conducted on each animal at the time of slaughter. The collection of the blood occurs before the head, hide, spinal column, or any other Specified Risk Materials (SRMs) are removed from the carcass.

In addition, international agencies do not consider blood to be a tissue of risk with regard to the transmission of BSE. Even in a country or zone with high BSE-risk, the importation and trade of blood products is not recommended to be restricted (Section 9.0, Tab K).

General Safety Concerns

First we would like to confirm that only USDA/FSIS-inspected and approved edible beef plasma is used in the production of our globulin product, ImmunoLin®. Proliant Inc.'s ImmunoLin® is manufactured exclusively from the bovine plasma of North American cattle. These animals have been slaughtered in USDA/FSIS-inspected and approved facilities where both ante- and post-mortem have been performed. These animals must pass both ante- and post-mortem inspection to be acceptable for human consumption.

To date, no native-born cattle in the United States have been found that have the disease Bovine Spongiform Encephalopathy (BSE). Only one case of BSE has been found in an imported animal from Canada in December 2003. The United States has an extensive BSE surveillance program in which more than 263,115 animals having neurological symptoms or other signs, suggesting the animal has BSE, have been tested by either histopathology or immunohistochemistry. Only the one imported animal, from Canada, was found to have BSE.

The tissues from BSE-infected cattle known to be the most infectious are those of the central nervous system. The new USDA/FSIS mandated program for meat for human consumption, and therefore the plasma collected from these animals, requires all SRMs (Specified Risk Materials) be removed from the carcass before processing. These extraordinary measures also help assure the safety of our product. The tissues used as the raw material for our ImmunoLin® product, namely whole blood, are deemed safe since they are not those known to harbor the infective agents of BSE. The global scientific community does not consider meat, blood and milk as risk tissues in regards to BSE, and therefore their consumption as food is not restricted.

Heavy Metals

Throughout the 20 years of manufacturing plasma for both human and animal consumption, standard quality testing has never revealed any increases in heavy metals or lead due to our processing methods. Analysis

of our ImmunoLin® product has also not revealed any increases in heavy metals. Test results can be found in Section 9.0, Tab L.

Pesticides

The USDA routinely tests for pesticides and their data reveal only occasional incidents of levels found above U.S. tolerances. Since the blood of a single animal is commingled with that of more than 1,800 other animals during a single day of collection, there would be little chance of the blood or the plasma having levels in excess of tolerances. If an animal ingested feed containing high levels of pesticides, assuming the presence of the pesticide could be detected in the blood, the dilution factor would reduce the level to below the detection limits of most laboratory testing equipment.

Antibiotics

The USDA Food Safety and Inspection Service (FSIS) has a residue program in operation to assure that antibiotics and other substances do not contaminate meat products. Since USDA oversees the collection of the blood and transportation of the plasma to our facilities, we would be notified of any problems and could retain the liquid plasma or spray-dried powder for residue testing prior to release for sale. As you may know, there is no such inspection system or residue program in place for the approval or testing of colostrum products. We have an additional margin for safety in ImmunoLin® because of the dilution factor and the method of processing. The plasma from more than 1,800 beef cattle is used to produce a single lot of ImmunoLin® powder. In the event that a single animal would test positive for a residue, the 1,800-fold dilution would reduce the residue to below the detection limits of our most sophisticated equipment.

Hormones

Recombinant Bovine Somatotropin (rBST) is not approved for use in beef cattle in the United States. Recombinant BST is only approved for use in U.S. dairy cattle. Proliant Inc.'s ImmunoLin® is only sourced from beef cattle.

Microbiological Hazards

In addition, Proliant Inc. utilizes a dramatic change in pH and dialysis in the processing of ImmunoLin®. The pH change, dialysis and spray drying serve to improve the microbiological characteristics of the product.

Peer Review

This document has been peer-reviewed by Dr. Jim Pettigrew, Ph.D. of the University of Illinois, Urbana, Illinois and Mr. Paul Flakoll of Iowa State University, Ames, Iowa with no adverse comments with regard to the safety of our product. A copy of their comments can be found in Section 9, Tab M.

The following chapters of this GRAS self-affirmation/petition request cover the required topics of concern in greater detail.

Bibliography

We have included a bibliography of related and supporting documents at the end of this petition (Section 10.0). Additional documents are on file at Proliant Inc. and copies of all referenced documents can be obtained by contacting Proliant Inc.

Proliant

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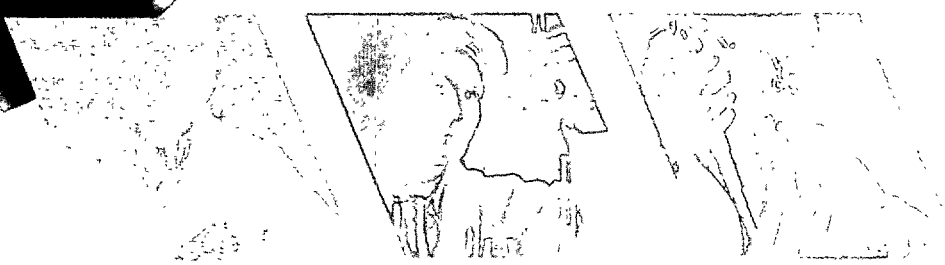
The Science of Protein.



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Proliant Inc. manufactures and markets protein products for the food, nutrition, human health, nutraceutical, diagnostic, life science research, biopharmaceutical and veterinary industries.

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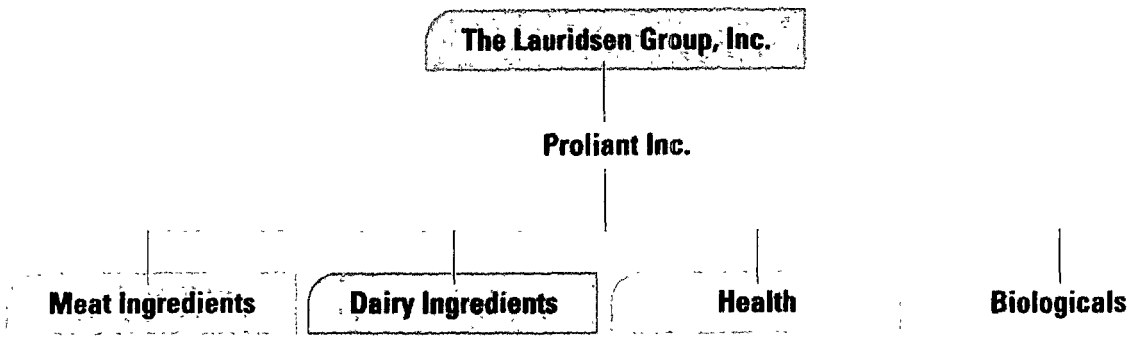
company

Proliant is committed to providing high quality products, exceptional service and advanced technology to our customers. Through careful management and exacting product standards, we create consistent products and long-term relationships that are world-renowned. Proliant has offices in the United States and Europe and an extensive global network of distributors. Proliant Inc. has four divisions, each focusing on specific market segments:

- **Proliant Meat Ingredients** — serving the food industry, including the meat, savory, prepared foods and snack markets.
- **Proliant Dairy Ingredients** — serving the food and nutrition industry, including the nutrition, dairy, bakery, confection, snack, meat and savory food markets.
- **Proliant Health** — serving the health and nutrition industry, including the dietary supplement, clinical nutrition, consumer health and functional food markets.
- **Proliant Biologicals** — serving the diagnostic, life science research, biopharmaceutical and veterinary markets.



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company

Proliant's success can be attributed to the company's ability to convert significant research and development into consistent quality commercial products.

1981 — The roots of our company go back nearly a century to Danish immigrant Hans Lauridsen, who came to Iowa to set up a butter and egg business.

1984 — Hans' son, Wally Lauridsen, and Wally's son, Nix, evolved the company into meat processing, then to related proteins with the founding of American Meat Protein Corporation, based in Lytton, IA.

1987 — Expansion of the Lytton plant is completed and manufacturing of meat stocks and broths begins. Construction is completed at the Lytton, IA facility and the production of beef plasma and edible red cells begins.

1989 — Construction is completed on the new company headquarters located in Iowa State University's Research Park in Ames, IA. Company headquarters is moved to a temporary location in Ames, IA to take advantage of Iowa State University's world-famous meat laboratories and food science research facilities.

1991 — American Meat Protein Corporation continues to grow, forming a joint venture with Hilmar Cheese Company of Hilmar, CA. This partnership adds a range of dairy protein products, including whey protein concentrate, to the company product line.

1992 — American Meat Protein Corporation shortens its name to AMPC, Inc. in recognition of the broader product offering. The corporate headquarters is expanded, allowing for growth in all areas of servic and personne

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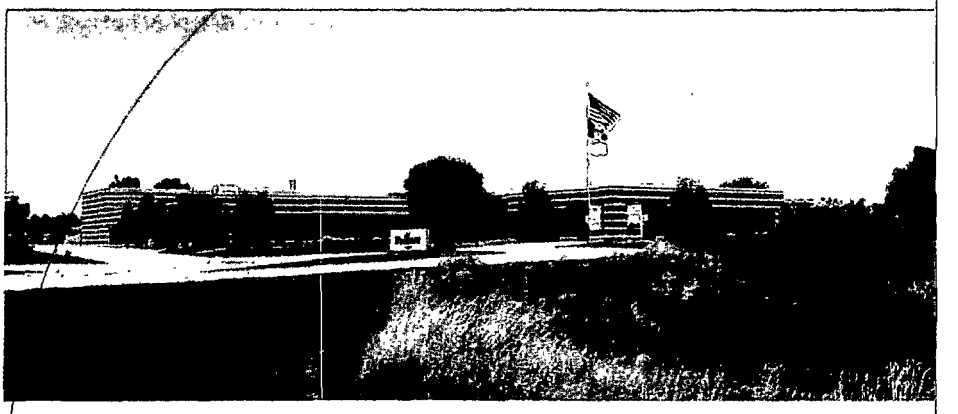

1993 — The manufacturing facility in Fremont, NE is completed and beef plasma manufacturing is switched from the Lytton, IA facility.

1995 — AMPC establishes a Fractionation Research Group for developing purification processes for proteins.

1995 — Serum production begins at Fremont, NE facility.

1996 — AMPC extends its product line to include lactose from its partnership with Hilmar Cheese Company.

1996 — An 11,000 sq. ft. R&D facility is completed at the Ames, IA headquarters. It houses eight separate laboratories and a pilot plant.

1997 — AMPC acquires Aprocat, a meat protein company in Granollers, Spain, and opens its European headquarters office.

1998 — The manufacturing facility in Harlan, IA is purchased and begins production of dehydrated functional meat proteins.

1998 — LG Laboratories is established, focusing on human health and nutrition.

1999 — AMPC Biologicals is established to develop, manufacture and market high-purity plasma fractions.

2000 — AMPC, Inc., including its Food Products and Biologicals divisions, and LG Laboratories, renamed Proliant.

2002 — To accomodate growth, Proliant Ingredients Division divides to become the Meat Ingredients and Dairy Ingredients Divisions.

2002 — A new state-of-the-art 24,000 sq. ft. Biologicals plant is constructed in Boone, IA for increased production capacity.

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the

customer partnerships, driven by service, grounded in innovative applied protein sciences.

When customers use our products, they receive unmatched quality and the benefits of excellence in service:

Total Quality Assurance

We believe one of the best ways to earn the confidence of customers is through delivery of products that meet, or exceed, even the most stringent quality standards. Quality raw material sources, USDA inspection, state-of-the-art equipment and complete dedication by our manufacturing facility personnel assure consistent, reliable protein products. Proliant's QA/QC Department implements rigorous testing procedures throughout the entire product handling and distribution process, to ensure that all products meet specification requirements.

Technical Sales Representatives

Proliant's technical sales staff works with our skilled research and applications scientists to make sure our customers get the products that best fit their unique needs. Our sales people help customers with formulation and marketing challenges, coordinating on-site research and providing insight on industry trends. Our sales representatives and distributors are located around the globe providing unsurpassed service.

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Responsive Research and Development Department

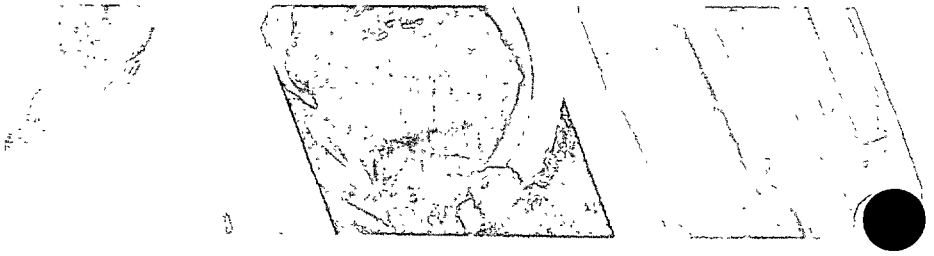
Proliant sees research and development as the gateway to helping our customers add value to their products. R&D scientists develop new specialty ingredients, improve existing products and respond to specific customer needs. Our 11,000 square-foot corporate R&D facility houses eight labs and a pilot plant, which are used to develop and test products for our customers on site. Additionally, Proliant has strong technical ties to several leading universities which give us access to additional resources to compliment our in-house capabilities. The Proliant approach to R&D reflects our commitment to growth through development and use of new technologies, resulting in genuine added value to our customers' products.

Innovative Applications Assistance

Proliant's applications scientists assist customers with their formulation and processing needs. With technical backgrounds in the industries they service, our application team have a practical understanding of the challenges and opportunities our customers face. Our applications scientists are ready to assist with on-site formulation work, applications seminars specific to customer needs, and offer in-plant visits.

Outstanding Customer Service/Logistics

The focus of our customer service/logistics department is to continually provide quality service by exceeding the expectations of our customers. Proliant's customer service professionals take special care to ensure that they have a complete understanding of specific order requirements. Product delivery is managed by our team of logistics professionals who specialize in prompt and dependable service.



proliant division

Proliant Meat Ingredients Division assists leading food developers on every continent with their ingredient needs. We offer more than 20 years of experience in the development of specialty meat protein ingredients, their practical application, and the continuous improvement of a broad variety of existing protein products. Our ingredients are ideal for meat, savory, prepared foods, and snack applications.

Proliant's line of ingredients provides several features and benefits, including improved flavor, increased functionality, added nutritional value, improved texture, increased cook yield, while reducing drying time, formulation costs, and purge. Our ingredients not only improve products; they improve the bottom line. Our ingredients include:

- Stocks, Broths, Extracts
- Functional Meat and Poultry Proteins
- Flavor Enhancers
- Savory Flavors
- Animal Fats
- Other Specialty Products

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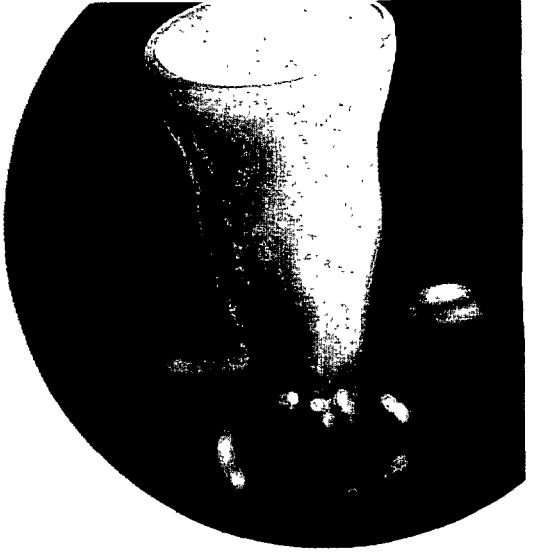

proliant division

Proliant's Dairy Ingredients Division provides specialty dairy proteins and assists with practical applications to a multitude of food industries worldwide. With an intense commitment to specialty protein ingredients, Proliant formed a joint venture with Hilmar Cheese Company of Hilmar, California, in 1991. Hilmar Cheese Company provides the high quality raw materials and state-of-the-art manufacturing for our whey protein concentrates, whey protein hydrolysates, lactose and specialty protein ingredients. These ingredients provide several features and benefits, including enhanced flavor, increased functionality, texture modification, and added nutritional values for dairy, bakery, nutrition, confection, snack, meat and savory applications.

- Whey Protein Concentrates
- Whey Protein Hydrolysates
- Lactose
- Specialty Proteins

Hilmar Cheese Company was founded in 1984 by Jersey dairy farmers from the Central Valley of California. It is the largest integrated cheese, whey and lactose production operation on a single site in the world, and it's still growing. The partnership between Hilmar Cheese Company and Proliant results in a synergy that provides customers with quality products and exemplary service.

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proliant

division

Proliant's Health Division specializes in development of ingredients from natural sources for the health and nutrition markets. We use exciting new technologies to concentrate or isolate bio-active proteins and peptides that play vital roles in human health. The most well-known of these are immunoglobulins.

ImmunoLin®, immunoglobulin is designed for the dietary supplement industry. ImmunoLin® provides immediate immune support quickly and effectively. It is suitable for use in tablets, capsules, drink mixes, lozenges and bars.

Our ingredients are unsurpassed in innovation and quality, which allows them to be used in the most sensitive nutritional applications. Our products include:

- Immunoglobulin concentrates
  - ImmunoLin®
  - ImmunoLin®-WP
  - NutraGammax™
- Heme iron concentrate
  - VitaHeme™

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proliant division

Proliant's Biologicals Division manufactures bovine serum albumin (BSA, also known as Fraction V), lipids, plasma diagnostic matrices and purified immunoglobulins for diagnostics, research and cell/microbial culture. Proliant is the only manufacturer of bovine plasma fractionation products to control the process from collection to final product, all subject to USDA regulations and inspection. Our products are manufactured in a state-of-the-art, GMP plant using a closed system to maximize reproducibility and ensure purity.

Traceability is of primary concern to facilitate customer regulatory requirements. Our raw material is derived exclusively from Proliant-controlled collection sites in the mid-west of the United States. The European Directorate for the Quality of Medicines (EDQM) has recognized Proliant's high level of traceability by granting our BSA a TSE Certificate of Suitability for minimizing the risk of transmission of BSE.

Our products include:

- Bovine Serum Albumin
  - –Standard Grade
  - –Reagent Grade, Fatty-Acid Free
  - –Culture Grade
  - –22% and 30% Solutions, isotonic or water-based

  All albumins are available as low endotoxin, low IgG and protease-free preparations.
- Bovine Lipoprotein Fraction
  For the culture of hybridoma, myeloma, CHO and other lipid-dependent cells in serum-free media
- Bovine Transferrin
  For the culture of hybridoma, myeloma, CHO and other lipid-dependent cells in serum-free media
- Bovine IgG
- Plasma Diagnostic Matrices
  For the replacement of human-based calibrators and controls
- Custom plasma or serum-derived products

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APC

Nutritional Health-Innovative Research-Natural Products

PLASMA PROTEINS

Manufacturing, Quality, Benefits and Uses

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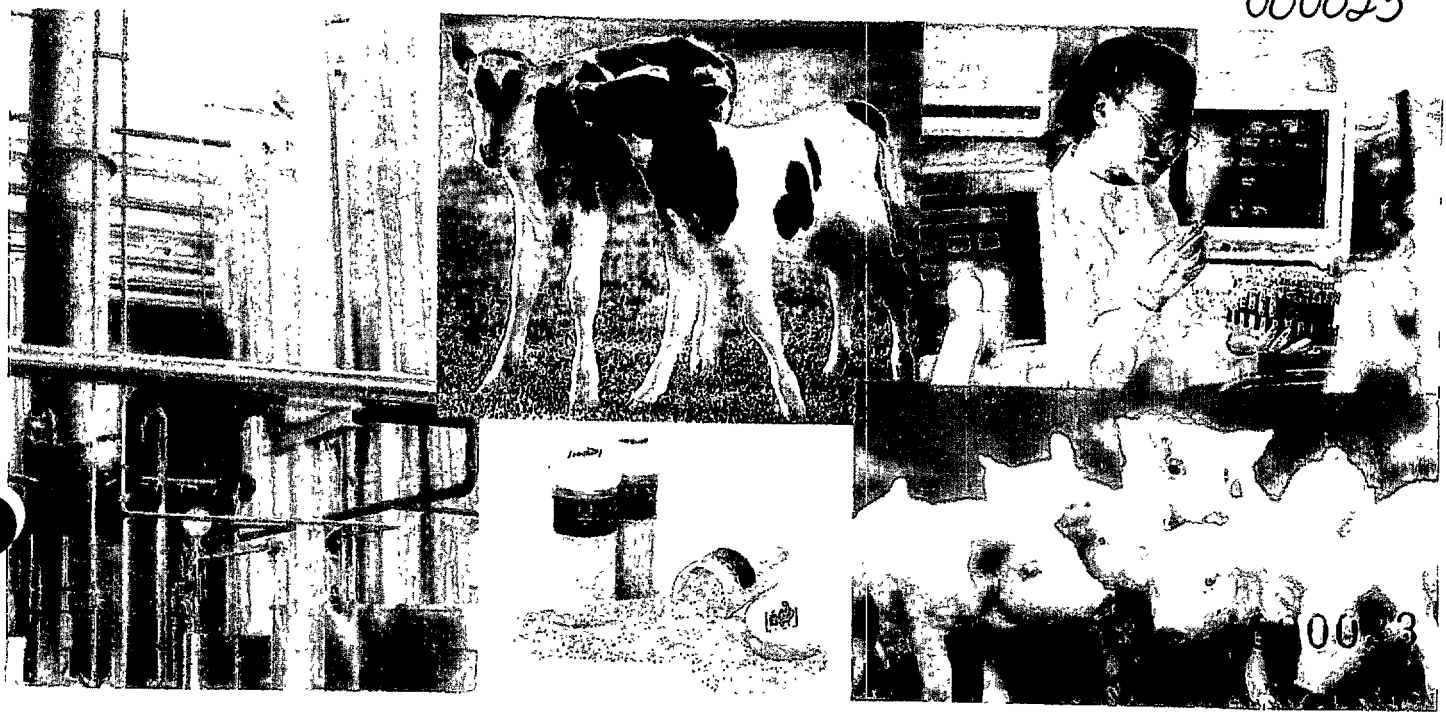


100 % NATURAL PRODUCTS

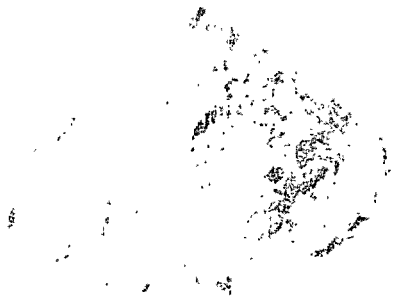
Introduction

During the last 15 years APC Spray Dried Plasma Proteins have been efficiently used as feed ingredients due to their high nutritional value and health supporting properties. The discovery of Spray Dried Plasma started in 1987 due to results from research conducted at Iowa State University, later confirmed by many research institutions worldwide.

Since then Spray Dried Plasma has proven to be **the best protein source for weaning pigs.** Spray Dried Plasma Proteins increase feed intake and growth consistently when compared to all other traditional proteins including milk, fish meal, potato protein and whey concentrates. It also decreases diarrhoea incidence and medication costs. All these aspects contribute to make pig production more profitable.

Plasma is a 100 % natural source of functional and bioactive proteins

Recently, there has been much scientific interest in the physiological as opposed to just "nutritional" properties of foods and human food. It is now well established that proteins and peptides arising from digestion of food and feed proteins can have specific biological activities. A new and exciting research area in Functional Food is developing and is anticipated to continue to develop, centred on elucidating the biological properties of food and feed.



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In the early 1990's, Spray Dried Plasma Proteins from livestock began to revolutionise swine nutritional programs. Diets of nearly all weaned pigs now include this product, **always showing beneficial effects on general health conditions of the animals.**

Spray dried plasma is a nutritious, safe and healthy ingredient

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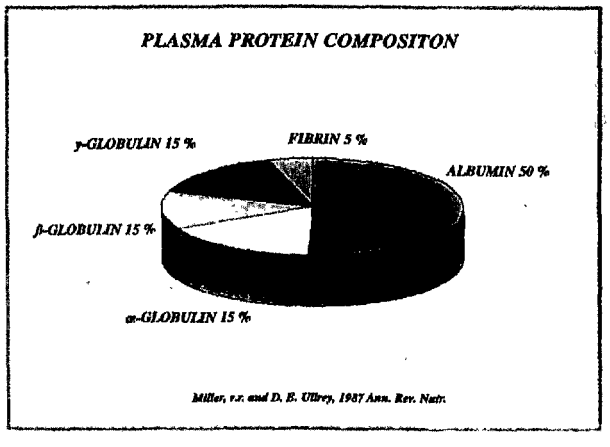
RESEARCH

The function of antibodies

○ **The scientific term for Antibody is immunoglobulin.** The body produces antibodies naturally as a defence against viruses, bacteria, toxins and other foreign agents. Antibodies help the body to fight against these "enemies".

Antibodies stick to the unknown "body" and avoid the growth of bacteria and viruses. By binding to these strange objects the organism is able to inactivate them.

When pathogenic organisms enter the bloodstream the specific antibodies will recognise the strange organism and will bind to it immediately, decreasing the potential infectivity of the agent.



Applying the science of proteins to nutritional health needs today

Antibodies are also produced at the gut level. In the intestinal tract antibodies bind to strange organisms and prevent aggressions to mucosae and intestinal epithelium.

○ Antibodies against many bacteria, viruses and toxins have been identified in plasma proteins. **Plasma is the best source of antibodies**, because of its consistency in antibody content and its broad antibody spectrum.

The benefits of antibodies used orally have been described in a great number of scientific journals.

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The properties of plasma proteins allow the reduction of medication in piglet diets

BROAD APPLICATIONS

Blood: a versatile liquid

○ Blood is an incredibly versatile liquid. If we look at the function of blood, it is obvious why blood has so often been referred to as "the fluid of life". The most important function of blood is the transportation of oxygen from the lungs to the rest of the body.

○ Another important function of blood is the transportation of nutrients, vitamins, antibodies (immunoglobulins or also called "defence molecules"), bioactive peptides like growth factors, repairing factors and minerals. **In blood there are more than 100 different compounds each with a specific function in the body. More than 40 different kinds of proteins and peptides have been isolated.**

Plasma proteins are being used in the Pet food industry due to their gelifying properties worldwide. The red cells obtained during the process of plasma production are used in Poultry feed, Aquaculture and in Dry Pet foods and ruminant by-pass proteins.

Blood proteins and other blood compounds are used in different kinds of industries all around the world.

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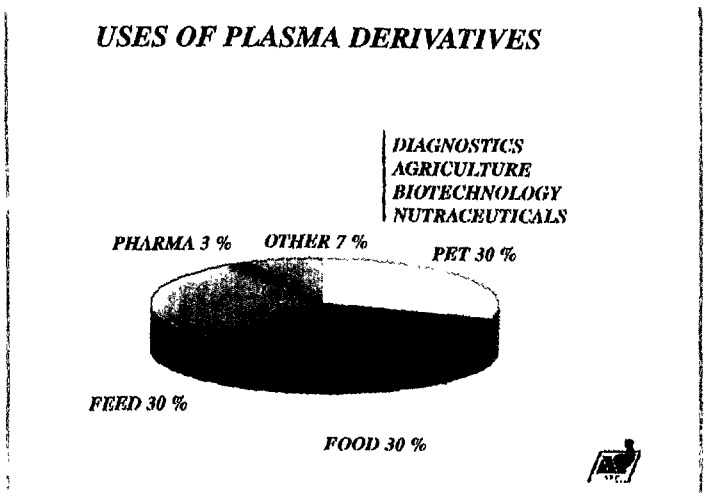

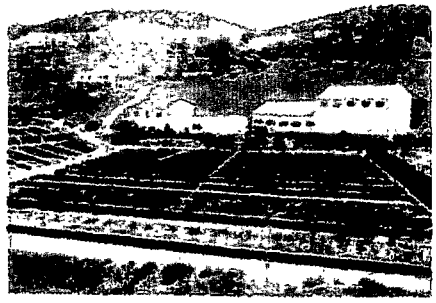

HEALTH & NUTRITION

What are Spray Dried Plasma Proteins?

Approximately 60% of blood is plasma, a light yellow clear liquid and 40% are red cells (responsible for the red color of blood). The liquid plasma consists of 90% of water with the remainder being important functional proteins, vitamins, minerals, sugars, amino acids and many other molecules of important biological value. The amino acids profile of plasma proteins is excellent, **similar to milk protein, considered as a nutritional model**. (Tybor, 1973).

Spray Dried Plasma is a light beige coulored, dry and odourless powder that contains a high level of quality proteins (70-80%, depending of manufacturing process). The spray drying process preserves its functional and biological properties. Spray dried plasma is recommended by many animal nutritionists, as a safe and efficient source of nutrients.

Milk and plasma are, from a physiological point of view, biological fluids with similar characteristics. Both deliver nutrients. Plasma offers vital nutrients to the body's cells and tissues and milk delivers nutrients to the newborn. Both fluids are characterised by containing functional proteins, including **defence proteins**, vitamins, minerals, sugars, several small **bioactive** peptides, amino acids and growth factors to name a few.

Many components of milk are not synthesised in the mammary gland. Rather these elements are directly absorbed from plasma. Plasma and colostrum are even more similar. **The immunoglobulin molecules of colostrum are the same as those found in the bloodstream, the IgG type**. In many animals, colostrum immunoglobulin is absorbed into colostrum directly from the bloodstream. The immunoglobulin rich colostrum is then transferred to the newborn offspring. In other words plasma could be considered the raw material of colostrum and milk.

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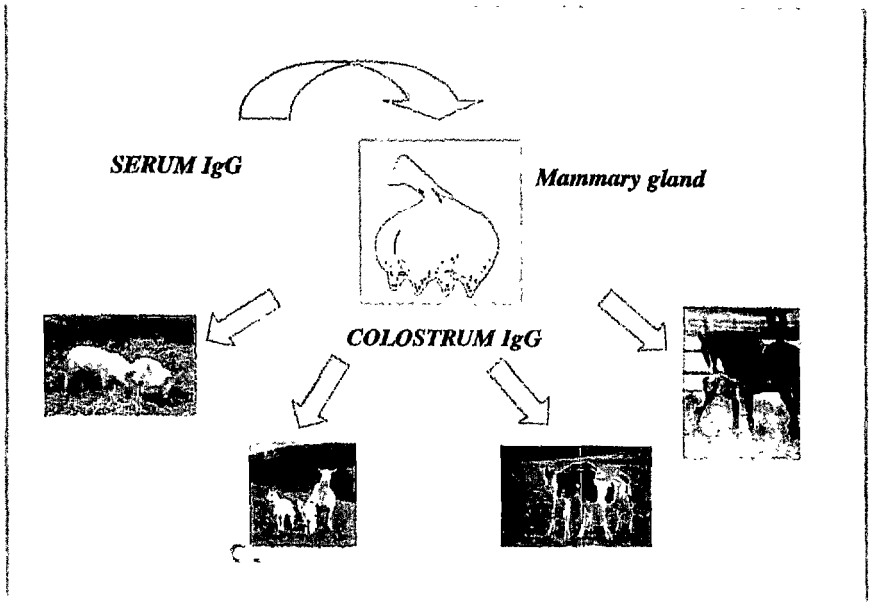


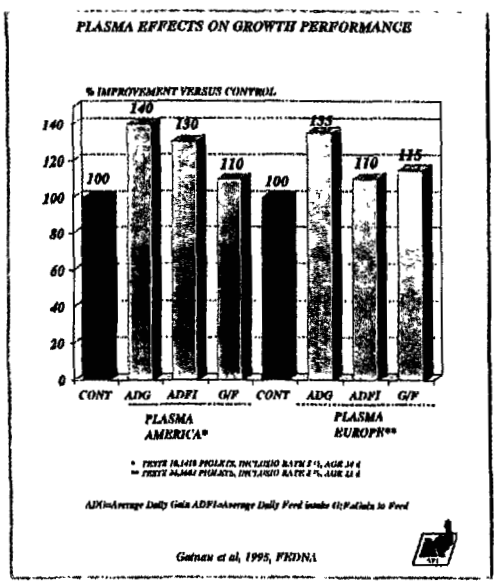
VITAL PROTEINS

Manufacturing of Spray Dried Plasma Proteins

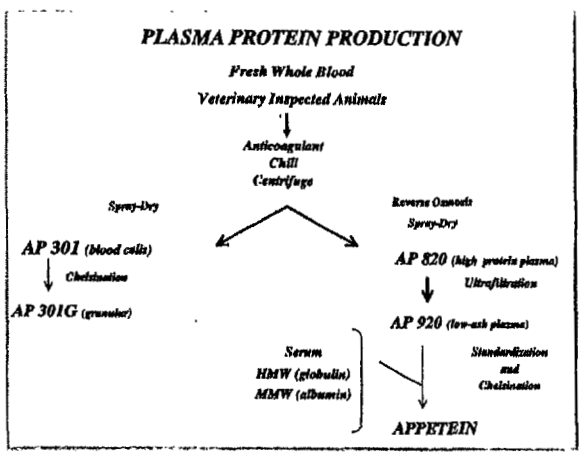
○ The milk processing industry is very similar to the plasma protein processing industry. Dedicated trucks, equipment, processing methods and quality control standards are nearly identical in both industries. **Dedicated stainless steel, food grade equipment is used.** Plasma is only obtained from animals that have been inspected and health approved by veterinary authorities.

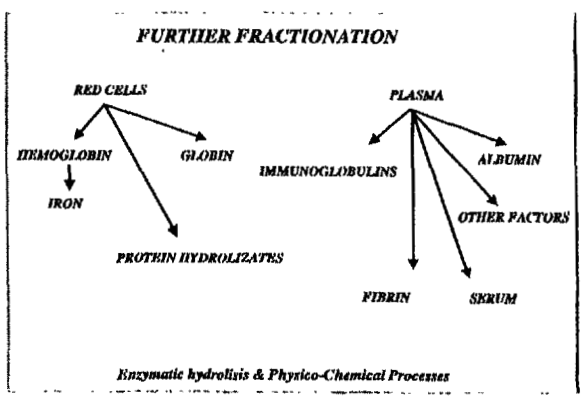
Plasma, as a biological fluid, is sterile in healthy animals.

Plasma is obtained by centrifugation of blood that has been hygienically collected and immediately refrigerated. After collection, it goes to stainless storage tanks and transported in refrigerated tanks. These stainless steel tank trucks are only used for the transportation of these products. They are routinely cleaned automatically by a Cleaning in Process System (CIP) before they leave the plants. Once the blood arrives to the plant it is unloaded and Quality Control and Quality Assurance (QC&QA) is test it on temperature, colour, pH and clotting. If QC&QA give their approval the blood is unloaded. It is important to notice APC controls the entire production process from collection to the final products. Only closed systems are used.



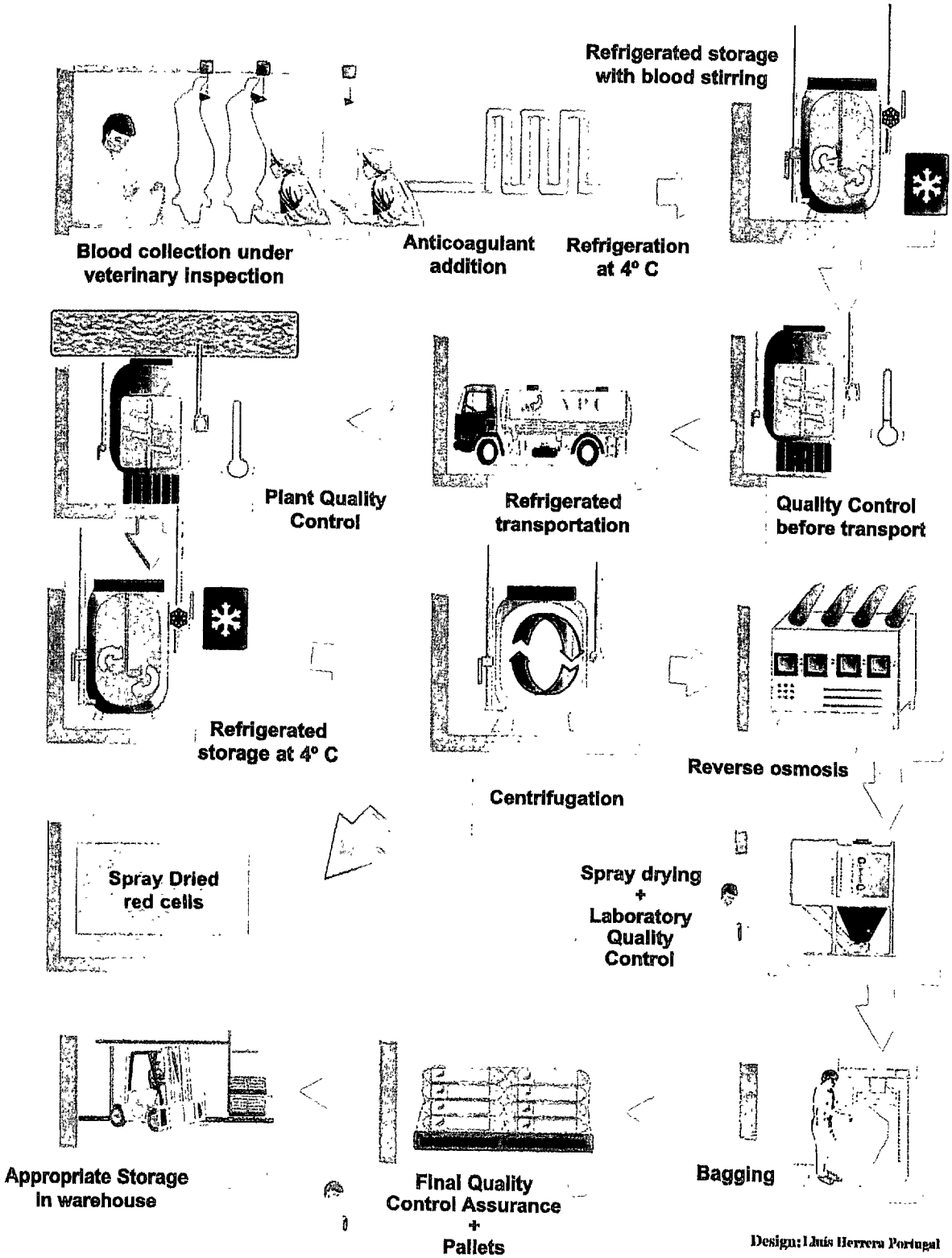
○ The plasma fraction is then submitted to a process of **ultrafiltration** or reverse osmosis to remove water and concentrate. Finally, the plasma is spray dried. Briefly, this process consists in micronization of plasma in small droplets (10-100 μm), achieved by using very high pressure (200 atm), in a tower with very high temperatures during a short time (Filková 1987). **The inlet temperatures are above 200° C and the outlet temperatures are 90° C.** This drying system allows proteins to remain functional and yet they keep the hygienical quality of plasma. The biosafety of plasma protein is due to the whole production process. The dried plasma with very low moisture content is then bagged in new polylined paper bags and stored. A rigorous Quality Control ensures the quality of the final product.





TECHNOLOGY

Plasma Protein Production



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QUALITY

Quality of Spray Dried Plasma

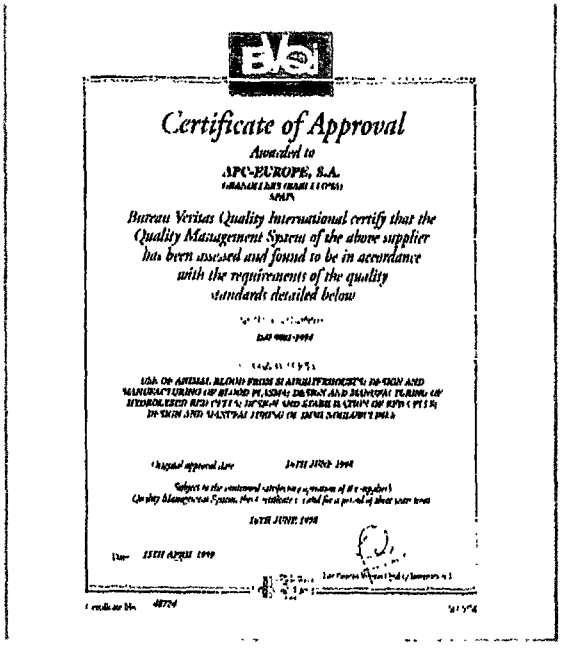
The definition of the quality of a food or feed product includes:

1. Safety
2. Healthfulness
3. Nutritional value
4. Organoleptic characteristics
5. Shelf life

An additional component of quality in the mind of the product user is, of course, the convenience.

In the case of Spray Dried Plasma there is a very good balance between all these factors affecting the quality of the product.
Plasma is a safe, healthy and nutritional ingredient. Spray dried plasma's exceptional palatability enables feeds containing plasma to be highly accepted by animals. Additionally, the manufacturing process, the low moisture and the low fat content of the final product provides a shelf life of more than 2 years at room temperature.

An optimised manufacturing process is the one that preserves as much as possible the biological activities of all those bioactive components, reducing the amount of pathogens and microorganisms to a level of complete safety.



Certificate of Approval

Awarded to

APC-EUROPE, S.A.

Bureau Veritas Quality International certify that the Quality Management System of the above supplier has been assessed and found to be in accordance with the requirements of the quality standards detailed below

APC EUROPE ISO 9001 CERTIFICATE

APC Europe's R & D and QC

○ APC's Quality Control includes the analysis of protein content, ashes, water content, microbiological parameters, organoleptic characteristics, solubility, gelifying capacity, water binding capacity, mineral content, nutritional value, aminogram and protein characterisation.

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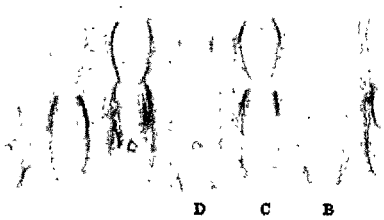
QUALITY

Immunoelectrophoresis



Agarose electrophoresis

QC Analytical methods for protein characterization

PROTEIN COMPOSITION
- Agarose electrophoresis
- Polyacrylamide gel electrophoresis (SDS-Page)
- Immunoelectrophoresis

ORIGIN:
- Western blotting
- Ouchterlony (Double immunodifussion)

IMMUNOGLOBULIN CONTENT:
- Radial immunodifussion (MANCINI)
- Turbidimetric Immunoassay (TIA) (COBAS)
- Bioactivity against pathogens (ELISA)

○ Since April 1999, APC has been developing and publishing a comprehensive set of risk analyses for their products. The aim was to inform authorities responsible for food and feed safety and the consumer that **any identified risks are managed in a responsible and reliable way, and in compliance with the law.**

The benefit of these reports is threefold:

It will provide information, data and a risk analysis on the sourcing, processing, use and bio-safety of spray dried plasma and other animal blood derivatives for the benefit of:

✓ Government and other agencies concerned with regulating the biosafety of these products, assisting them in giving practical advice to the industry, and to develop practical and enforceable regulations should this be deemed necessary.

✓ APC's customers enabling them to further improve upon their formulations and expand their knowledge of the safety and effectiveness of spray dried plasma proteins.

✓ Veterinary, scientific and nutritional research groups and other parties interested in the sourcing, collection, processing and use of animal-derived protein in animal feeds.

With these objectives in mind, an international and interdepartmental group was formed, consisting of experienced professionals in different fields: biologists, pharmacists, **epidemiologists**, veterinary surgeons, nutritionists and engineers, coming from various divisions in the APC company such as Animal Nutrition, Research and Development, Quality Control, Human Nutrition, Diagnostics and Biological, Marketing and Production.

As part of the Total Quality Assurance System, APC has intensively worked on a risk assessment program to investigate the risk of spreading known and unknown diseases by utilisation of blood derived proteins. This assessment usually adopts standards accepted by all EU member countries and thus has international validity. It is frequently done in the Pharmaceutical industry to quantify the risk associated with the use of a product.

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BIOSAFETY

Safety of Plasma

Factors in the production of plasma proteins which significantly contribute to risk reduction are:

- ✓ Blood is collected only from healthy animals that have been veterinary inspected.
- ✓ Blood is only recovered from approved abattoirs in which animals undergo veterinary inspection.
- ✓ The plasma manufacturing system is always closed.
- ✓ All production equipment is stainless steel.
- ✓ Blood is refrigerated and transported in isothermal trucks.
- ✓ Trucks are cleaned and only used for this purpose.
- ✓ Blood from many different animals is pooled in the plants. **This drastically reduces any risk of disease transfer due to the high dilution factor.**
- ✓ Ultrafiltration or reverse osmosis is used.
- ✓ Spray Drying with pressure (200 atm) and inlet temperature of 240° C and outlet of 90° C is used.
- ✓ Plasma is bagged in new polylined paper bags.
- ✓ **Storage is long due to the low humidity content of plasma.**
- ✓ Dry plasma pooling.
- ✓ Quality control check points from the abattoir, through production and packaging of finished product.
- ✓ All machinery is subjected to regular cleaning schedules.
- ✓ **Plasma is included at low doses in feed (5%) during 15 days.**
- ✓ Mixing and Pelleting.
- ✓ **Route of administration always oral.**
- ✓ Total consumption of 150 grams that is 10 grams per pig, per day, or 2.5gr/kg (Metabolic Body Weight)

All these processes make the risk for known and unknown disease spread negligible. Many worldwide scientists support these studies which attest to the biosafety of plasma products.

RISK REDUCTION QUANTIFICATION

ACTION OR PROCESS	*REDUCTION FACTOR*
VETERINARY INSPECTION AT FARM AND ABATTOIR	⬇⬇⬇
BLOOD POOLING	⬇
SPRAY DRYING	⬇⬇⬇
STORAGE	⬇
MIXING	⬇
ORAL ADMINISTRATION	⬇

NEGLIGIBLE RISK

BSE (Bovine Spongiform Encephalopathies) Risk Assessment.

Although APC already knew from published articles, EU Reports and other official documents that blood and **blood derivatives were considered low risk material for BSE transmission,** a biosafety task force investigated if there is a potential risk and analysed the production processes.

To evaluate the potential risk of transmitting BSE, APC worked with experienced specialists who had been involved in the aetiology of the disease and the characteristic of the infective agent, its transmission, its resistance, and in general its behaviour through different infectious routes.

CURRENT RECOMMENDATIONS BY THE WHO (World Health Organization) FOR THE PROTECTION OF PUBLIC HEALTH FROM AGENTS CAUSING TSE (WHO, 1996)

- No part or product of any animal that has shown signs of BSE should enter any food chain (human or animal).
- Countries should not permit tissues that are likely to contain the BSE agent to enter any food chain (human or animal).
- Bovine blood clot and serum is placed in CATEGORY IV (No detectable infectivity) (WHO, 1997). This categorisation was endorsed by the Scientific Steering Committee of the EC (SSC, 1997).
- In the European Agriculture Minister Council meeting, celebrated in June 2000, an agreement on SRM (Specific Risk Material) was approved. Blood was again excluded from this list of risk materials.

Factors determining the safety of plasma proteins regarding BSE.

Six main factors have to be considered:

✓ Geographical origin of the animals. **Animals come from BSE free zones.**

✓ The age of the animal. **Only cattle under 35 months enter the food chain.**

✓ **The Collection procedure of raw material avoids any contamination with brain tissue.**

✓ **Pigs do not develop and transmit BSE.**

✓ APC risk assessment has shown that the risk is negligible.

✓ **Plasma is one of the safest ingredients** (Bradley, 2000).

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HEALTH & NUTRITION

Most frequent questions about spray dried plasma

Q. What are spray dried plasma proteins used for in animal feeding?

A. They are used because their inclusion in piglet diets increase feed intake and growth consistently when compared to all traditional proteins including milk, fish meal, potato protein, soy proteins and whey concentrates. It also decreases diarrhoea incidence and decreases medication costs. All those aspects contribute to make pig production more profitable.

Q. What is plasma?

A. Plasma is the liquid part of circulating blood already separated from red cells, white cells and platelets. Plasma is a translucent, straw-coloured fluid that constitutes approx. 60% of total blood. Liquid plasma is 90% of water and contains important functional proteins, vitamins, minerals, sugars, amino acids and many bioactive peptides of important biological value. The amino acid profile of blood proteins is similar to milk proteins, which are considered as nutritional models.

Q. What is Spray Drying?

A. Spray drying is a process used in many industries whereby a liquid or cellular product is pumped through a fine nozzle at high pressure into a heated environment in order to remove water, inactivate microorganisms and stabilise the resultant powder that has good keeping quality.

Q. What temperatures are used?

A. APC exposes liquid plasma to a high temperature for a very short period of time during the Spray Drying process. The input temperature is usually above 200° C and the output temperature is 90° C. This high temperature treatment ensures the safety of the final product. The precisely short time the plasma is exposed to these temperatures ensures the heat does not damage the plasma's biological functionality. More aggressive treatment could deteriorate the quality of the final product.

Q. How are the cells separated from the plasma?

A. By centrifugation.

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Most frequent questions about spray dried plasma

Q. *From what sort of animals is blood collected?*

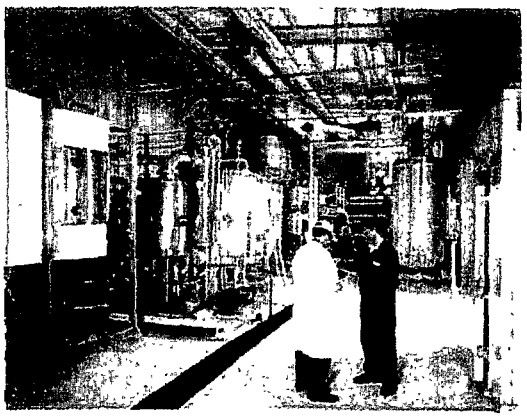
A. Blood is only collected from healthy pigs and cattle that have passed an official inspection and have been slaughtered in a licensed slaughter-house supervised by the competent veterinary authority.

Q. *What happens between collection and Spray Drying?*

A. The separated cells and plasma are transported to the blood processing plant as individual raw materials. Plasma is subjected to osmosis (which removes the water) and nanofiltration (which removes anticoagulant). It is then ready for spray drying.

Q. *What happens after Spray Drying?*

A. The dried plasma is bagged as a batch which is labelled and identifiable. Quality control checks are done for microbiological analysis and nutritional components in the end product. The processing parameters for each batch must reach the minimum standards set by APC.



Q. *Is there any risk of transferring viral diseases such a classical swine fever or foot and mouth disease?*

A. The risk in blood from OIE List A diseases (those can be a serious hazard to livestock of the appropriate species because of their contagious nature or their transmission by biting insects) is regarded as NEGLIGIBLE (Hueston, 1999). This is because all these diseases have obvious clinical signs and are noticeable to the competent veterinary authority. The antemortem inspection is an important safeguard. Pooling has a reduction in potential contamination, Spray Drying at high temperatures has shown to inactivate viruses and bacteria.

APC has already conducted several trials on viral inactivation by Spray Drying. The results show no detectable viral activity after the Spray Drying in artificially contaminated samples.

Also during the last 15 years APC has been analysing more than 750 different product batches for more than 10 different animal viruses. The results have always showed no detectable viral infection in the final product.

Q. *During the last months some news and articles have been published in general and specific journals dealing with the potential risk of BSE infection of blood. Is there any risk of BSE (Mad cow disease) transmission through Spray Dried Plasma?*

A. Blood has never been reported to be infective in naturally occurring animal BSE. Blood, as

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Most frequent questions about spray dried plasma

milk, are considered low risk materials regarding BSE (Directive 96/449 or Directive 97/582). The most important is to avoid cross contamination with nervous tissues during blood collection. The collection procedures implemented by APC ensure that there is absolutely no blood contaminated with nervous tissues.

Q. *It had been suggested that certain methods used for stunning cattle can cause brain material to enter the blood stream. How is this prevented?*

A: This has only been reported following the use of a particular stun gun that injects air under pressure into the cranial cavity or to a lesser degree following pitting. APC does not collect blood from animals stunned by either of these methods. From October 2000, the EU has banned the use of these methods in each country member and asks for an official certificate stating that animal origin products imported from third countries are not coming from animals killed by such methods.

Q. *Is there any risk of dioxin contamination in plasma?*

A. Dioxins are chemical substances that accumulate in fat. The content of fat in plasma is lower than 0.3%. Many analyses performed in plasma samples have never shown detectable amounts of dioxins.

Q. *Have blood products prepared as described above ever been the source of disease?*

A. No, during more than 15 years of using Spray Dried Plasma as a feed ingredient there has never been an instance when plasma has contributed to disease. The low moisture of Spray Dried Plasma prevents the growth of any micro-organisms responsible for a food borne disease.

Annually there are more than 200 million pigs receiving an average of 150 grams of plasma in more than 50 countries. During the last 15 years, even with an increasing rate of usage, no disease problem has been detected. Conversely, it is typical to observe an improvement in the health status as a result of improved nutrition and gut protection.

During the last 15 years in Europe and the USA millions of cases of food borne diseases have been reported, some with serious consequences.

During this same period not a single case of contamination due to Spray Dried Plasma has been reported.

Q. *Blood and Plasma have been reported to be transmitters of infectious diseases, how do you explain that Spray Dried Plasma used in the feed industry have never been reported to contaminate animals?*

A. People know that blood transfusions can present risk of transmission of diseases if the donor is infected and the recipient not. This is because transfusion is the most efficient route of transmission: there is usually no dilution factor, the blood is used untreated and the transfusion is done directly to the blood stream, without primary protection of mucosal defence. These are the main reasons why "blood" is considered to be a potential carrier of diseases. By contrast with fresh blood, spray dried plasma and blood derivatives come from healthy animals that have passed veterinary inspection, are heat-treated products

Most frequent questions about spray dried plasma

derived from multiple animal donors and are included as a small proportion of the total diet by the oral route. This means that an important dilution has taken place, a proven heat-treatment with two main effects: heat inactivation and drying has been applied to the blood derivatives. These are the main reasons why Spray Dried Plasma is so safe.

Q. Can the use of plasma proteins in animal diets be considered a kind of cannibalism?

A. No. This question has been clearly answered by the EU Scientific Steering Committee in September 1999.

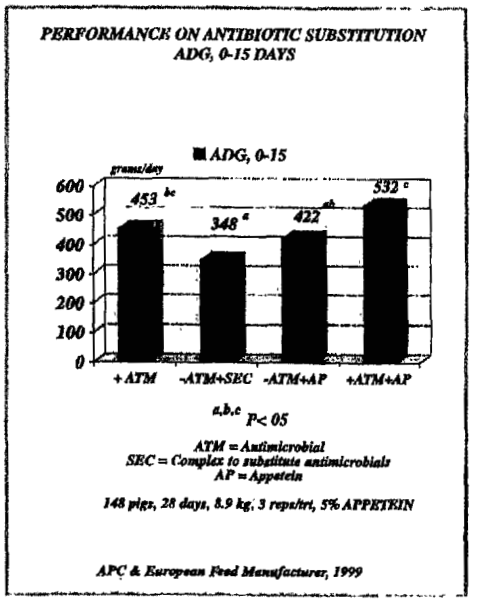
"Recycling of animal co-products processed into basic biochemical substances as fat and protein is recognised as an effective way to re-use valuable materials. When an animal is discomposed of this material is not anymore recognised as being inter-species recycling".

According to this statement, spray dried plasma proteins used as ingredients in feed cannot be considered any kind of cannibalism. In the same way, the ingestion of human proteins, like hormones derived from human biological fluids, oral human administration of immunoglobulins to infants, ingestion of growth factors obtained from human placenta, is not considered cannibalism.

Q. What are the main benefits of plasma proteins and fractions?

A. The main benefits of plasma proteins that have been observed in animals and humans are:

- ✓ Increased feed intake
- ✓ Improved weaning process
- ✓ Quick reduction of diarrhoea
- ✓ Strong anti-inflammatory effect
- ✓ Improved nutrient utilisation by the stimulation of tissue repair and increasing absorptive area
- ✓ Normalised bowel function
- ✓ Intestinal immune support
- ✓ Intestinal epithelium repair
- ✓ Growth promotion



PLASMA IMPROVES INTESTINAL MORPHOLOGY

Touchette, K. et al., 1999.University of Missouri

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1.0 Identity and Composition of Additive

1.1 Definition

Bovine Globulin is a spray-dried powder product obtained by removing the fibrin and albumin proteins from liquid bovine plasma by chemical and mechanical processes. The globulin is concentrated by membrane filtration and then spray dried into an off-white powder.

The brand name for Proliant Inc.'s bovine globulin is ImmunoLin®. Future documents will use this brand name when referencing the globulin.

1.2 A Product Information Bulletin

This document outlines the general description and use of the product, physical characteristics and information pertaining to the labeling, packaging and storage of the product.

Proliant
HEALTH INGREDIENTS
The Art of Service.
The Science of Protein.

ImmunoLin®

Description:
ImmunoLin® is a purified immunoglobulin protein isolate that is manufactured in the US and contains a minimum of 45% IgG. It is engineered to deliver the most concentrated and consistent immune system support available. ImmunoLin® contains very low levels of saturated fat and no lactose. It is also free of milk proteins, casein and β-lactoglobulin, which are not well tolerated by all people.

Physical Characteristics:
ImmunoLin® is a spray-dried, cream colored powder with a neutral flavor and odor.

Recommended Use:
The recommended dosage of immunoglobulin is 500 mg to 1,500 mg per day of IgG in powder, capsule, or tablet form. This is the equivalent of 1,100 to 3,300 mg of ImmunoLin® per day.

Labeling:
ImmunoLin®: immunoglobulin protein isolate or immunoglobulin isolate

Supplement Facts Serving Size = 1.1 gram	
Amount per Serving	
Total Protein	940 mg
Immunoglobulin G	500 mg †
† Daily value not established	

Storage:
Store in a cool dry environment. Rotation of stock is recommended. ImmunoLin® is stable for two years when stored in a sealed container under recommended conditions.

Packaging:
ImmunoLin® is packaged in 25 kg drums and bagged in polyethylene liners.

2425 SE Oak Tree Court • Ankeny, Iowa 50021 • (866) 440-1797 • Fax (515) 289-4360 • www.proliantinc.com • Products of USA

1.3 Product Specification

Provides the chemical and microbiological profiles of the product and ordering information.

Proliant
HEALTH INGREDIENTS
The Art of Service.
The Science of Protein.

Product Specification
ImmunoLin®

Test	Specification	Test Method	Reference
Protein (Dry Basis)	≥90%	A.O.A.C. 15th Ed.	992.15
IgG	≥45%	Internal Method	Etzel et al 1997
Fat	≤1.5%	Acid hydrolysis	
Moisture	≤8%	A.O.A.C 15th Ed.	934.01
Ash	≤8%	A.O.A.C. 15th Ed.	942.05
Heavy metals	<10 ppm		
Arsenic	<1.5 ppm		
Microbiological			
Std. plate count, cfu/g	≤10,000	A.O.A.C. 16th Ed.	990.12
Salmonella	ND	FDA BAM (8th Ed.)	Chap. 5
Listeria	ND	FDA BAM (8th Ed.)	
Staph. aureus	ND	A.O.A.C. 16th Ed.	975.55
E. coli	ND	A.O.A.C. 16th Ed.	992.30
Typical bulk density	0.35 g/cc		

ND=None detected in sample

Order Information:
ImmunoLin® has a product SKU number of 56000 and is shipped FOB from Iowa. Call Proliant Health Ingredients toll-free (800) 369-2672 or email health@proliantinc.com

Note: This product contains proteins of animal origin

This information is presented for consideration in the belief that it is accurate and reliable, however, no warranty, either expressed or implied, is made and no freedom from liability from patents, trademarks, or other limitations should be inferred. Any data listed are averages only and are not to be considered as guarantees, expressed or implied, not as a condition of sale.

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1.4 Typical Nutritional Profile

Typical profile of the product including mineral and vitamin content.

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ImmunoLin®
Spray-Dried Bovine Serum Globulin
Typical Nutritional Profile

Protein	89.9
Moisture	6.6
Ash	2.0
*Carbohydrates	1.3
Sucrose	<0.1
Dextrose	<0.1
Glucose	<0.1
Fructose	<0.1
Lactose	<0.1
Maltose	<0.1
Fat	0.2
Cholesterol (mg/100g)	14
*Total Calories (K Cal/100g)	367
MINERALS (mg/100g)	
Calcium	147
Chloride	110
Magnesium	26
Phosphorus	543
Potassium	40
Sodium	461
VITAMINS	
Vitamin C mg/100g	<1.0
Vitamin B_1 mg/100g	0.1
Vitamin B_{12} mg/100g	<0.1
Vitamin B_2 mg/100g	<0.1
Vitamin A IU/100g	835

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Typical Amino Acid Profile

	*Typical (g)
Alanine	4.3
Arginine	5.0
Aspartic Acid	9.1
Cystine	2.7
Glutamic Acid	11.3
Glycine	4.1
Histidine	2.6
Isoleucine	2.8
Leucine	7.8
Lysine	8.4
Methionine	2.0
Phenylalanine	4.5
Proline	5.6
Serine	8.9
Threonine	7.3
Tryptophan	1.6
Tyrosine	5.0
Valine	7.2

Amino acids as % of total amino acids

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Fatty Acid Profile

SATURATED	FORMULA	%
CAPROIC	6:0	0.4
CAPRYLIC	8:0	0.8
CAPRIC	10:0	0.3
UNDECANOIC	11:0	<0.1
LAURIC	12:0	0.4
TRIDECANOIC	13:0	<0.1
MYRISTIC	14:0	1.2
PENTADECANOIC	15:0	0.3
PALMITIC	16:0	16.9
MARGARIC	17:0	0.3
STEARIC	18:0	16.9
EICOSANOIC	20:0	<0.1
BEHENIC	22:00	0.6
TRICOSANOIC	23:00	0.2
LIGNOCERIC	24:0	<0.1

MONOUNSATURATED	FORMULA	%
MYRISTOLEIC	14:1	0.6
PENTADECENOIC	15:1	0.5
PALMITOLEIC	16:1	1.5
MARGAROLEIC	17:1	0.9
ELAIDIC	16:1	<0.1
OLEIC	18:1	23.5
GADOLEIC	20:1	0.3
HENEICOSANOIC	21:0	0.8
ERUCIC	22:1	0.3
NERVONIC	24:1	<0.1

POLYUNSATURATED	FORMULA	%
LINOLEIC	18:2	29.2
LINOLELAIDIC	18:2	<0.1
LINOLENIC	18:3	1.6
GAMMA LINOLENIC	18:3	<0.1
EICOSADIENOIC	20:2	1.0
EICOSATRIENOIC (2 forms)	20:3	0.5
ARACHIDONIC	20:4	0.8
EICOSAPENTAENOIC	20:5	0.2
DOCOSADIENOIC	22:2	<0.1
DOCOSAHEXAENOIC	22:6	<0.1

SATURATED FATTY ACID	-	**TOTAL**	38.3
MONOUNSATURATED FATTY ACID	-	**TOTAL**	28.4
POLYUNSATURATED FATTY ACID	-	**TOTAL**	33.3

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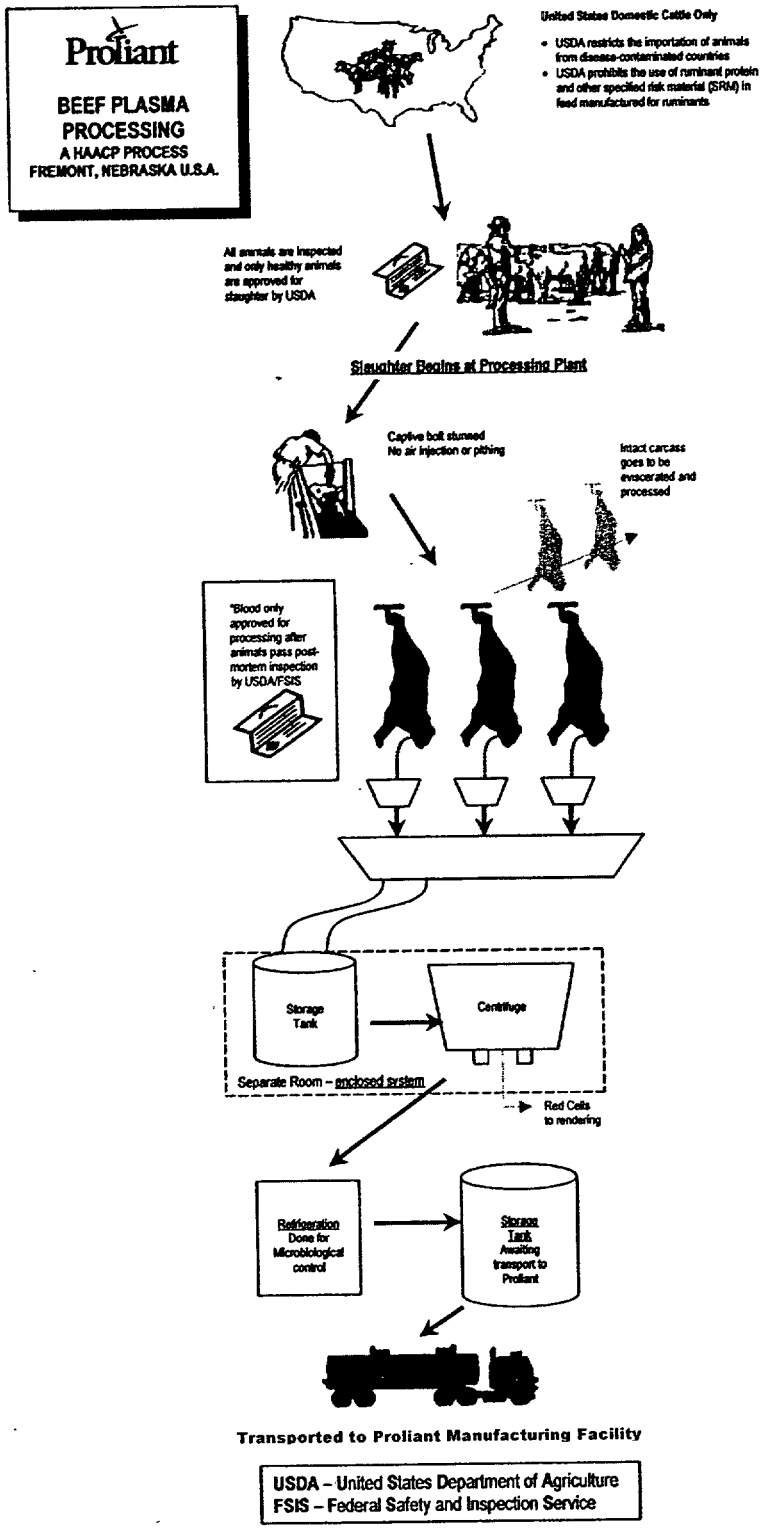
1.5 Process Flow Charts

Specific details explaining each step follow the flow charts. Although some details are proprietary, we have outlined all the meaningful steps in the process so the reviewer will have a better understanding of the processing steps.

1.5.1 Process flow chart for edible blood collection and transportation to processing facility.


Proliant
BEEF PLASMA PROCESSING
A HAACP PROCESS
FREMONT, NEBRASKA U.S.A.
United States Domestic Cattle Only
• USDA restricts the importation of animals from disease-contaminated countries
• USDA prohibits the use of ruminant protein and other specified risk material (SRM) in feed manufactured for ruminants
All animals are inspected and only healthy animals are approved for slaughter by USDA
Slaughter Begins at Processing Plant
Captive bolt stunned
No air injection or pithing
Intact carcass goes to be evacerated and processed
*Blood only approved for processing after animals pass post-mortem inspection by USDA/FSIS
Storage Tank
Centrifuge
Separate Room – enclosed system
Red Cells to rendering
Refrigeration Done for Microbiological control
Storage Tank Awaiting transport to Proliant
Transported to Proliant Manufacturing Facility
USDA – United States Department of Agriculture
FSIS – Federal Safety and Inspection Service

Beef Plasma Processing Steps

1. Animals are inspected and approved for slaughter by USDA/FSIS inspectors.

2. Animals are stunned by captive bolt and hung by their hind feet with head down.

3. Technician slits the throat open and places a cleaned and sanitized stainless steel bucket containing liquid sodium citrate on the animal's neck to catch the blood.

4. Animal's blood drains into the bucket while carcass travels the line for the further processing.

5. Bucket is removed from the neck and placed on conveyor. When animal passes post-mortem inspection, the bucket of blood from that animal dumps into the collection system. If the animal is condemned, the bucket of blood from the animal along with the bucket of blood <u>before</u>, and the bucket of blood <u>after</u> the identified container are discarded into a rendering trough.

6. The blood is centrifuged to separate the red cells from the plasma. The plasma is cooled to below $\leq$45°F by running it through a plate chiller.

7. The plasma is pumped into an insulated silo and held for pick up.

8. Plasma is loaded into a cleaned, sanitized and dedicated Proliant Inc. trailer and brought to either the Fremont, Nebraska facility or the Boone, Iowa facility with seal identified by number on USDA shipping documents.

1.5.2 Process flow chart for the manufacture of spray-dried bovine globulin.

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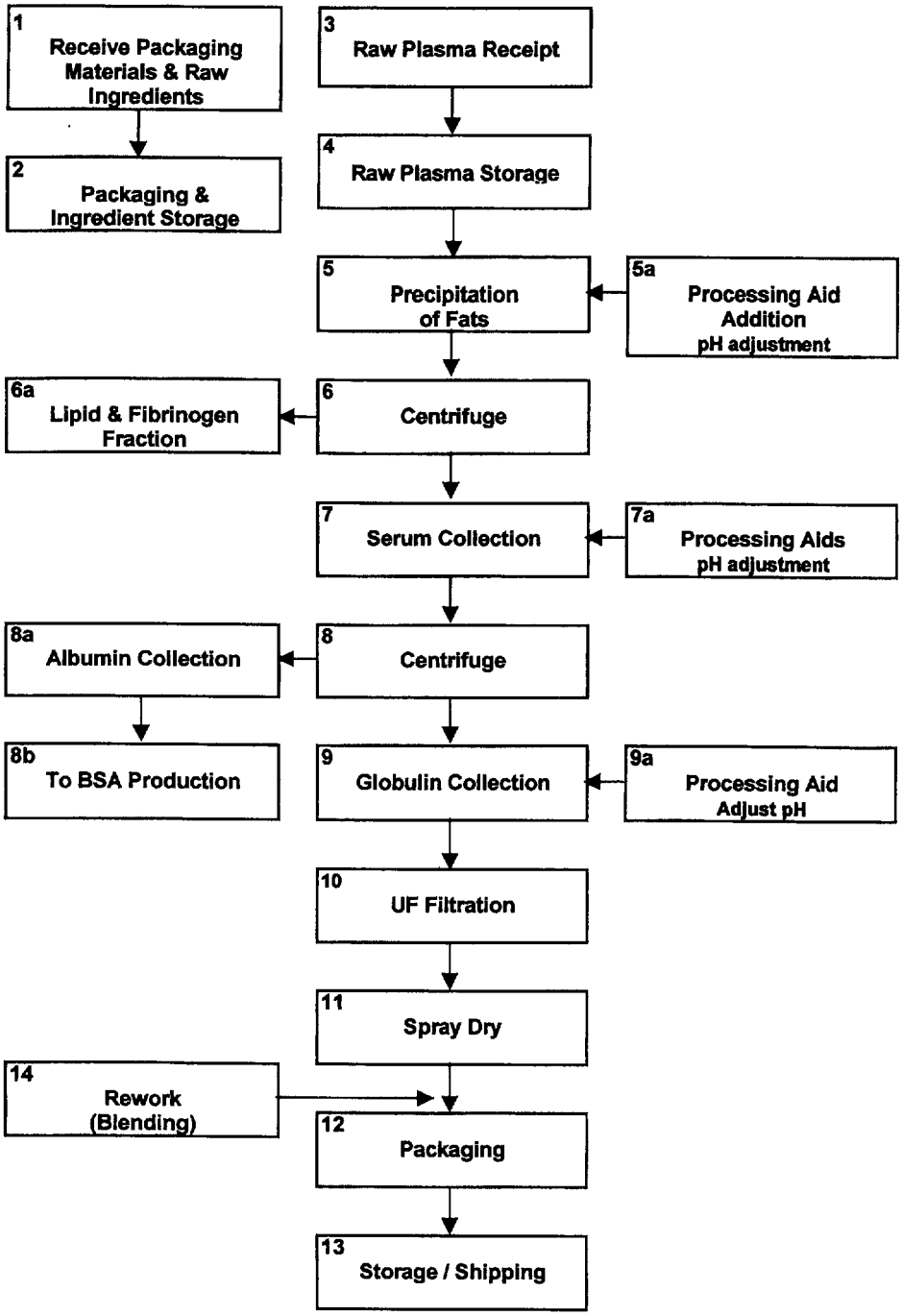
Spray-Dried Bovine Gloublin
Process Flow Chart



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1.6 Description of Manufacturing Process — Spray-Dried Bovine Globulin

Liquid beef plasma is collected from USDA approved slaughter plants, which were mandated by FSIS (Food Safety and Inspection Service) to have a HACCP (Hazard Analysis Critical Control Point) plant installed in their plants beginning on January 26, 1998. Raw plasma that has been chilled to a temperature of ≤45°F is loaded into a previously cleaned, sanitized and inspected tanker that is inspected by a USDA inspector prior to loading. It is then transported to Proliant Inc.'s plant in Boone, Iowa for further processing.

1.6.1 Packaging Material and Raw Ingredient Receipt

Only USDA/FDA approved packaging materials and ingredients are received and stored in this facility.

1.6.2. Packaging and Ingredient Storage

Packaging materials and shelf-stable ingredients are stored in the warehouse under ambient conditions.

1.6.3 Raw Plasma Receiving/Unloading Procedures

Upon arrival at Proliant Inc., each tanker of raw plasma is backed into the unloading room, where the tanker of plasma is checked for temperature. The thermometer that is used is calibrated once per day. This temperature and other tanker information is recorded and signed by the unloading operator. Proliant will not unload the plasma if the recorded temperature is >45°F. Should any load exceed 45°F, Proliant will reject the plasma load. This rejected tanker will be shipped to an inedible plasma facility.

1.6.4 Raw Plasma Storage

Once the tanker has been approved for unloading, the operator will pump the liquid plasma through a previously cleaned and sanitized (CIP) (Cleaned in Place) stainless steel pipe to a stainless steel holding tank.

1.6.5 Precipitation of Fats and Fibrinogen

Plasma is pH adjusted. A processing aid is added to precipitate the fat and fibrin fractions.

1.6.6 Centrifugation

Lipids and fibrin are separated from plasma by centrifugation.

1.6.7 Serum Collection

Additional processing aids are added to liquid serum. Solution is pH adjusted. This process helps separate globulin from albumin.

1.6.8 Centrifugation

Globulin and albumin are separated.

1.6.9 Globulin Collection

Supernatant (globulin) is collected in the holding tank, processing aid(s) are added and pH is adjusted.

1.6.10 UF Filtration

All processing aids are removed. Globulin is concentrated.

1.6.11 Drying Procedure

The dryer temperature is monitored by the operator and recorded every hour on the Drying Report Form. Once the globulin has been dried in the dryer chamber, it passes into the holding silo until packaged.

1.6.12 Packaging

The samples are collected and given to the Quality Control (QC) department for further testing. Powder is manually weighed and then placed in a poly-lined, USDA-approved bag. Product is labeled, coded, and placed on a pallet.

1.6.13 Warehouse / Shipping

Finished product is tested by Quality Control. All of the bagged, dried globulin products are stored in an approved facility. Release of product is done by Corporate QA personnel.

1.6.14 Rework / Blending Procedures (Optional)

On occasion, Spray-Dried Globulin may be re-worked when quality issues are not related to product safety.

The dried globulin may be introduced into the blender and mixed with different lot codes of globulin for

achieving a particular level of ImmunoGlobulin content (quality parameter only).

1.7 Product Formula

The formula is the document used by Proliant Inc. to manufacture the spray-dried globulin.

Proliant ORIGINAL PRODUCT FORMULA

FORMULA NO. F 2351.BB

TECHNICAL [ ] EDIBLE [X]

Product Brand Name:

Bovine Serum Globulin, Low Fat

Description: Spray Dried Bovine Serum Globulin

Ingredient #	Proliant Ingredient	Description [1] (Fibrin Is Not Removed)	Weight (kg)	% of Formula
	Bovine Plasma, microfiltered*	Proliant Fremont	**	80-90%
	Collodial Silicon Dioxide	NALCO 1115		3-6%
	Edetate Disodium 6.25% solution	Dow Chemical Versene® NA chelating agent		<1%
	Sodium Hexametaphosphate 33.3% solution	FMC, Solutia, Astaris		<1.5%
	Hydrochloric Acid 2N solution	Bayer, Interstate Chemical Company		3-7%
	Sodium Hydroxide 15% solution	Bayer, Interstate Chemical Company		0.5-1.5%
	Total Batch Weight			

* Microfiltering of plasma is optional.
** Weights will vary depending on actual plasma weight.
[1] See Approved Biologicals Vendors List for Additional Approved Suppliers

BEST ORIGINAL COPY

Yield

100% Theoretical (Dry Powder)	725 kg	29	units (25 kg drum)
Actual (Dry Powder)	218 kg	8.7	units (25 kg drum)

Note: Calculations based on 50.0% BIgG level.

SKU	PRODUCT	NET WT.
55000	NutraGammax™ 40	25 kg drum
56001	ImmunoLin™ (without flow agent)	25 kg drum

APPROVALS:

(b) [redacted] 7/26/01 Date
V.P. Research & Development

(b) [redacted] 7/23/01 Date
Res. Proliant Biologicals

(b) [redacted] 7/25/01 Date
Plant Q.C.

(b) [redacted] 7/30/01 Date

(b) [redacted] 7/24/01 Date
Dir. Proliant Health

(b) [redacted] Date

APPROVED JUL 30 2001

Issue Date _____ Issue No 3

Supercedes Issue Issue #2 - August 25, 2000

cc: VP Operations

Plant Location (s) Biologicals Plant Boone, Iowa

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1.8 Structure and Function of ImmunoGlobulin

ImmunoGlobulin (IgG) is an integral part of human blood produced by the human body. Bovine ImmunoGlobulin is a secondary source of this concentrate for the use in nutritional supplements, protein supplements and food products. Although the species is different, the chemical structure of the IgG molecule is very similar.

Pages 000061-000062 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

IMMUNOGLOBULINS:

The immunoglobulins are a complex group of proteins that make a significant contribution to the protein content as well as exerting an important immunological function, especially in colostrum. The structure and general function of bovine immunoglobulins has been reviewed by Larson (1992) and Buttler (1973,1983).

The immunoglobulins found in bovine whey and colostral whey include:

IgA and secretory IgA
IgG_1, IgG_2 and IgG fragments
IgM
IgE
J-chain or component
free secretory component

The nomenclature of the immunoglobulins is based on their immunological cross reaction with reference proteins, preferably of human origin and proposed by the World Health Organisation (Buttler, *etal.*, 1971).

All the immunoglobulins are glycoproteins that are monomers or polymers of made up of two light chains (~20,000 Dal) and two heavy chains (~50,000 - 70,000 Dal) linked together with sulphide bonds (Buttler, 1974, Lascelles, 1979). In the molecules of a class or subclass, the two have chains are identical – with a constant and a variable region. The light chains are also identical with constant and variable regions. The variable N-terminal regions of both the heavy and light chains provide the antigen binding sites, whereas, the complement fixation, membrane transport, species specific and class specific antigenic determinants are related to the constant region of the heavy chains (Buttler, 1974, Lascessels, 1979).
The variable carbohydrate moiety bound to the heavy chain is considered to be important in the specificity of the Igs.

000063

2

000063.001

2.0 Proposed Use in Dietary Supplements and Foods

The bovine globulin, when added in recommended quantities to dairy, juice, snack, and beverage systems provides a value-added dietary supplement of ImmunoGlobulin (IgG) for intestinal well being.

Proliant Inc. has been selling spray-dried bovine whole plasma as a food substance since 1992. It has been added to surimi (artificial crabmeat) in Japan, hot dogs in China and has been sold for general meat applications worldwide. Edible plasma has been produced in a USDA/FSIS inspected facility (Est. 13524) since 1992 in Fremont, Nebraska. Bovine globulin is simply a fraction of the bovine plasma.

Bovine globulin is a derivative of edible plasma presently manufactured in a FDA bulk pharmaceutical manufacturing facility in Boone, Iowa. It has been sold into the dietary supplement industry since November 1999 as a purified ImmunoGlobulin (IgG) (containing a minimum of 20-45% IgG). The recommended dosage of this ImmunoLin® is 1.0 to 10 grams per day in powder, capsule or tablet form.

A list of products containing this material is located in Section 3.0.

2.1 Dietary Supplements

Dietary supplements and foods as permitted by government regulations in which bovine serum is to be used:

Dietary supplements (as defined by DSHEA Provision of 1994) include:

- a vitamin
- a mineral
- an herb or other botanical
- an amino acid
- a metabolite, extract or combination of any of the above

A letter of notification was sent on November 18, 1999 to Special Nutritionals, Center for Foods Safety and Applied Nutrition regarding the future sale of our spray-dried globulin, NutraGammax™ (Section 9.0, Tab N).

2.2 Population Expected to Consume Product

The populations expected to consume this substance are those individuals who currently consume dairy products, beverages, nutritional bars, snack foods, and sports nutrition protein supplements. The majority of consumers eat these products on a daily basis.

The individuals consuming this globulin would be the general public seeking nutritional supplements. This ranges from children and infants to older adults, depending on dosage. Infants consume these nutritional supplements to support physical growth and well being.

2.3 Religious Concerns

The Hindu religion prohibits the consumption of bovine products, however the species will always be clearly identified on our labeling.

There should be no other religious issues relating to the use of this food additive.

This product is neither Halal nor Kosher approved.

000067.001

3.0 Level of Use in Foods

3.1 Consumption Data – Recommended Levels of Use

Product	Recommended Usage
Dairy products	1-10 grams/day
Juices / beverages	1-10 grams/day
Nutritional bars	1-10 grams/day
Snack foods	1-10 grams/day
Sports nutrition protein supplements (nutrient supplement)	20% of formula by weight *

* This formula was used in a chocolate drink for a cholesterol study

If an individual consumed a tablet/capsule, food and drink containing the recommended maximum dosage daily, he/she would ingest 30 grams of spray-dried bovine globulin per day. This is equal to .22 grams per kilogram of body weight (average weight 150 pounds).

Additional Consumption Data – the following information is based on our present customer usage.

Table 2 – ImmunoLin® Intake Data

Products Containing ImmunoLin® (Globulin IgG) as of 09/01/2004

Product Name	Manufacturer	Recommended Servings per Day	Grams of ImmunoLin® per Serving	Total Grams ImmunoLin® per Day
Schiff ImmunAssure	Weider Nutrition	1	1.25	1.25
Swanson Ultra IgG Concentrate	Swanson Health Products	1-2	0.5	0.5-1.0
Swanson Condition Specific Immune Essentials	Swanson Health Products	2	0.1	0.2
ImmunoSense	NutraSense	1	2.5	2.5
IgG 2000DF	Xymogen	1	5	5
GF-Pro	Ergo Pharm	2-3	1.4	2.8-4.2
ImmunAdvantage	Now Foods	1-3	0.45	0.45-1.45
Advanced Therapeutics	Nature's Plus	1	0.6	0.6
Eclips Humatropil	Boss	1	1	1
ImmnoGro	Black Star Labs	1-3	3	1-3
ImmunoWhey	Black Star Labs	2-4	1.4	2.8-5.6
Ever-Well	Life-Line Vitamins	1	0.5	0.5
First Defense	Vibrant Health	2-6	0.5	1.0-3.0
Might Gulp	Mighty Health	1	0.2	0.2
Torque	Protein Factory	1	4.6	4.6
Bovine Immunoglobulin Concentrate	Protein Factory	1	5	5
Palumbo's Anobolic Elixir	Protein Factory	1	4	4

3.1.1 Estimated Daily Intake (EDI)

Proliant recommends a daily intake of 1 to 10 grams of powdered globulin. Drinks containing .91 to 52.5 grams/per kilogram of body weight per day were used in child human studies. Therefore, a 50-pound (22.7-kg) child would ingest 57 grams per day.

Estimated Average Daily Intake of Spray-Dried Bovine Globulin Estimates Compared to Actual Usage & Test Data					
Means of Consumption	Proliant's Maximum RDI for 150 lb (68 kg) person [1]	For Human Supplement	Consumption to date in approximately 900 million newly weaned pigs	Consumption to date in approximately 3 million newborn calves	Consumption in 10 children weighing approximately 8 kg for 21 days
Capsule	4.5 grams	0.18 grams			
Food/Feed	4.5 grams	0.18 grams	0.3-0 72 grams		1.74-3.48 grams
Drink	4.5 grams	0.18 grams		2.0-2.5 grams	
TOTALS	13.5 grams	0.54 grams	0.3-0.72 grams	2.0-2.5 grams	1.74-3.48 grams

[1] Based on maximum recommended usage level

Since Proliant Inc.'s spray-dried bovine globulin is only of value in products marketed for their supplemental value, the greatest perceived excessive use would result from a person consuming multiple supplements containing our product. As noted in the table above, even if a person consumed our product in all three forms daily, (tablet/capsule, food/feed, drink) the total would be 0.54 grams of globulin. This total amount falls below the amount consumed by animals on a daily basis and this value is below the levels used in the referenced documents and the feeding trial of Guatemalan children.

3.1.2 Acceptable Daily Intake (ADI)

Proliant knows of no publications or data regarding the acceptable levels of ingestion of this globulin supplement. We see no reason to restrict its intake since no known adverse effects have been reported.

The consumer might restrict the use of globulin more on its palatability acceptance rather than any negative health effects.

3.2 Limitations of Conditions of Use

Proliant sees no need to limit the conditions of use for this globulin protein.

000072.001

4.0 Data Establishing the Intended Effect

Proliant Inc. has positioned plasma products as a source of high-protein for use in food matrix systems. One of its most successful traits has been the documented inactivation of the protease enzyme found in surimi. The Japanese have used our spray-dried plasma for many years in their surimi industry. The gelling property of edible plasma has been successfully applied in the meat and poultry industries to increase the firmness of muscle tissue.

The new plasma derivative, bovine globulin, has been shown to be effective in maintaining intestinal wellbeing and delivers 40% IgG (ImmunoGlobulin) per dose or serving to the recipient. Proliant intends to market this product as a nutritional supplement and single food additive.

The following statements are used in labeling and related documents for use as a dietary supplement:

Bovine globulin:

- actively supports and maintains gastrointestinal health
- provides intestinal wellness support
- helps maintain gastrointestinal balance and supports normal nutrient absorption
- promotes normal gastrointestinal health by:
    - promoting intestinal function
    - increasing intestinal absorptive surface area
    - optimizing the utilization of nutrients
    - to promote intestinal health in humans
    - provides gastrointestinal and immune support while
    - optimizing the uptake of nutrients

    - helps promote and maintain intestinal health function

- promotes gastrointestinal balance:

    - supports normal digestive absorption for stable gastrointestinal function

This bovine globulin can be used in foods as a high protein replacement ingredient for soy or dairy proteins to avoid allergic reactions.

4.1 Animal Studies

The animal feed industry has been using technical sourced bovine plasma since the 1980's as an additive to pig starter feeds and calf milk replacers with great success. The death rate of newborn pigs has been significantly reduced through the use of plasma. Over 100,000 animals per year are saved by consuming our plasma products.

4.1.1 Porcine Animal Studies

Coffey, R.D. and Cromwell, G.L., *Use of spray-dried animal plasma in diets of weanling pigs,* Pig News and Information, 2001, Vol. 22 No. 2 39N-48N (Section 9.0, Tab O)

Straw, B.E., D'Allaire, S., Mengeling, W.L., Taylor, D.J., *Diseases of Swine, 8th Edition,* 1999 Iowa State University, Chapter 69, 1045-1055 (Section 9.0, Tab P)

Hansen, J.A., Nelssen, J.L., Goodband, R.D., and Weeden, T.L., *Evaluation of Animal Protein Supplements in Diets of Early-Weaned Pigs,* J. Anim. Sci. 1993. 71:1853-1862 (Section 9.0, Tab Q)

Kats, L.J., Nelssen, J.L., Tokach, M.D., Goodband, R.D., Hansen, J.A., and Laurin, J.L., *The Effect of Spray-Dried Porcine Plasma on Growth Performance in the Early-Weaned Pig,* J. Anim. Sci. 1994. 72:2075-2081 (Section 9.0, Tab R)

Gomez, G.G., Phillips, O. and Goforth, R.A., *Effect of Immunoglobulin Source on Survival, Growth, and Hematological and Immunologial Variables in Pigs,* J. Anim. Sci. 1998. 76:1-7 (Section 9.0, Tab S)

Bosi, P., Casini, L., Finamore, A., Cremokolini, C., Merialdi, G., Trevisi, P. Nobili, F., and Mengheri E., *Spray-dried plasma improves growth performance and reduces inflammatory status of weaned pigs challenged with enterotoxigenic Escherichia coli K88,* J. Anim. Sci. 2004, 82:1764-1772 (Section 9.0, Tab T)

Quigley, J.D., J.M Campbell, Hammer, C.J., Crenshaw, J.D., and Russell, L.E., *Effects of Spray-Dried Plasma in the Diets of Pigs and Poultry,* February 2004 (Unpublished, Section 9.0, Tab U)

The document by Coffey and Cromwell, is an excellent review of the studies conducted in young pigs. The use of animal plasma as a source of immunoglobulin in the stressful postweaning period is a common practice used to improve the growth performance and health of these young animals.

Over 30 million pounds of plasma products have been used worldwide in swine farrowing operations. 90% of all swine are fed plasma immediately after weaning.

4.1.2 Bovine Animal Studies

Quigley, J.D., J.M Campbell, Hammer, C.J., Crenshaw, J.D., and Russell, L.E., *Effects of Spray-Dried Plasma in the Diets of Dairy Calves,* February 2004 (Unpublished, Section 9.0, Tab C)

Poulsen, K.P., Collins, M.T., and McCuirk, S.M., *Field Trial of an Artificial Bovine Colostrum Replacer*, PowerPoint presentation, School of Veterinary Medicine, University of Wisconsin-Madison (Section 9.0, Tab A)

Calf Note # 98 – BSE and bovine blood proteins, Dr. J. Quigley, CalfNotes.com, 2004 (Section 9.0, Tab V)

This same material being fed to pigs is being fed to calves at a dosage rate of 75g/dose. Death loss has been significantly reduced in both cases.

Based on these animal studies, evidence suggests that similar dosages of globulin would be a safe and excellent nutrient for humans.

4.2 Human Studies

Several human studies have been conducted during the past 15 years showing no adverse effects and documenting improved health of the study participants.

Numerous clinical human feeding trials were conducted between 2002 and 2004 with very encouraging results. The information on each of these trials (listed below) follows this page.

4.2.1 Adult Human Studies

Nash, G.S., MacDermott, R.P., Schloemann, S., Bertovich, M.J., O'Neal, J., Porter, L., and Kulczycki, A., *Bovine IgG1, but not IgG2, binds to human B cells and inhibits antibody secretion,* Immunology, 1990 69 361-366 (Section 9.0, Tab W)

Copelan, E.A., Bechtel, T.P., Klein, J.P., Klein, J.L., Tutschka, P., Kapoor, N., Featheringham, N.C., and Avalos, B.R., *Controlled trial of orally administered immunoglobulin following bone marrow transplantation,* Bone Marrow Transplantation, 1994, 13:87-91 (Section 9.0, Tab X)

4.2.2 Child Human Studies

Lembcke, J.L., Peerson, J.M., and K.H. Brown, *Acceptability, Safety, and Digestibility of Spray-Dried Bovine Serum Added to Diets of Recovering Malnourished Children,* Journal of Pediatric Gastroenterology and Nutrition, 1997, Volume 25(4), pp. 381-384 (Section 9.0, Tab F)

> Children (8.017 kg BW) received either 8.7 or 17.4 g of bovine serum which is equivalent to 1.74 or 3.48 g of immunoglobulins. No adverse effects were reported. Serum biochemical parameters were within normal range during each diet period.

Melamed, MD., I., Griffiths, MD., FRCPC, A.M., and Roifman, MD., C.M., *Benefit of oral immune globulin therapy in patients with immunodeficiency and chronic diarrhea,* Journal of Pediatrics, Sept. 1991, Vol. 119, No. 3 (Section 9.0, Tab Y)

Hanning Ph.D., R.M., Drew Ph.D., M., *Bovine Immunoglobulin Feeding Trial* (Unpublished, Section 9.0, Tab I)

> Twelve healthy adults participated in a short-term feeding study that supports the safety and acceptability of bovine immunoglobulin when administered at a rate of 10 g per day.

Brown, K.H., Santizo, M.C., Begin, F., Torun, B., *Effect of Supplementation with Multiple Micronutrients (MMN) and/or Bovine Serum Concentrate (BSC) on the Growth of Low-Income, Peri-Urban Guatemalan Infants and Young Children* (Currently in draft form only, Section 9.0, Tab Z)

> 259 children (6-14 months of age) were enrolled and 171 completed a long-term feeding study in which they were fed daily a pudding fortified with 3.7 g bovine serum (0.74 g immunoglobulin). No adverse effects on anthropomometry[1] were reported.

Haskell, M.J., Santizo, M.C., Begin, F., Torun, B., Peerson, J. M., and Brown, K.H., *Effect of Supplementation with Multiple Micronutrients (MMN) and/or Bovine Serum Concentrate (BSC) on Serum Retinol (R) and α-Tocopherol (TL) Concentrations of Low-Income, Peri-Urban*

Guatemalan Infants and Young Children, (Currently in draft form only, Section 9.0, Tab Z)

> 259 children (6-14 months of age) were enrolled in an 8 month feeding study in which they were fed multiple micronutrients and whey protein concentrate; bovine serum concentrate; multiple micronutrients and bovine serum concentrate; or whey protein concentrate only. No adverse effects on anthropomometry[1] were reported.

Earnest, Ph. D., C.P., Jordan, MS., A.N., Safir, RD., M., Weaver, Ph.D., E., *The effect of a bovine serum immunoglobulin fraction bIg) on blood lipid indices in mildly hypercholesterolemic subjects*, (Currently in draft form only, Section 9.0, Tab H)

> 52 male and female adult (25-70 years) volunteers with moderate hypercholesterolemia (210-270 mg/dL) participated in a double blind, placebo controlled study, receiving 5g of bIg. Consumption of bovine IgG appears to positively modulate major lipid indices associated with cardiovascular disease.

[1] human body growth measurements

4.3 Mechanics of Action

As may be noted in the enclosed published papers, Proliant Inc.'s technical sister company, APC, Inc., has carried out years of animal studies showing the efficacy of plasma. The actual mechanics of this vital IgG component seems to be the colonization of the gut preventing other harmful bacterial from attaching and causing diarrhea.

Since the gut is a primary source of exposure to pathogens, healthy immunity begins with the mucosal membrane system in the intestinal tract.

We have traditionally considered the globulin protein for its nutritional value as a source of the building blocks of protein, the amino acids. However, past animal studies have shown a reduction in deaths when bovine plasma is included in animal diets. It seems reasonable to assume that this phenomenon may also occur in humans. To this end, Proliant has produced its immunoglobulin, bovine globulin for intestinal wellness. Some animal studies have suggested that it may assist in tissue healing, i.e.: ulcers in swine.

As a food ingredient, our globulin is a high protein replacement ingredient for soy and dairy proteins to avoid allergic reactions.

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5.0 Quantitative Detection Methods in Food

5.1 Determination of IgG SOP 2800-400-129 Radial Immune Diffusion

This method will determine the quantitation of bovine IgG in serum or plasma (Standard Operating Procedure # 2800-400-129-00).

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A. PURPOSE

The purpose of this document is to describe the procedure for the preparation of Radial Immunodiffusion (RID) to determine the quantitation of Bovine or Porcine IgG in serum or plasma.

B. SCOPE

This procedure applies to all employees in the Quality Assurance (QA) department at Proliant Inc. It is the responsibility of the QA Manager, or designee, to coordinate and oversee this procedure.

C. RELATED DOCUMENTS

SOP# 2800-400-112 pH Water Method For Plasma, Blood-Based Powders, Lyophilized BSA, and Whey Protein Products.

D. DEFINITIONS

Radial Immunodiffusion is based on the diffusion of antigen from a circular well into a homogeneous gel containing specific antiserum for each particular antigen.

E. REFERENCES

1. The Binding Site, Immunobiological Reagents 1997/1999.
2. Triple J Farms, Bovine and Swine IgG Test Kit – 200 to 3000 mg/dl.
3. AD001 Electronic Plate Reader, User's Manual
4. Accumet AB15 User Manual, Fisher Scientific

F. SAFETY

None.

G. MATERIAL/EQUIPMENT

1. Radial Immunodiffusion Plates
2. Electronic Plate Reader
3. Calibration Plate
4. Bovine and Porcine calibrator
5. 50 ml centrifuge tubes and rack
6. 15 ml centrifuge tubes and rack
7. Microcentrifuge tubes and rack
8. Accumet AB15 pH meter or equivalent
9. pH 4.00 and 7.00 calibration buffers
10. 10% NaOH and 2 M HCl
11. Nanopure water
12. Saline (NaCl 0.146 M) filtered

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13. Spoonula
14. Analytical balance
15. Various pipets and tips to dispense amounts between 5 ml and 5 ul
16. Rocker
17. Vortexer

H. PROCEDURE

1.0 Reagents and Media

1.1 10% NaOH. IE: Weigh 100 g NaOH into a 1 L of volumetric flask. Dilute to volume with nanopure water and mix well. Other similarly scaled amounts may be prepared.

1.2 2 M HCl. IE: Add about 50 ml of nanopure water into a 100 ml volumetric flask. Add 16.7 ml of conc. HCl (12 M) and dilute to volume with nanopure water and mix well. Other similarly scaled amounts may be prepared.

1.3 Saline (NaCl .146 M). IE: Dilute 8.5 g NaCl with 1 L of nanopure water. Then filter saline through an encapsulated filter (0.22 um) with pump or vacuum filter.

1.4 Bovine Calibrator, Midland BioProducts, IgG concentration 34.4 mg/ml.

1.5 Porcine Calibrator, Midland BioProducts, IgG concentration 25 mg/ml.

2.0 Sample Preparation

2.1 Powder plasma samples: re-hydrate 1:10 with saline. IE: 1.00 g sample and 9.00 g of saline. Vortex until slightly mixed (3-5 seconds). Place on a rocker for a minimum of two hours until samples are completely dissolved. If the sample does not go into solution, a homogenizer may be used.

2.2 Note: If further dilutions are necessary, after rocking, use diluted rehydrated sample. Mix well by inverting.

2.3 Record the pH for all samples after dilutions are completed. (Reference #SOP 2800-400-112). If the pH is not between 6.5 – 7.5, add the appropriate amount of 10% NaOH or 2 M HCl to adjust the pH and

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record the new pH. Also record the amount of 10% NaOH or 2 M HCl used, if any, for the adjustment. This amount, along with any previous dilutions made on the sample, will be added into the final dilution factor.

3.0 Standard Preparation

3.1 Bovine Standards: Total of 200ul in each standard.

Conc. mg/ml	%	Calibrator ul	Saline ul
3.44	10	20	180
6.88	20	40	160
13.76	40	80	120
20.64	60	120	80

3.2 Porcine Standards: Total of 200ul in each standard.

Conc. mg/ml	%	Calibrator ul	Saline ul
2.5	10	20	180
5.0	20	40	160
10.0	40	80	120
15.0	60	120	80

3.3 Note: The concentration of the Bovine and Porcine Standards may change due to the variation in lots.

4.0 Plate Care

4.1 Plates must be stored upside down in the refrigerator. Remove plates from the refrigerator to room temperature for approximately 30 min. before filling the radial wells. Do not open bag until ready to use.

4.2 If excess moisture is present, remove plate from bag and remove cover until excess moisture has evaporated from the surface and wells. Replace cover until used.

4.3 Shrinkage of gel or oval shaped wells indicates drying and the plate should not be used.

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4.4 Unused sections may be run on a later day if the plate has been stored between 2 to 8°C between incubations in its plastic bag. Check carefully for evidence of drying.

5.0 Method

5.1 Fill out date of completion, read time, type of protein, and the appropriate sample and standard on each radial well.

5.2 Remove plate from the bag and remove cover from the plate.

5.3 Place 5 ul (do not overfill) of each standard and sample in duplicate into the radial wells by placing the pipette tip at the bottom of the well. Avoid bubbles to ensure proper volume and diffusion of sample.

5.4 Replace cover and replace plate in bag and reseal carefully.

5.5 Incubate plate upright on a flat surface at room temperature for at least 24 hours for End Point readings.

6.0 Calibration

6.1 Turn the micrometer-adjusting knob on the AD001 Electronic Plate Reader clockwise slowly to its maximum reading position.

6.2 Using the pointer on the knob as a guide turn the micrometer adjusting knob counter clockwise two full turns.

6.3 Using the calibration control knob set the liquid crystal display (LCD) reading to 12.64. The instrument is now calibrated.

6.4 Check the calibration against the two standards. (The standards consist of metal discs cemented to an empty RID plate.) The reading on the LCD display should be within +/- 0.08 mm of the standard values.

7.0 Calculations

7.1 Use the QA RID spreadsheet found in Excel.

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7.2 Enter the four standard ring diameters, then check the R squared value (Correlation Coefficient). The R squared value should be as close to 1.000000 with a minimum of .990000.

7.3 Enter the control sample first and check to see if the % average falls within the designated minimum and maximum limits. If the control does not fall between this range then the test fails and needs to be retested.

8.0 Notebook / Documentation

8.1 Record the analyst's initials, date, name of the test, SOP number, balance used, lot number of gel, and pH calibrations.

8.2 Record plant initial, lab number, pallet number, SKU number, and final dilution factor.

8.3 Record the ring diameters of all standards, samples, and the control in duplicate. Then record the average of the two ring diameters.

8.4 Record the initial pH values and the pH adjusted values of the control and samples.

I. ATTACHMENTS

None.

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Originator	(b) [redacted]	Date	7/2/02
QA Lab Supervisor Corporate	Position Open	Date	
QA Manager Corporate	(b) [redacted]	Date	7-2-02
Director of QA Corporate	(b) [redacted]	Date	7-8-02
Vice President of QA Corporate	(b) [redacted]	Date	7/9/02

5.2 Speciation Test (for non-bovine origin products) PCR (Polymerase Chain Reaction)

The presence of bovine DNA can be detected in food materials by PCR methodology. Proliant Inc. uses Genetic ID for speciation/identification testing of our products. Sensitivity is at <10 ppm.

Revised June 2002

Method for detecting animal DNA in animal feeds by PCR (Polymerase Chain Reaction)

This method consists of detecting and confirming the existence or not of mammal, ruminant, cow, pig or chicken tissues in composite foods or animal feeds through detection of the typical mitochondrial DNA for each animal in the corresponding tissues.

1. Samples and test subjects

1.1 Test foods

Composite foods for animals and materials used in animal feeds.

1.2 Test animals

Mammals, ruminants, cows, chicken, sheep, goats, etc.

The animals that can be detected by each primer are shown below.

In this revision, primers from pigs and plants have been added.

List of test animals

Primer type (sources)	Animals for which detection is available
1. Mammal detector primer (anicon5, anicon3) *1	Cows, pigs, sheep, goats, horses, deer, hares and whales (does not detect humans, fowl, fish or shellfish #)
2. Ruminant detector primer (rumicon52, rumicon32)*1	Cows, goats, sheep, deer (does not detect pigs, horses, hares, whales, fowls, fish or shellfish)
3. Cow detector primer (cow52, cow32)*1	Cows (does not detect pigs, sheep, goats, horses, deer, hares, whales, chicken, fish or shellfish)
4. Cow detector primer (L8129, H8357)*2	Cows (does not detect pigs, sheep, goats, horses, deer, whales, chicken, fish or shellfish)
5. Chicken detector primer (SIM, chicken primerC)* 3	Chicken, quails (does not detect cows, pigs, sheep, horses, deer, whales, fish or shellfish)
6. Sheep and goat detector primer (SIM, sheep primers)*3	Sheep and goats (does not detect cows, pigs, horses, deer, whales, chicken, fish or shellfish)
7. Pig detector primer (pig5-3, pig32-2)*1	Pigs (does not detect cows, sheep, goats, deer, hares, whales, chicken, fish or shellfish)
8. Plant detector primer (placon5, placon3)*1	Maize, rice ear, soy, beetroot, wheat (does not detect cows, pigs, chicken, fish or shellfish)

*1 Substances developed by the Biomaterial Research Institute and this center. 1., 2. and 3. are supplied by the Animal Food and Fertilizer Inspection Center. 7. and 8. will be supplied in the future.

*2 Marco Tartaglia, et al., Journal of Food Protection 61 (5) 1998.

*3 T. Matsunaga, K. Chikuni, R. Tanabe, S. Muroya, K. Shibata, J. Yamada and Y. Shinmura, Meat Science 51, 143 (1999).

For fish and shellfish, cod, salmon, sardine, crab, prawn and clam are already confirmed.

1.3 Detectability

The detectability of cow tissues in food products and feeds by this method is as follows (in percent)

1. Mammal detector primer 0.1 ~ 0.01%
2. Ruminant detector primer 0.1 ~ 0.01%
3. and 4. Cow detector primer 0.1 ~ 0.01%
5. and 6. the detectability of these substances has not yet been studied.

The detectability of meat and bone tissues sometimes depends on the percentage composition of animal materials in feeds, the manufacturing methods (conditions in which they are heated) and the percentage content of meat and bone tissues.
This method for the detection of animal DNA is used not only in animal tissues but also in materials with skimmed powdered milk or plasma proteins that contain this DNA.

2. Sample obtainment and milling

2.1 Sample obtainment

In principle, the samples will be obtained from wrapped materials that cannot be contaminated by other materials or by other circumstances during storage.
The samples will be obtained using the appropriate method for later performing an organism detection test and taking great care not to contaminate them with foreign substances.
Approximately 500 grams of sample will be taken, using plastic gloves and a sterilized spatula and placing in sterilized collection bags.

2.2 Milling, etc.

The powdered meat, bones and fish are not milled (as there is a high risk of contamination if they are milled). If there is any unusual particle size, they are sieved (1 mm), cleaned and what has gone through the sieve is taken.
In the case of composite foods, they are milled until they can pass through the 1-mm sieve. In this case, the mill is changed or cleaned after each sample, to prevent cross-contamination between samples.

2.3 Sample storage

The samples are kept cooled. If the milled samples are kept for long periods (more than one week), they will be kept in the freezer (-20°C).

3. Test

3.1 Obtaining the test medium

If not specified otherwise, water passed through the ion exchanger or distilled water will be used. To obtain the liquid used in the PCR reaction and to dilute the DNA extracted, as in the cases mentioned below, purified water will be used (RO water purified by reverse osmosis film or distilled water purified by Milli-Q, etc.), until the comparative resistance of 17MΩ/cm is attained) after sterilizing in the autoclave (for 15 minutes at 121°C).

1) Apparatus for the extraction of mitochondrial DNA from tissues and cells. A mtDNA extractor CT assembly (Wako Junyaku) or similar will be used.
2) 0.5M EDTA solution
   Add 18.6 g of disodium ethylenediaminetetraacetate dihydrate to 80 mL of purified water and, after adjusting to pH 8.0, add about 2 g of sodium hydroxide in grain, add more purified water sterilized in the autoclave for 15 minutes at 121°C, until a total volume of 100 mL is obtained.
3) 1M tris hydrochloric acid regulating solution
   Add 12.1 g of tris hydroxymethylaminomethane to 80 mL of purified water and add about 4.2 mL of hydrochloric acid after adjusting to pH 8.0, followed by purified water to give a total volume of 100 mL. Autoclave for 15 minutes at 121°C.

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4) Electrophoresis regulating solution (TAE regulating solution)
Dilute 4.84 g of tris hydroxymethylaminomethane, 1.14 mL of acetic acid and 2 mL of EDTA solution in water to give a total volume of 1000 mL.
5) TE regulating solution
Dilute 1 mL of tris hydrochloric acid regulating solution and 0.2 mL of EDTA solution in water to give a total volume of 100 mL. Autoclave for 16 minutes at 121°C.
6) Agarose gel
Add 2.5 g of extrapure agarose (electrophoresis grade) (Sawady Technology) or an equivalent substance to 100 mL of electrophoresis regulating solution, heat until dissolved and then cool to 50~60 ℃, pour into the mold so that the gel thickness is 3-4mm, and insert the comb, making sure that no bubbles are formed between the gel and the comb, and leave at room temperature so that it can solidify.
7) Electrophoresis dye solution
Dissolve 25 mg of bromophenol, 25 mg of xylene cyanol and 3 g of glycerin in purified water and autoclave for 15 minutes at 121℃, obtaining a final volume of 10 mL.
8) DNA size marker (50~1000 pb) (FMC, Takara Shuzo, etc.).
9) Ethidium bromide solution (Aldrich Chem)
Prepare an undiluted solution with 10 mg of ethidium bromide (Aldrich Chem.) and 500 μL of purified water. To make the test solution, dissolve 25 μL of this solution in 1000 mL of electrophoresis regulating solution.
Store this undiluted ethidium bromide solution in a cool, dark place since it decomposes when exposed to light. Ethidium bromide is carcinogenic. Consequently, gloves must be used when handling and, after completion of the test, it must be filtered through an absorbent filter and discarded. The agarose gel stained by ethidium bromide must also be discarded after immersing in a 5% sodium hypochlorite solution and exposing to direct sunlight to promote decomposition.
10) Zirconium beads (1.5 mm diameter)
Sterilize the YTZ Bead (Nikkato Co.) or similar in the autoclave for 15 minutes at 121℃.
11) Detector primer
   1) Mammal detector primer (anicon5, anicon3): the band size is 176 pb
   2) Ruminant detector primer (rumicon52, rumicon32): 104 pb
   3) Cow detector primer (cow52, cow31): 120 pb
   4) Cow detector primer (L8129, H8357): 271 pb
   5) Chicken detector primer (SIM, chicken PrimerC): 227 pb
   6) Sheep and goat detector primer (SIM, sheep PrimerS): 331 pb
   7) Pig detector primer (pig5-3, pig32-2): 126 pb
   8) Plant detector primer (placon5, placon3): 140 pb

   1 Mammal detector primer, ② Ruminant detector primer ③ Cow detector primer are supplied by the Animal Food and Fertilizer Inspection Center, ⑦ Pig detector primer, and ⑧ Plant detector primer, will be supplied later on.
12) Enzyme for PCR reaction
Ampli Taq Gold (Applied Biosystems)
13) PCR regulating solution
PCR buffer II (Applied Biosystems), PCR Gold buffer (Applied Biosystems) or similar.
14) 2mM dNTPmix (Applied Biosystems)

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15) Positive control
Use mitochondrial DNA from cows, chicken, sheep, etc. extracted as described in section 3.3.

3.2 Apparatus and instruments

1) Thermal Cycler
PE9600, PE9700 (Applied Biosystems), PCR Thermal Cycler MP (TaKaRa) or similar.
2) Clean bench
3) Milling device with beads
2000 rpm revolution capacity and couples to 2-mL tube.
BC-20 (Central Kagaku Trading), Multi Beads Shocker (Yasui Kikai), FastPrep
FP120 (Funakoshi), Mixer Mill MM300(QIAGEN) or similar.
4) Autoclaves
5) Electrophoresis device
Mupid-2 (Cosmobio) or similar.
6) Polaroid camera
7) UV transilluminator
(Cosmobio, 365nm) or similar.
8) High-speed cooling centrifuge
Connect using 1.5-mL and 0.2-mL tubes, cool to 4℃, and spin at 12,000 x g.
9) Microtube micropipette
For 1000, 200, 100, 20 and 2 μL, digitally interchangeable.
10) Plastic tubes for 2 mL, 1.5 mL and 0.2 mL , and several micropipette chips (sterilized)

3.3 DNA extraction

We will now explain the method for extracting DNA from tissues and cells using mitochondrial DNA extraction devices (mtDNA Extractor CT kit)(Wako Junyaku); it is a method that is generally available on the market and, in our opinion, has given good results.
We also have reports of the methods for extracting DNA from feeds, such as the phenol chloroform method, the method which uses guanidine thiocyanate (Tartaglia et al. #2), and the method which uses synthetic resin (Wang et al. #4), etc.

1) Place 100 mg of sample in a 2-mL plastic tube with a screw top, add about 1.5 g of zirconium beads, add 1 mL of extraction regulating solution (included with the mtDNA extraction apparatus). Using the bead mill (*), stir for 1 minute at 2,000 rpm. After leaving to rest for 1 minute, stir again for 1 minute at 2,000 rpm and then cool with ice and spin for 2 minutes at 1,000 x g (4℃).
(*) This can be the usual method for homogenous extraction, taking precautions to prevent cross-contamination between the samples .
Take 1.5 mL of the supernatant, place in a plastic tube and spin for 10 minutes at 10,000 x g(4℃), then discard the top layer of the liquid.
2) Add 50 μL of DNA I extraction solution (included with the apparatus), leave in suspension for pipetting and add 100 μL of DNA II extraction solution (included with the apparatus) and mix; then cool with ice for 5 minutes.
3) Add 75 μL of DNA III extraction solution (included with the

apparatus and cooled with ice) and then vortex; cool immediately with ice.

4) After spinning (4°C) for 5 minutes at 12,000 x g, pour the supernatant into the new 1.5 mL tube.
5) Add to this solution 300 µL of sodium iodide solution (included with the apparatus) and mix; add 500 µL of isopropanol and mix.
6) After spinning for 10 minutes at 12,000 x g (room temperature), discard the supernatant.
7) After adding and mixing 1 mL of 70% ethanol, spin for 5 minutes at 12,000 x g. Add a further 1 mL of 70% ethanol and mix, spin for 5 minutes at 12,000 x g, discard the supernatant and after drying by decompression, dissolve the DNA by adding 20 µL of TE solution. This DNA solution is kept frozen.

3.4 PCR reaction

The usual procedure for the PCR method is followed.

It is advisable to use the anicon5, anicon3 primer set for detecting mammal DNA, and rumicon52, rumicon32 for detecting ruminant DNA, cow52, cow31 or L8129, H8357 by Tartagli et al. (#2) for detecting cow DNA.

The reaction conditions for each primer have been described in the enclosed sheet 1{DNA REACTION CONDITIONS}.

The procedure to be followed is summarized as follows.

1) The test solution consists of 18 µL of PCR reaction solution and 2 µL of extracted DNA solution diluted 10-fold, mixed in a 200-µL plastic tube.
2) The positive control solution consists of 18 µL of PCR reaction solution mixed with 2 µL of cow DNA solution diluted 10-fold. The negative control solution consists of 18 µL of PCR reaction solution mixed with 2 µL of water.
3) Pour the test solution, the positive control solution and the negative control solution into the thermal cycler, and carry out the PCR reaction in the conditions described in the enclosed sheet 1.

3.5 Electrophoresis

1) Pour agarose gel into an electrophoresis bowl containing electrophoresis regulating solution and perform the preparatory electrophoresis at a constant voltage of 100V for 10 minutes.
2) Add 1 µL of electrophoresis dye solution to 5 µL of DNA size marker and mix, pour the entire mixture into the agarose gel slot.
3) After completing the PCR reaction, mix the test solution with 5 µL each of positive control and negative control solutions and about 1 µL of electrophoresis dye solution and pour the entire mixture into the agarose gel slot.
4) Perform the electrophoresis at 100V and continue until the bromophenol blue moves 3 -4 centimeters from the slot.
5) After completing the electrophoresis process, immerse the agarose gel in the ethidium bromide solution for about 30 minutes so that it can stain.
6) Place the agarose gel in the UV transilluminator and expose to ultraviolet radiation at 365 nm. Compare the position of the DNA band detected in the test solution with the position of the size marker band and determine the size of the DNA fragment. Photograph with a black and white Polaroid camera.

3.6 Decision method

When the size marker band is detected in the positive control but not in the negative control and a band of the same size as in the positive control is detected in the sample, the test is considered POSITIVE.

When the band is not detected in the positive control and is detected in the negative control, it is possible that the PCR has reacted incorrectly and the test must be repeated.

4 Inspection considerations.

4.1 Precautions during performance of the test.

The PCR method is very effective and detects very small quantities of DNA. For this reason, when performing the analysis, it is very important to prevent any other DNA becoming mixed with the DNA being analyzed. As the DNA is decomposed by microorganisms or by enzymes and is found on human skin, etc., appropriate sterilization precautions must be taken.

1) The micropipette chip must be sterilized and a new one must be used in each operation. It is preferable to use a chip that has a filter.
2) For the analysis, use plastic gloves and also sterilize with alcohol.
3) Sample obtainment and milling, performance of the PCR and electrophoresis must be carried out in separate places. The PCR reaction solution must be prepared in a clean place.

4.2 Precautions for ensuring accuracy of the inspection.

1) Two DNA samples must be taken simultaneously from a material and both DNA samples must undergo the reactions also simultaneously.
2) After performing the PCR reaction, the positive control and negative control must be performed simultaneously.
3) For the double check, it is advisable to use more than two primers. For example, to distinguish between cow, sheep, etc. species, it will be better to use a mammal detector primer and a detector primer for the particular species.

4.3 Decision on the result of the test.

The test can only be considered POSITIVE when two positive results are obtained from DNA samples taken from a material.

References

(#1) Not quoted

(#2) Marco Tartaglia, Ernestina Saulle, Simonetta Pestalozza, Luisella Morelli, Giovanni Antonuzzi and Piero A. Battaglia; Journal of Food Protection 61 (5), 513 (1998)

(#3) T. Matsunaga, K. Chikuni, R. Tanabe, S. Muroya, K. Shibata, J. Yamada and Y. Shinmura; Meat Science 51, 143 (1999)

(#4) R-F. Wang, M.J. Myers, W. Campbell, W-W. Cao, D. Paine and C.E. Cerniglia; Molecular and Cellular Probes 14, 1 (2000)

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6.0 Full Report on Safety Status – Basis for GRAS Determination

6.1 Historical Perspective

The basis of GRAS determination can be argued on both a practical and scientific basis.

6.1.1 Edible Whole Blood

Bovine blood has been commonly used in food for hundreds of years. Blood sausage is an excellent example of this application.

A meatless blood dish, "Blood Pudding" is a staple of England and Germany and "Blood Curd Soup" is a popular product in Asia. Articles on edible blood products, Section 9.0, Tab D.

The USDA recognizes these products in their Food Standards and Labeling Policy Book and does not restrict the amount of blood used in these products (Section 9.0, Tab E.

6.1.2 Edible Bovine Plasma

Edible, spray-dried bovine plasma has been sold by Proliant Inc. into the surimi industry for protease inhibition since 1993 in Japan. It has also been used as a seafood analogue, replacing surimi.

Spray-dried bovine plasma has been used for over 10 years to replace meat and maintain texture in emulsified products that are consumed heated or fried. It has been used to partially replace soy protein improving some properties of tumble/injected products like hams.

Manufacturers have found improved slice-ability in whole hams when the plasma is part of the formula. In addition, the use of spray-dried plasma has improved the cohesion of ground meat products that are thermally processed. United States manufacturers have been able to increase texture, replace other more expensive ingredients in processed meats and reduce purge in injected whole meat products.

6.1.3 Edible Globulin

Proliant Inc.'s spray-dried bovine globulin has been sold into the dietary/nutritional supplement industry since November 1999. Over 30,000 pounds of finished powder has been sold and consumed over the past four years with no reported adverse reaction from recipients or incidents of consumer complaints. Proliant (LGI Laboratories) notified the FDA (Food and Drug Administration) of its intention to sell this product in November 1999 (Section 9.0, Tab N). In addition, a letter of intent was sent to Dr. Robert Post, Director Labeling and Additives Policy Division, USDA/FSIS, Office of Policy, Program Development and Evaluation, stating the name for our product would be Bovine Serum Globulin (Section 9.0, Tab AA). Dr. Post had no objection to the label (Section 9.0, Tab AB).

Over the past four years, globulin has been used in tablets and nutritional drinks without incident concerning its safety and use (see Table 2 in Section 3.0). Since we have a new customer intending to use the globulin in a dairy product application, Proliant Inc. now wishes to seek GRAS status and enlarge our area of sales as a food additive as well as a dietary supplement.

6.2 Regulatory Status

Title 9 CFR § 310.20 - Saving of blood from livestock as an edible product.

This section states that blood may be saved for edible purposes at official establishments provided it is derived from inspected and passed livestock carcasses, and that the blood is collected and treated with an approved anticoagulant so as not to render it adulterated under the Federal Meat Inspection Act and other regulations pursuant thereto.

In addition, Title 9 CFR § 318.6 - Requirements concerning ingredients and other articles used in preparation of products (Section 9.0, Tab AC)

> "318.6 (b)(7) Blood from livestock prepared in accordance with § 310.20 of this sub-chapter may be used as an ingredient of a meat food product for which a standard is prescribed in part 319 of this subchapter, if permitted by such standard, and may be used in any meat food product for which no such standard is prescribed in part 319 of this sub-chapter if it is a common and usual ingredient of such product."

Blood and therefore its derivatives are deemed edible and fit for human consumption.

6.3 Heavy Metals

With regard to the concentration of heavy metals, there appears to be no increase in the lead content during the processing steps. Our early data shows values remain below 0.5 in the finished product (table below). This data compares favorably to data of dried milk and dried meat proteins and should pose no additional risk to foods in which this globulin is added.

Table 3 – 1999 Heavy Metals Data

Date Tested	Metal	Results
Sample 1 (Jan)	Lead	<1 ppm
	Arsenic	<1 ppm
	Mercury	<0.05 ppm
Sample 2 (May)	Lead	<1 ppm
	Arsenic	<1 ppm
	Mercury	<0.01 ppm
Sample 3 (Sep)	Lead	<1 ppm
	Arsenic	<1 ppm
	Mercury	<0.32 ppm

See additional data on the Silliker, Inc. Certificate of Analysis (Section 9.0, Tab L, data outlined in red) for 10 samples of Spray-Dried Globulin produced during 2004.

6.4 Pesticides

An organochlorine pesticide screen was conducted on a composite sample of ten lots of globulin (see Organochlorine Pesticide Screen on Silliker Certificate of Analysis (Section 9.0, Tab L, data outlined in blue) resulting in less that 0.1 ppm in all categories.

The reported value is below the tolerance of 2 ppm that USDA uses for acceptance of muscle meats.

6.5 Antibiotics

The same 10 samples of spray-dried powdered globulin were also tested for antibiotic residues. It is important to note that USDA/FSIS conducts daily checks at every slaughter facility for antibiotic residues in animals being slaughtered which assures that safe initial levels of antibiotics will be maintained in the whole blood we purchase (on Silliker Certificate of Analysis, Section 9.0, Tab L, data outlined in green).

6.6 Hazardous Substances from the Process

We cannot identify or foresee any detectable risks of hazardous substances resulting from our processing procedures. Most of the ingredients added at the beginning of processing are removed during processing.

All additives are approved for use in foods and carry GRAS status. This information is from Title 21 CFR § 182 Substances Generally Recognized As Safe.

The only additives/processing aids used to manufacture the globulin are as follows:

A. Defatting Agent

21 CFR Part 182 – Substances Generally Recognized As Safe - § 182.90 - Substances migrating to food from paper and paperboard products.

GRAS status additive/processing aid approved for use in foods as a filter column to separate fractions of blood plasma during purification process and separate out fats.

B. Edetate Disodium (EDTA) - VERSENE® NA

21 CFR Part 172 – Food Additives Permitted for Direct Addition to Food for Human Consumption, Subpart B – Food Preservatives § 172.135 Disodium EDTA

To stabilize trace minerals in aqueous solution
IFN 8-05-689

C. Food-Grade Phosphate

21 CFR Part 182 — Subpart G – Sequestrants - § 182.6760 – Phosphate

Generally recognized as safe when used in accordance with good manufacturing practices.

D. Food-Grade Acid

21 CFR Part 182 – Subpart B – Multiple Purpose GRAS Food Substances; § 182.1057 –Acid

Generally recognized as safe when use in accordance with good manufacturing practices.

E. Food-Grade Alkaline

21 CFR Part 184 – Direct Food Substances Affirmed as Generally Recognized as Safe — Subpart B – Listing of Specific Substances Affirmed as GRAS - § 184.1763 – Alkaline

These ingredients have been used in similar food systems without any apparent risks. Individually and in combination with foods, these ingredients form no hazardous compounds based on their previously approved GRAS status.

F. Sodium Citrate

21 CFR Part 184 Direct Food Substances Affirmed as Generally Recognized as Safe — Subpart B – Listing of Specific Substances Affirmed as GRAS - § 184.1751 – Sodium citrate

Anticoagulant added to whole blood during the blood collection process.

GRAS status additive approved for use as an anticoagulant in the blood collection process. This solubilized sodium citrate solution is added directly to the whole blood during the collection step to prevent coagulation and aid in the centrifugation/separation process.

6.7 Oxidation/Carbonation

Our thermal process (spray drying) does not elevate the temperature of the product to the degree that any degradation would occur, such as excessive oxidation or carbonation. This is verified by the amino acid and free fatty acid profile levels showing nearly identical values to those found prior to drying (taking into account the loss in moisture). Contact Proliant Inc. for validating data.

6.8 Microbiological Hazards

The hazards associated with the microbiological toxins or pathogens are controlled by the USDA/FSIS monitored HACCP Programs at our blood plasma suppliers and our FDA bulk pharmaceutical facility.

Incorporated into the collection facility HACCP Program is the sanitary method for blood collection and the rapid cooling of the separated plasma from the red cells. The plasma remains below 45°F at the collection facility and during transit in a dedicated tanker to the bulk pharmaceutical plant. Upon receipt, the plasma is immediately processed using centrifugation, refrigerated storage and spray drying/frozen packaging.

The microbiological integrity of the incoming plasma is monitored by the lab at the manufacturing facility. In addition, the spray-dried product is tested to show adherence to finished product specifications (Table 4, next page).

These microbial quality levels fall well within those found for whey protein products and are equal to or exceed those know to be safe for foods.

At no time do the processing conditions or equipment allow for pathogen growth. Such growth would be detected by either microbiological testing or routine QC/Sanitation checks.

Proliant Inc.'s globulin product is not released for sale until compliance with the specifications is confirmed by Corporate Quality Assurance.

Table 4 – 2004 Microbiological Data

ImmunoLin®

	(cfu/g) <100	(cfu/g) 100 - 399	(cfu/g) 400 - 999	(cfu/g) >1000	
SPC	61	65	11	5	
142 Total SPC tested					min = <100 cfu/g
%	43.0%	45.8%	7.7%	3.5%	max = 7400 cfu/g

	(mpn/g) <3.0	
Coliforms	87	
87 Total Coliform test		min = <3.0 mpn/g
%	100%	max = <3.0 mpn/g

	(mpn/g) <3.0	
E.coli	87	
87 Total E.coli test		min = <3.0 mpn/g
%	100%	max = <3.0 mpn/g

	(cfu/g) <10	
Yeast	87	
87 Total Yeast test		min = <10 mpn/g
%	100%	max = <10 mpn/g

	(cfu/g) <10	(cfu/g) 10	
Mold	82	4	
86 Total Mold test			min = <10 cfu/g
%	95.3%	4.7%	max = 10 cfu/g

	Negative	
Salmonella	86	
86 Total Salmonella test		min = Neg
%	100%	max = Neg

6.9 Water Activity

The water activity (during the Proliant spray drying process) of the spray-dried powder is reduced to below 0.50. As long as it is protected from the re-absorption of moisture during subsequent handling and storage, there can be no additional microbiological growth in the product.

The thermal spray drying process is responsible for the thorough destruction of pathogenic organisms. The moisture-protected, heat-sealed, poly-lined bag assures a safe means of storage and transfer of this concentrated nutritive protein.

6.10 Allergens

Neither meat nor blood products are listed in the U.S. nor EU as one of the top 8 major food or food groups considered to be allergens: milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans (Section 9.0, Tab J). Therefore Proliant Inc. does not consider this bovine product to be allergenic or problematic. Since the term bovine or beef is carried through on all labeling, the consumer would have proper information for decision making, whether cultural or dietary in nature.

6.11 Bovine Spongiform Encephalopathy (BSE)

Only USDA-inspected and approved edible beef plasma is used in the production of ImmunoLin® and NutraGammax™. Proliant Inc.'s ImmunoLin® and NutraGammax™ products are manufactured exclusively from the bovine plasma of North American cattle. These animals have been slaughtered in USDA/FSIS-inspected and approved facilities where both ante- and post-mortem have been performed. These animals must pass both ante- and post-mortem inspection to be acceptable for human consumption.

To date, no native-born cattle in the United States have been found that have the disease Bovine Spongiform Encephalopathy (BSE). Only one case of BSE has been found in an imported animal from Canada in December 2003. The United States has an extensive BSE surveillance program in which more than 263,115 animals demonstrating neurological symptoms which may indicate the animal has BSE, have been tested by either histopathology or immunohistochemistry with only the one imported animal (from Canada) found to have BSE.

The tissues from BSE-infected cattle known to be infectious are only those of the central nervous system. The new USDA/FSIS edible meat program requires all SRM (Specified Risk Materials) be removed from the carcass before processing. These regulations require that the raw materials we use in the production of these products do not contain the following materials: spinal column, vertebrae of the tail, the transverse process of the thoracic and lumbar vertebrae, and the wings of the sacrum from animals 30 months and older. Plasma is not an SRM and remains to be an edible tissue as do our ImmunoLin® and NutraGammax™ products. These extraordinary measures also help assure the safety of our products. The tissues used in our ImmunoLin® and NutraGammax™ products are deemed safe since they are not those known to harbor the infective

agents of BSE. The global scientific community does not consider meat or blood as risk tissues in regards to BSE, and therefore their consumption as food is not restricted.

6.12 Use of Blood for Packaging Materials

6.12.1 In 1961 FDA gave GRAS status for the use of blood in food packaging material.

A GRAS petition from the Adhesive Manufacturers Association of American for the use of "animal blood' was submitted and approved in 1961 and is now found in the Code of Federal Regulations, Title 21, Part 175—INDIRECT FOOD ADDITIVES: ADHESIVES AND COMPONENTS OF COATINGS. The FDA concluded that this type of glue was acceptable for use in food packages due to an ". . . .inconsequential amount of migration" shown in test data . . . ". (Published in the Federal Register, January 20, 1961, Pgs. 621-624)

6.13 Patent Information

On December 4, 2003 Proliant applied for the patent on our globulin protein for lowering cholesterol in humans. Patent is pending (Section 9.0, Tab AD).

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7.0 Proposed Tolerances

Proliant Inc. has determined that no tolerances are needed for the product due to the historical use of whole blood and it derivatives for centuries in a wide number of food matrixes.

We therefore request that no tolerances be set for this bovine serum/globulin product.

7.1 Concentration Effect of Solids During Processing

As it may affect the concentration of hazardous substances in the ingredient:

Estimated Average Composition of Raw Plasma

- Protein 7%
- Moisture 91-92%
- Ash .8-1.2%
- Fat <1.0%
- Solids 8-9%

Concentrated Globulin Fraction

- Protein 88-90%
- Moisture 5-8%
- Ash 1.5-2.5%
- Fat <0.5%
- Solids 92-95%

Heavy Metals

The net maximum concentration effect would be 10x any inherent hazardous substances in the raw plasma.

Antibiotics

Regarding antibiotics, the USDA routinely tests the residue in the meat from slaughtered animals, requiring their levels to be below detection levels (1-10ppm on average).

One shipment (a truckload is 48,000 lbs.) of our plasma product will easily be the composite of 1,800 animals (4 gallons equals approximately 32 pounds) and thereby provides a diluting effect of any animals that may contain antibiotic residuals. We do not believe that the plasma has any more potential for antibiotics than the meat consumed by the general public.

In addition, spray-dried technical bovine plasma has been fed to animals and pets at unrestricted levels for over 15 years. This would suggest that no adverse levels of concentrated hazardous materials have been recognized or reported by either Proliant Inc. or APC, Inc. or to our knowledge the FDA to date. These ingredients include spray-dried whole blood, red blood cells, bovine plasma, serum, and globulin.

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8.0 Environmental Information

Not applicable.

9

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9.0 Data Availability

The data and information in this report are the basis for the petitioner's GRAS determination and are available for the Food and Drug Administration's (FDA) review and copying at reasonable times at the address set out in this notice and will be sent to FDA upon request.

A

000121.001

Field Trial of an Artificial Bovine Colostrum Replacer

K.P. Poulsen, M.T. Collins, S.M. McGuirk

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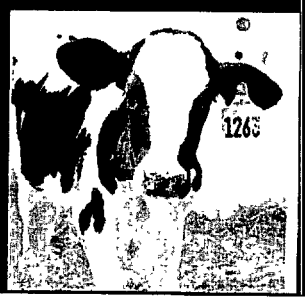
Importance of Dairy Heifers

- National shortage
- Average cull rates 30-35% per year
- For every 100 calvings 35 live heifer calves at weaning
- Dairy expansion increases heifer demand



Bacterial Contamination of Colostrum

- Industry standard: Total bacteria <100,000 cfu ml
- Data from problem herd investigations visits 36 samples from 8 farms
  - 19 (53%) bacterial counts >1,000,000 cfu/ml
  - 16:19 (84%) >10,000 fecal coliforms/ml

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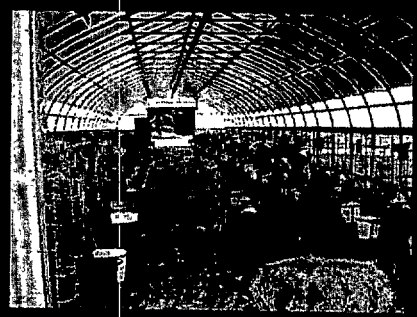
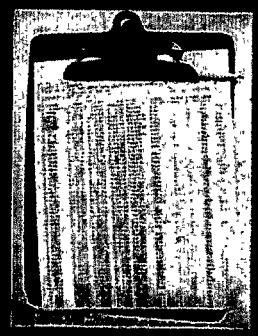
Colostrum as a Source of Infection?

- Farm dependent
- Bacterial contamination impairs passive transfer
- Important focus of calf health investigations
  - Maternity pens
  - Adult cows
  - Calf housing
  - Colostrum



Colostrum's Role in Johne's Disease Transmission

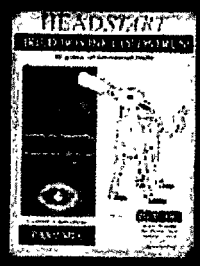
- *Mycobacterium paratuberculosis*
  - 22% of herds infected in USA
- Johne's disease control programs
  - Johne's positive dams cannot donate colostrum
    - How many truly negative cows are there?
  - Other disease control (BLV) and no colostrum used from springing heifers may create a shortage



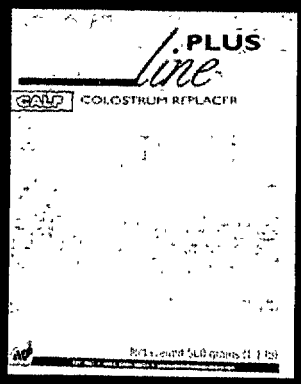
ACQUiRE
COLOSTRUM REPLACEMENT
Convenient. Safe. Effective

Lifeline Plus (Acquire)

Colostrum Supplement
- 30 g IgG per packet
- 70% whey protein



Colostrum Supplement
- 60 g IgG per packet
- Serum IgG @ 24 hours is 5 g/l
- PT serum IgG @ 24 hours requires 10 g/l
- No USA license



Colostrum Replacement
- 100 g IgG per packet
- 45% protein, 15% fat
- Vitamins and minerals
- Gets calves to PT

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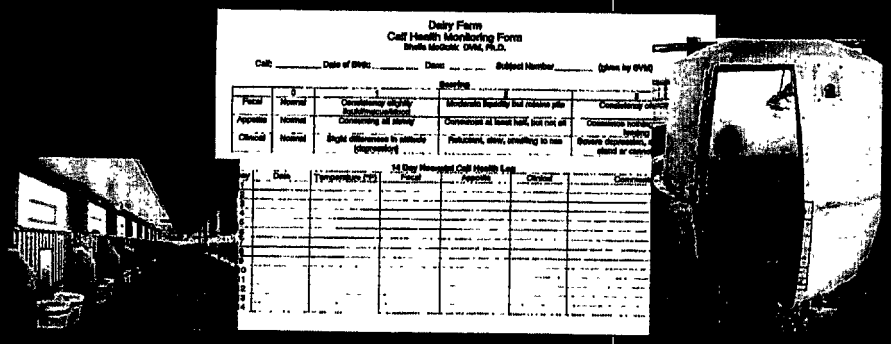
Acquire Quality Control

- Blood source is steers and is an edible collection plant
- Spray-drying effective pathogen kill step
- 3rd party pathogen monitoring
- Irradiated at 10 kiloGray to kill Johnes

On Farm

- Every other calf given Lifeline Plus within 6 hours of age and LifeLine at 12 hours of age
- Temperature, appetite, fecal, and attitude scored on 0-3 scale on sick calves only
- Blood drawn at 24 hours minimum age for TP measurement



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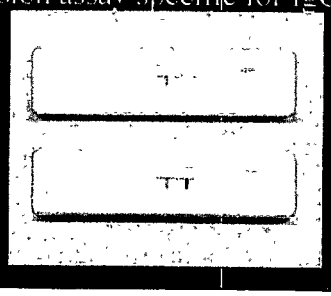
In the Laboratory

- Serum TP checked with refractometer
- Midland Quick Test done on all serum with <5.5 g/dl
- Failed Passive Transfer (FPT)= <5.5 g/dl + negative Midland Quick Test
- Radial Immunodiffusion assay specific for IgG



Preliminary Results: Passive Transfer

Based on serum TP and Midland Quick Test

Total Calves: 239 (Goal is 350 from 8 farms)

Colostrum Calves: 119. 8 Failed Passive Transfer

Acquire/Lifeline Calves: 120. 3 Failed Passive Transfer

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Preliminary Results: Serum IgG Levels

Work in Progress

Artificial Colostrum on the Farm

- Colostrum Shortages
- Poor Quality Colostrum
- Disease Control

VETERINARY COLUMN
School of Veterinary Medicine, University of Wisconsin
Drs. Sheila McGuirk
Ken Nordlund
Simon Peek

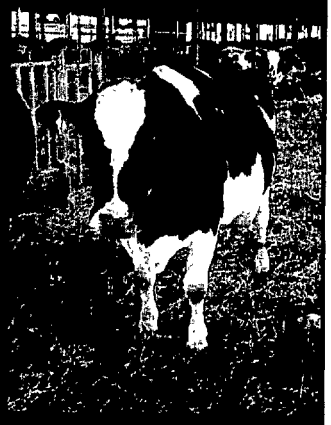
SHORT OF COLOSTRUM

We are trying to control Johne's by feeding colostrum only from cows with a negative ELISA blood test. At times, we struggle to have enough colostrum for our calves.

I heard that we should not feed colostrum from first-calf heifers or from adult cows that produce more than 18 pounds colostrum.

MINNESOTA S.K.

...e is the ...
...y product of its ...
and is a *safe an...*
...*ective colost...*



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Acknowledgements

Funding Provided by:

Merck-Merial, American Protein Corporation, the Wisconsin Milk Marketing Board and the University of Wisconsin School of Veterinary Medicine

Special Thanks to:

Carter Manley

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B

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2325 North Loop Drive
Ames, Iowa 50010
Phone: 515/268-2529
Fax: 515/268-2606
Email: mel.vandenberg@amerprocorp.com

APC, Inc.

MEMO

TO: Joe Crenshaw, Gerry Almburg, Jerry Frankl, Larry Hoelting, Wiley Houchins, Roger Jacobsen, Mark Kuennen, Gary Manning, Jim Quigley, Luciene Ribeiro, Louis Russell, Duane Suess,

FROM: Mel Vanden Berg, Vice President of Quality Assurance
Bonnie J. Humm, Director of Quality Assurance

DATE: April 10, 2003

RE: Vlot Brothers Complaint – No. 3-1030C

CC:

This memo is written in keeping with the procedures outlined in our *Recall Manual*. The complaint alleged that at first 90 calves died and later between 200 and 300 calves died as a result of being fed APC, Inc. LifeLine product. The symptoms cited were bloat and impaction. Upon hearing of this situation, the following information was provided to Corporate Quality Assurance:

1. Vlot Brothers started using LifeLine on December 22, 2002
2. Vlot Brothers alleges they stopped using LifeLine on February 11, 2003
3. While at a Tulare, California farm show Don Gasper of Vlot Brothers states on February 11, 2003 that LifeLine is a "wonderful" product and supports its use.
4. Complaint received March 14, 2003 cites problems back to December 2002
5. Luceine Ribeiro checks with other major users of the LifeLine in California to see is any others are experiencing similar problems. No similar situations found.
6. An unopened bag of the LifeLine product in question was received from Vlot Brothers with the indication that it is one that was causing deaths. This product has been tested by the Corporate Quality Assurance department, outside laboratories and APC, Inc.'s Calf Ranch.

Micro testing resulted in SPC <1,000 cfu/g and no Coliform, *E. Coli, Salmonella, or Listeria.*

7. The Calf Ranch feeding of calves did not result in bloat or impaction.

8. All QA testing of the lot after production was within specifications and records indicated product as acceptable and safe.

Since the bag of LifeLine had been irradiated, the micro results reflected the low or absent micro counts that would be expected. ISU Veterinary Lab tests reveal no aerobic and anaerobic growth from the sample.

Based on the facts and results, we have determined that there is no need to initiate a Recall Standby. Unless further information becomes available which contradicts these findings, we consider this situation to be caused by circumstances outside of those of our LifeLine product.

MVB/BJH:pjm
30000292 R1

C

000130.001

Effects of Spray-Dried Plasma in the Diets of Dairy Calves

J. D. Quigley[1], J. M. Campbell, C. J. Hammer, J. D. Crenshaw and L. E. Russell
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Date Prepared: 21 January, 2004

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Executive Summary

Spray-dried animal plasma (**SDAP**) is a product produced by careful collection, processing and spray-drying blood collected from government inspected abattoirs. Methods use to collect and process SDAP result in a product that contains functional proteins, including immunoglobulin G (IgG), which can provide intestinal immunity when administered orally to calves following cessation of macromolecular transport.

Spray-dried animal plasma contains approximately 15% IgG, which is composed of antibodies with titers against economically important diseases. These antibodies are partially resistant to digestion and a significant proportion reach the intestine. Antibodies (IgA and IgG) are an important component of the intestinal immune system and research has shown that circulating IgG moves from the circulation into the lumen of the intestine to inhibit binding of pathogens. Research in calves, pigs, rodents and humans indicates that intestinal Ig (produced locally or transported into the intestine) are a key part of the overall intestinal immune response. In addition, SDAP contains other functional proteins, including growth factors such as insulin like growth factor 1 and transforming growth factor beta, which have been shown to improve cellular growth, particularly in animals following intestinal damage.

Spray-dried animal plasma is widely used in diets for young animals, particularly young pigs. It has been estimated that over 90% of early weaned pigs in the U.S. are fed SDAP in their weaning diets. Many veterinarians and nutritionists consider SDAP an essential ingredient to reduce weaning stress, improve intake, reduce the incidence and severity of diarrhea and replace antibiotics in the diet. The functional proteins in SDAP serve as a "first line of defense" against enteric pathogens and work with the immune system to reduce effects of these common challenges. Calves have also been fed SDAP as a source of IgG to support intestinal health during the milk feeding phase, when calves are most susceptible to enteric infection.

Studies published in the scientific literature indicate that SDAP or bovine serum (derived from plasma) improved survival, health, and growth of calves reared in normal herd conditions. When exposed to oral challenge with pathogens such as *Escherichia coli*, coronavirus or *Cryptosporidium parvum*, SDAP reduced morbidity and mortality associated with the disease and improved rate of animal recovery.

Reducing the reliance on sub-therapeutic antibiotics is a goal of many industries. Many calf raisers have identified the use of bovine serum and SDAP as a means of reducing their use of antibiotics without impairing health or growth of animals. The continued availability of SDAP and bovine serum to provide enteric immunity is crucial to continued economic development of the dairy and beef cattle industries.

Introduction

Blood and plasma proteins have long been recognized as high quality feed ingredients for farm animals, including swine, cattle, and poultry. Blood proteins are readily available and have an excellent amino acid profile and high digestibility. Indeed, plasma has been recommended for use in animal diets for hundreds of years – not only as a source of protein, but also as a source of immunological support. Prior to the development and widespread use of antibiotics in animal agriculture, proteins such as egg yolk, blood and colostrum were used as a source of immunologically active proteins to help animals recover from disease. Further, use of blood proteins in animal diets removes this by-product stream from environmental contamination and can increase the value of the animal to the farmer.

Traditionally, proteins were considered simply sources of amino acids for the animal. However, increasingly, scientists recognize that some proteins retain biological activity in the animal – these are called *functional proteins*. Functional proteins are recognized as having some function in the animal that elicits a physiological response. Spray-dried animal plasma (**SDAP**) is an ingredient widely used in animal feed applications. Several important functional proteins are retained in SDAP that can improve animal survival, health and performance of animals.

Method of collection

Blood proteins have traditionally been collected and heated to high temperatures in the manufacture of blood meal, which destroys the functional components. Conversely, spray-drying preserves the functional characteristics of the proteins, including biologically active peptides, such as albumin and IgG. In this process, blood is collected into stainless steel tanks, troughs or other devices containing an anticoagulant in abattoirs under government inspection. Blood is only collected from animals determined to be fit for slaughter by veterinary inspection. The blood is then transferred to stainless steel tanks, followed by centrifugation to separate plasma from the cellular fraction and chilling to 4-5°C. Chilled plasma is then transported by dedicated equipment to separate and dedicated facilities where it is spray-dried to produce a light brown, freely flowing powder that may be used in feed applications. Recently, the North American Spray-Dried Blood and Plasma Producers Association was formed to establish policies and GMP procedures for the manufacture of high quality and safe products (Russell, 2001).

Characteristics of Plasma

Functionality. The method of processing will influence the degree of biological activity that a protein will retain. Spray-drying plasma maintains the functionality of important proteins in plasma, including protease inhibitors, hormones, growth factors and immunoglobulins. Because plasma contains approximately 15% IgG, the functionality of this fraction is biologically meaningful to the animal consuming SDAP. Further fractionation can be used to increase IgG concentration to greater than 90%. Of course, the method of processing and preparation have important effects on the degree of functionality of the proteins.

The IgG in plasma are maintained and are biologically active. The Ig titers reflect the combined immunological history of the animals from which blood was collected and typically reflect both

the vaccination and exposure history of the animal. Plasma is collected into large lots, therefore, wide swings in Ig titer are avoided. It is of interest that the immunological history of animals (swine and cattle) will change over time; therefore the Ig titers in plasma will change also. Spray-dried animal plasma typically contains a wide array of specific antibodies that can be measured by standard serology tests. Plasma as a source of IgG will always reflect the immunogenic history of cattle.

Much research has been done to determine whether Ig from one species of animal may provide immunological support in another species of animal. In the feed industry, bovine, porcine and mixed plasma are available. In several studies, improvements in animal performance have been reported in animals fed swine or bovine plasma (Campbell et al., 2003; Quigley et al., 2003). Additionally, a tremendous number of published literature is available that documents the use of IgG (primarily bovine IgG or chicken IgY) as oral therapy in humans. More recent studies have evaluated the use of bovine colostral IgG as a means of improving athletic performance.

Role of IgG in intestinal immunity

Oral IgG are partially resistant to digestion (Roos et al., 1995) and can be measured in the feces of animals consuming SDAP. This suggests that intact and immunologically active IgG from SDAP may support the intestinal immune response of animals.

In addition to IgG provided by SDAP in the diet, researchers have shown that circulating IgG move from the circulation into the lumen of the intestine, where they can also support the intestinal immune system. Besser et al., (1988a) injected I^{125} labeled IgG into the jugular vein of calves. The researchers monitored production of I^{125} (total and protein-bound) in urine and feces following the injection. Approximately 2.5% of the I^{125} was excreted daily in the urine, and only about 3% of this was bound to protein. In addition, about 1.5% of the total I^{125} injected was excreted in the feces; however, 82% was still bound to protein. The researchers concluded that a portion of the IgG injected into the jugular vein moved into the intestine, where it was then excreted in the feces. In a subsequent study, Besser et al., (1988b) reported that the subcutaneous injection of colostral whey containing high titers of rotavirus antibody protected colostrum deprived calves from an oral rotavirus infection. Again, the authors concluded that circulating IgG were an important source of IgG to support intestinal immunity.

Use of plasma in animal diets

Spray-dried animal plasma has been included in the diets of commercially reared early weaned pigs for at about 20 years. Today, the vast majority of all feeds fed to pigs immediately after weaning (pig starters) in the U.S. contain SDAP at the rate of 2 to 7% of the formulation. Most research has reported improved intake, animal growth, feed efficiency and animal health in animals (including pigs and calves) when animals were fed SDAP (Gatnau et al., 2000; Morrill et al., 1995; Kats et al., 1994; Quigley and Bernard, 1996; Quigley et al., 2002, 2003; Van der Peet-Schwering, 1995, 1997). Others have compared SDAP to antimicrobials in diets of pigs (Coffey and Cromwell, 1995; Conde et al., 2000; Torrallardona et al., 2002, 2003) and calves (Quigley and Drew, 2000). We have summarized 47 research trials that used plasma in diets of pigs immediately after weaning – the average improvement in body weight gain, feed intake and

feed efficiency were 34, 23 and 9%, respectively. Pigs are very susceptible to anorexia immediately after weaning, therefore, a highly palatable ingredient could make a tremendous difference in animal performance (van Dijk et al., 2001a). The availability of SDAP in weanling pig diets allowed growers to reduce the age at weaning, and although the overall cost of the weaning ration increased with the inclusion of SDAP, overall return to the producer was improved.

Animals fed SDAP under low antigenic conditions (excellent hygiene and low exposure of the pigs to enteric pathogens) showed little improvement in performance when plasma was fed to pigs. On the other hand, when pigs were raised in a more "conventional" environment with typical exposure to enteric pathogens (indicated in the study by greater incidence of scours), animals fed plasma grew much faster than those fed the control ration. Coffey and Cromwell (1995) confirmed this finding when they fed pigs diets without or with plasma in "low antigenic" or "high antigenic" conditions. Pigs exposed to greater environmental stressors responded more dramatically to the inclusion of SDAP in the diet. These findings suggest that components in SDAP reduced effects of environmental stress. The authors suggested that the IgG fraction was critical to the overall response.

The value of animal plasma in the diets of herd replacement calves has been evaluated experimentally (Morrill et al., 1995; Quigley and Bernard, 1996). Morrill et al. (1995) reported improved body weight gain in calves fed plasma (25% of protein) compared to control (whey protein concentrate). All diets in this study were medicated with neomycin / oxytetracycline. Animals in this study were purchased from sale barns and transported to a commercial calf ranch in Kansas. Amount of stress in these calves was significant, as was shown by loss of body weight for the first two weeks of the study. Under these conditions, SDAP (bovine or porcine) resulted in significantly greater BW at 6 wk of age compared to calves fed control. By the end of 6 wk, calves fed milk replacer containing plasma had consumed 4.15 kg more calf starter than calves on control.

Quigley and Bernard (1996) reported no significant effect of bovine plasma (25% of protein) on animal growth, intake, or efficiency. Animals in this study were derived from dairy farms, raised under excellent management conditions and exposed to little stress. Rates of body weight gain in this study were greater than the study by Morrill et al. (1995) and were indicative of excellent management conditions.

Quigley et al. (2002, 2003) reported that the inclusion of SDAP or bovine serum in calf milk replacers improved the health and survival of Holstein bull calves (n = 360) purchased from dairy farms or sale barns. Bovine serum was used as an additive to milk replacer for the first 15 days of the study; bovine or porcine plasma was blended into the milk replacer formula and fed throughout the milk feeding period. Calves were not challenged with a specific pathogen, but incidence of diarrhea was significant, due to exposure to pathogens after birth and during transport. In all experiments, most calves had low concentrations of circulating IgG and had been exposed to significant immunological challenge from transport and commingling. The authors reported lower mortality, reduced incidence and/or severity of diarrhea and reduced use of therapeutic antibiotics during the first four weeks of each study when SDAP or bovine serum were included in the diet (Quigley et al., 2002, 2003).

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Plasma reduces effect of enteric challenge

Numerous studies have been conducted in many classes of animals that indicate that plasma reduces mortality and morbidity associated with enteric challenge.

Pig challenge studies. Studies have evaluated the use of SDAP in the diet of pigs challenged with enteric pathogens, particularly *Escherichia coli* and rotavirus, which are economically important pathogens to the swine industry. While a thorough review of all of these studies is outside the scope of this presentation, several trials are particularly instructive. Researchers in the U.S. reported that the administration of water-soluble SDAP reduced the effects of oral challenge with *E. coli* (Borg et al., 1999). Italian researchers (Bosi et al., 2001) challenged pigs with an enteropathogenic strain of *E. coli* at weaning. Pigs were fed for 15 days and intake, gain, feed efficiency and mortality were determined. Feeding SDAP reduced mortality, improved growth and efficiency following oral challenge.

A follow-up study by the same researchers (Bosi, et al., 2004) also reported improved performance in pigs fed 6% SDAP or antibiotic (250 mg/kg colistin + 500 mg/kg amoxycycline) in the diet and challenged with *E. coli* (10^{10} CFU *E. coli* K88 on d 3 after weaning). These researchers also measured the amount of specific K88 antibody in both the plasma and saliva of the pigs on d 15 after challenge. Interestingly, production of K88 specific *E. coli* antibodies in plasma and saliva were reduced when either the antibiotic or SDAP was included in the diet. These data suggest that animals were not exposed to the challenge organism, and therefore, did not produce antibody.

Calf challenge studies. Quigley and Drew (2000) challenged 36 colostrum-deprived Holstein bull calves with *E. coli* K99 at 3 d of age. Calves were fed commercial calf milk replacers containing no additive, an antibiotic (neomycin and oxytetracycline) or bovine serum at 3.3% of the formula. All calves showed signs of enteric infection following oral challenge; however, calves fed either antibiotic or bovine serum had lower mortality, morbidity (number of days with diarrhea) than calves fed the control milk replacer.

Arthington et al. (2002) fed Holstein bull calves milk replacer containing 0 or 80 g/d of bovine serum as a therapy following oral challenge with bovine coronavirus on d 0. Feeding bovine serum improved average packed cell volume and respiration rate compared to calves fed diets without serum. The authors concluded that supplementation of milk replacer with bovine serum improved the rate of recovery in calves following coronavirus challenge.

Hunt et al. (2002) challenged 24 calves orally with 10^8 cfu of *Cryptosporidium parvum* at 8 days of age. Calves were fed milk replacer with added bovine serum or soy protein concentrate. Oral challenge caused significant fecal shedding of *C. parvum* oocysts, diarrhea, increased intestinal permeability, reduced villus surface area and reduced intestinal lactase activity. Calves fed bovine serum had a 33% reduction in oocyst shedding and a 33% reduction volume of diarrhea produced, improved intestinal morphology and more rapid recovery following challenge. The authors (Hunt et al., 2002) recommended the use of bovine serum as a possible therapy in human cryptosporidiosis.

Researchers from Belgium (Nollet et al., 1999) reported that the presence of glycoprotein glycans in bovine plasma may have an antimicrobial role in animals. Calves were assigned to receive no added SDAP or bovine or porcine plasma added to milk replacer to 14 days of age. Plasma powder was manufactured by collection of blood from EU approved slaughterhouses. Blood was centrifuged to collect plasma, which was then pasteurized at 50°C for 15 min to destroy antibodies. Calves were infected with 10^{10} CFU of *E. coli* F5+ or F17+ strains at 12 to 24 h of age. Subsequent evaluation to determine titers to F5+ or F17+ recovered none. After pasteurization, plasma was concentrated and spray dried. The researchers concluded that inclusion of bovine plasma improved survival of challenged calves, reduced incidence and severity of diarrhea, improved overall health of calves and reduced excretion of F5 organism by 2 to 4 logs due to inhibition of adhesion. The concept of carbohydrate moieties (oligosaccharides) to non-reversibly bind bacteria has been shown experimentally (Grizard and Barthomeuf, 1999; Le Blay et al., 1999) and is used in some animal industries (Oli et al., 1998).

Other effects of SDAP

Recent research suggests that the functional proteins in SDAP may play important roles in other biological functions of the animal.

Researchers in Spain fed weanling pigs (21 days age weaning) diets containing 0 or 7% SDAP and measured concentrations of caecal microflora following challenge with *E. coli* (Torradellona, 2001). Counts of Clostridium were reduced from 1.56 to 0 log CFU/g of caecal contents when pigs were fed SDAP. Further, concentrations of Lactobacilli were increased with SDAP in the diet (8.83 vs. 8.01 log CFU/g).

Modification of bone mass in pigs fed SDAP after weaning has also been reported (Jiang et al., 2000), as well as a significant reduction in intestinal weight in rats (Moreto, 2001, unpublished data) and pigs (Jiang et al., 2000). Jiang et al. (2000) also reported reduced urea N concentrations when pigs were fed SDAP postweaning, and suggested that changes in intestinal mass and cellularity was related to changes in circulating urea N concentrations. Plasma or purified globulin fed to pigs has been shown to reduce the concentration of circulating IgG in pigs (Campbell et al., 2000, unpublished data) and Bosi et al. (2001) reported that the inclusion of SDAP protein or antibiotics reduced concentrations of IL-8, TNF-α and IFN-γ in the jejunum in pigs challenged with *E. coli*.

When taken collectively, these data suggest that dietary SDAP may serve as a "first line of defense" against enteric pathogens, including viruses and bacteria. This "passive enteric immunity" reduces stimulation of the immune system under modern commercial animal production. The animal's immune system is not stimulated, and as a result, pro-inflammatory cytokines are not secreted by macrophages. Cytokines secreted by macrophages affect many tissues and are responsible for liberating nutrients for use in supporting an immune response. Recent reports (Touchette et al., 2002) indicate that feeding SDAP reduced secretion of TNF-α, IFN-γ, and IL-6-β. When the immune system is not overstimulated, more nutrients are available for tissue accretion. According to many researchers, SDAP may serve an antimicrobial role in the intestine (Jiang, et al., 2000), thereby reducing the overall enteric challenge.

Constituents in SDAP have also been reported to improve repair of damaged intestinal epithelial tissue in vitro (Rhoads et al., 2000) and in humans (Lembcke et al., 1997). Reports of improved intestinal integrity in pigs challenged with enteric pathogens (Torrallardona et al., 2002; van Dijk, 2001b) are also consistent with these observations. It is not yet clear which component(s) are responsible for the observed improvements in regrowth of damaged tissue.

Research continues to elucidate an important role for the functional proteins in SDAP in animal agriculture and potentially, in human nutrition and health. The urgent need to reduce the overuse of antibiotics in diets of animals demands that viable alternatives be found. Numerous published research trials have documented the value of SDAP in reducing the effects of experimental and on-farm enteric challenges.

Summary

Use of SDAP is well accepted in animal agriculture. The value of functional proteins, including IgG, to support enteric health, reduce the effects of pathogenic challenge and reduce morbidity and mortality are well documented in both scientific and popular literature. Although not reviewed in this paper, the use of functional proteins (especially IgG) to improve human health is also well documented. Continued availability of SDAP to dairy and beef farmers will improve animal health, reduce the need for antibiotics and improve the efficiency and safety of beef and dairy products.

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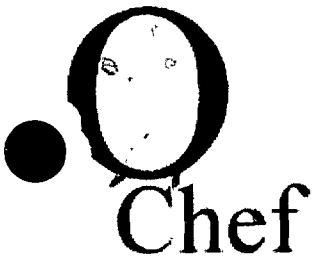

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What is the Origin of Blood Sausage?

Q. I would like to know the country of origin for blood sausage. I think I am correct in saying black pudding is Ireland's version of blood sausage, but who invented — if that's the right term — blood sausage?

A. We will answer your question if you will tell us where pigs were first killed for meat. Because the practice of not wasting any portion of the animal has been in effect since the earliest days, and that includes the blood, which is nutritious but highly perishible. Of course, there is no record of who first used other ingredients to soak up the blood and make a kind of sausage, but Jeffrey Steingarten, writing in the exceptionally informative and periodically hilarious *It Must've Been Something I Ate* (Canada, UK), says his sources indicate that blood pudding was invented (the very word he uses) by Aphtonite, a cook in ancient Greece.

There is also a reference in Homer's *Odyssey* to a stomach stuffed with blood and fat and roasted over a fire, that dates from approximately 1,000 B.C. The oldest blood sausage recipe dates from the first centuries A.D., and included hard-boiled egg yolks, pine nuts, onions, and leeks in a pig's intestine.

Even though blood pudding is not in the mainstream in this country, there are many versions made throughout the world.The French *boudin noir* is essentially an equal mix of onions, pork fat, and blood, with flavorings, although it may be stretched with cream or bread. There are also variations that include apples, brandy, spinach, currants or raisins, butter, eggs, and other ingredients. Black pudding in England and Ireland are often stretched with oatmeal or occasionally rice. In Sweden it is often made with rye meal and raisins. A version in Spain includes almonds, pimentos, and parsley.

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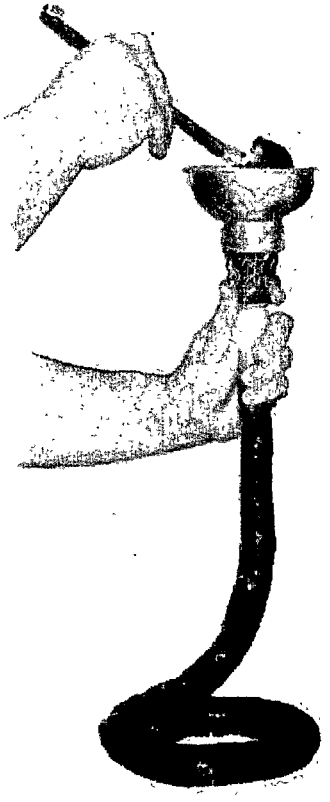

Boudin noir — blood sausage — is one of France's oldest charcuterie preparations. It's part of a sausage making tradition in France that has lasted well over 2000 years. "There is evidence of the activities of the *lardarius,* the Gaulish charcutier, in a number of bas-reliefs and inscriptions found at Narbone, Bordeaux and Cologne, and at Rheims, where a bas-relief shows two of these specialists at work... All the items of charcuterie shown in the relief are easily identifiable [blood sausages], strings of sausage, joints of pork, pig's heads."1 Although the raising and subsequent slaughter of the family pig was common in the French provinces up to the Second World War, today few families in the countryside raise their own pigs.2 The slaughtering process, usually performed in late-November, required the whole family plus a few neighbors to work from dawn to dusk to first kill and bleed the animal, and then to butcher the carcass. The majority of the pieces were used to produce meat items that would sustain the family through the winter.3,4 These items would include hams, sausages, pâtés, and other preparations which preserve the pig flesh long past the time where fresh meat would have spoiled.

When bleeding the pig, it was necessary to constantly move one of the forelegs to facilitate drainage and avoid the formation of clots that would prevent the blood from flowing.5 The warm blood had to be stirred to keep it from coagulating. This task often fell to the children. Today in France, the blood is removed from the pig with a vacuum system and immediately centrifuged to prevent it from solidifying.6 Pork blood consists of 77% water, 7.2% albumin, 14.5% globulins, 0.3% fibrin, 0.2% fat, and 0.8% other substances. Centrifuging breaks down the fibrin and inhibits coagulation. The blood will still coagulate if cooked to between 70 and 80 °C (158 to 176 °F).7

Blood sausages are very perishable and are often produced on a daily basis in French charcuteries.8 The sausage is produced in long undivided lengths from which the patron purchases a cut piece, or in individual links. In the home kitchen, these sausages should be eaten within a day or two of preparation, or frozen until needed.

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Each producer of blood sausages has his own recipe where the ingredients and proportions differ from those of the shop down the street. Traditional *boudin de Paris* will contain equal quantities of blood, fat, and cooked onions, but the proportions can vary widely. Different seasonings may be used, fruit or vegetables may be added, and various aromatics may be included. Common additions include apples and chestnuts.9

For the most part, the preparation of blood sausage is similar to making other types of pork sausage. The details of sausage preparation were published previously on this web site. The recipe

accompanying this article leaves out many of the details already covered on that page.

One notable difference between the making of blood sausages ar other types of sausages is that a stuffer is not used for filling blood sausages. For blood sausages, the casing is slid over the spout of a funnel and the sausage mixture is ladled into the funnel. In France, metal funnels appropriate for stuffing are available, but I was not ab to find a suitable one in my neighborhood. However, I was able to fashion a usable system from the combination of a canning funnel, a disposable pastry bag, and a standard sausage stuffing tube. To mak this sausage you will need a wide-mouth funnel with a straight spou

The basic recipe for *boudin noir* presented herein is based on a recipe for the sausage presented by Charles Barrier, a Michelin-starred chef from Tours in the Loire Valley at the Ecole de Château d'Amondans in the mid-1990s. Chef Barrier's original recipe didn't use apples, but when I learned this recipe fro Chef Frédéric Médigue at the Château, apples had been added and the fat reduced.

Blood sausages are commonly eaten fried or grilled and accompanied by potatoes and/or apples. In *boudin grillé en compote de pommes citronnée et fruits secs*, the sausage is simply grilled and served with a warm compote prepared from apples, lemon juice, anc dried fruits. In *émincé de boudin noir aux pommes (et aux pommes c terre)*, the blood sausage is sliced before grilling and is served with caramelized apple wedges and fried potatoes. In *tarte au boudin noi et aux deux pommes*, the same ingredients along with onions are combined into a pleasant tart. In *bouchées croustillantes au boudin noir* and *feuilletés aux pommes et boudin*, the sausage is used with apples to produce bite-size portions suitable for serving as an ***amuse bouche***. Lastly, *crème de boudin noir*, a soup, is the preparation that set me on my course of study to learn how to make my own blood sausages. The dish was originally called ***boudin maison servi comm un velouté, relevé au pigment antillais et quelques des d'ananas*** — homemade blood sausage prepared as a cream soup and seasoned with red peppers and pineapple — when I learned the recipe from Chef Médigue of Le Château l'Amondans in October, 2001. (I thought some simplification of the name was in order.)

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4. Grigson, Jane. *Charcuterie and French Pork Cookery.* New York Penguin Books, 1967, 7.
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7. Marcel Cottenceau, Jean-François Deport, and Jean-Pierre Odeau *The Professional Charcuterie Series.* Translated by Anne Sterling

New York: Van Nostrand Reinhold, 1991, 1:198.
8. Ibid., 200.
9. *Larousse Gastronomique* (in English), New York: Clarkson Potter/Publishers, 2001, 120.

home

boudin noir

The following recipe produces about 700 grams (24 ounces) of finished sausage. Depending on the diameter of the casing used, the final length will be between 60 and 75 centimeters (24 and 36 inches). It can be prepared in a single length or twisted into individual links prior to poaching. Note: click on the illustration below for a slide show about making *boudin noir*.

1.25 meters (4 feet) hog casing, 34 to 36-mm diameter

stuffing:

350 grams finely minced onions
90 grams lard
75 grams (1/2 large) peeled, cored, and minced apple
100 grams pork fatback
150 grams lean pork
6 grams minced garlic
10 grams minced flat-leaf parsley
12 grams (2-1/2 teaspoons) fine salt
2 grams (1 teaspoon) ground black pepper
1/4 teaspoon ground nutmeg
1/4 teaspoon *quatre épices*
90 milliliters whole milk
1 tablespoon calvados
375 grams fresh pork blood

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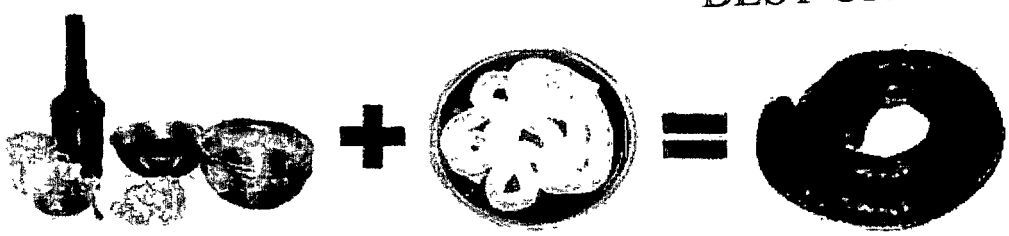

1. Sweat the onions in 75 grams lard over medium-low heat until they start to melt, about 30 minutes. Drain. Cool to room temperature before mixing with the other ingredients.
2. Sweat the apple in 15 grams lard over medium heat until it starts to color, about 10 minutes. Cool to room temperature before mixing with the other ingredients.

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3. Grind the fatback and meat through a fine blade.
4. Soak sausage casing in warm water for 15 minutes. Rinse inside of casing with cold water.
5. Combine the cooked onions and apples with all the remaining ingredients, except the blood, in the bowl of a stand mixer fitted with a paddle. Use the lowest speed to thoroughly mix the ingredients. Add the blood and mix. Immediately stuff mixture into the casing. Do not overstuff the sausage.
6. Place the sausage in a large pot. Fill the pot with cold water. Cover the sausage with a drop lid. Heat the water to 85 to 90 °C (185 to 195 °F) and maintain it in this range. Poach the sausage for 18 minutes and until the internal temperature reaches at least 74 to 77 °C (165 to 170 °F). Carefully lift the sausage out of the water and place on a baking sheet. Rinse the sausage with cold water. Cool it in a refrigerator. Wrap sausage in plastic wrap until ready to use.

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USDA

Food Standards And Labeling Policy Book Changes 98-01

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A semi-dry type sausage. The maximum MPR is 3.7:1.

BLOOD:

See: 318.6(b)(7)
317.8(b)(31)

BLOOD AND TONGUE SAUSAGE:

Same as blood sausage, except cured and cooked pork or beef tongues are used.

BLOOD PUDDING:

A nonspecific product made with livestock blood. The product name must be followed by an ingredients statement.

BLOOD SAUSAGE:

A cooked sausage formulated with blood and some meat. Usually contains pork skins and/or pork jowls. May also contain sweet pickled ham fat, snouts, and lips. If the product does not contain meat, it must be labeled as "Blood Pudding."

BOHEMIAN PEESKY:

Made of salt-cured pork trimmings, seasoned with garlic, pepper, and salt. The label must show true product name, e.g., "Seasoned Salt Cured Pork."

BOINGGHETTI:

This label must show a true product name, "Spaghetti with Chicken Sauce." The product must contain at least 6% cooked chicken meat.

BOLO:

Contains beef and pork, water, nonfat dry milk, and flavoring. The label must show a true product name, i.e., "Beef and Pork, Water and Nonfat Dry Milk Product."

BOLOGNA, LEBANON:

See: Lebanon Bologna

BOLOGNA: (Cooked Smoked Sausage)

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Effects of bovine serum concentrate, with or without supplemental micronutrients, on the growth, morbidity and micronutrient status of young children in low-income, peri-urban Guatemalan communities

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ABSTRACT

Background – Nutritional stunting and micronutrient (MN) deficiencies are common among young children in lower income countries, and additional information is needed on possible interventions to reduce growth faltering and improve their MN status.

Methods – We completed a randomized, double-masked intervention trial among young Guatemalan children residing in poor peri-urban communities to determine the effects of dietary supplements containing bovine serum concentrate (BSC – a source of immunoglobulins), multiple micronutrients (MMN), both BSC and MMN or whey protein concentrate (WPC – control group) on the children's growth velocity, rates of common infections, and MN status

Results –There were no significant differences in the growth velocity or rates of morbidity by treatment group. Children who received MMN had lower rates of anemia and (in the group that received MMN and WPC) less of a decline in serum ferritin than those who did not, but there were no differences in other biochemical indicators of micronutrient status by treatment group.

Conclusions – MMN supplementation protected against anemia and iron deficiency in this population, but there were no differences in other biochemical indicators, growth or morbidity in relation to the MMN content or the source of protein in the supplements.

Funding was provided by LG Laboratories (Ames, Iowa), which produces the bovine serum concentrate used in this study. The members of the research team have no financial involvement in this company.

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KEY WORDS

Multiple micronutrient supplementation, growth, micronutrient deficiencies, malnutrition, bovine serum concentrate, children, diarrhea, Guatemala

INTRODUCTION

More than 250 million pre-school children in lower income countries are affected by nutritional stunting, defined as length-for-age <-2 SD with respect to the international reference population median (1). In Guatemala, for example, nearly 50 % of children less than five years of age have low height-for-age (2), and most of the height deficit of Guatemalan adults can be attributed to growth failure during early childhood (3).

Inadequate dietary intakes and high rates of infections are believed to be the principal post-natal biological factors that limit children's growth, although the specific nutritional causes of growth stunting remain uncertain (4). Several reports indicate that particular aspects of dietary quality, especially micronutrient density and bioavailability, as well as the proportion of nutrients obtained from animal source foods may be important determinants of children's growth (5). A trial in Guatemala found that supplementation with zinc alone increased the growth of stunted, but not of non-stunted, infants (6), findings which are consistent with the results of a meta-analysis of the effect of zinc supplementation on children's growth (7). A double-blind, placebo-controlled trial of multiple micronutrient (MMN) supplementation conducted in Mexican infants found positive effects of supplementation on growth, suggesting that deficiency of one or more these micronutrients was limiting the physical development of these infants (8). Likewise, the growth of older children in Tanzania was greater when they received a MMN mixture (9). In Vietnam, daily and weekly MMN supplementation increased growth only among children who were stunted at baseline (10). Contrary to the results of these foregoing studies, MMN supplements did not affect the growth of children in other settings (11, 12), possibly because the study subjects were less stunted initially or not lacking in any of the specific micronutrients that

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are critical for growth.

Experimental trials in newly weaned farm animals have demonstrated that incorporation of spray-dried bovine and porcine blood plasma into their feeds increases their dietary intakes and produces greater rates of weight gain compared with control diets (13,14), possibly because the higher concentrations of immunoglobins in the plasma-fortified products protected against infection. We therefore proposed that similar benefits might occur in young children in populations that experience high rates of diarrhea and other infections. Food-grade bovine serum concentrate (BSC) prepared from hygienically collected and processed blood is now available commercially as a spray-dried powder and for use in the preparation of processed meats. A preliminary clinical study found that BSC incorporated into an infant formula was well tolerated by small group of young Peruvian children recovering from malnutrition (15).

The present research was completed to determine whether dietary supplementation with MMN, BSC, or both would enhance the physical growth and micronutrient status of Guatemalan infants residing in a low-income community. We hypothesized that infants who received a small, daily supplement of a maize-based pudding containing: 1) BSC, 2) BSC and MMN, or 3) whey protein concentrate (WPC) and MMN for six to eight months would have increased linear growth and accrual of body weight compared with those who received the same pudding supplemented only with WPC. We further postulated that the children who received the supplements containing MMN would have higher levels of selected biochemical indicators of micronutrient status at the end of the period of supplementation. Finally, we assumed that these outcomes would be mediated at least partially through reductions in infectious diseases in the groups that received BSC and/or MMN, although the available sample was only sufficient to

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permit detection of fairly large differences in morbidity rates.

METHODS

Study design - The study was a randomized, double-masked, community-based trial of dietary supplements that contained either WPC (control group), BSC, WPC+MMN, or BSC+MMN in a small amount of a maize-based pudding that was provided daily (six days per week under field worker supervision, with one dose left with the caregiver for administration on the seventh day).

Study site, subjects, and randomization procedures - The study was conducted in nine low-income communities ("asentamientos") in the area of El Mezquital on the outskirts of Guatemala City. A census was completed initially and five and ten months later to identify all infants <5 months of age who were eligible to participate in the study. Children were excluded from consideration if: 1) they were severely malnourished (defined as length-for-age <-3 Z or weight-for-length <-2 Z with respect to WHO/NCHS/CDC reference data (16); 2) they had evidence of congenital abnormalities that might interfere with growth or predispose to infection; or 3) their parents planned to move from the study community within the next few months or did not consent to participate. The study protocol was approved by the institutional review boards of the University of California, Davis and the Institute of Nutrition of Central America and Panama; a parent of each child provided written, informed consent.

Previous observations in the study community indicated that more than half the infants were anemic (defined as altitude-corrected hemoglobin <10.3 g/dL, corresponding to a measured hemoglobin <11.0 g/dL) at six months of age. Therefore, all children who were enrolled in the

current study were given daily supplements of 10 mg iron (as ferric ammonium citrate) as a preventive measure during the period from four to five months of age. At five months of age hemoglobin levels were measured in capillary blood by using a portable hemoglobinometer (HemoCue Inc., Mission Viejo, CA), and any child who was anemic was given 20 mg iron per day during the following one to two months. Children whose hemoglobin was still <11.0 g/dL at seven months were dropped from the study and referred for further evaluation and treatment.

When the children became five months of age, disease surveillance was initiated by a field worker, who visited the home six days per week to inquire about specific symptoms of illness during the previous one or two days, as described below. Once the children became six to seven months of age, those who were eligible for the study were stratified by sex and randomly assigned to one of 12 letter codes, using a block randomization scheme, with block length of 12. Each of the code letters corresponded to one of the four treatment groups. Baseline anthropometric and biochemical assessments were completed before treatments were initiated. The identity of the individual treatments remained masked until all data collection, laboratory assays, and preliminary analyses of data were completed.

Supplements – Children <12 months of age were provided with ~14.2 g (dry weight) of the supplements each day, using a pre-calibrated measuring spoon. The supplements were composed of maize flour, maltodextrins (corn syrup solids), sugar, flavoring agents, WPC or BSC, and either a vitamin/mineral supplement or additional maize flour (Table 1). In the two groups that received BSC, IgG accounted for approximately 22% (0.68 g) of the 3.1 g of BSC (NutraGammax 20; LG Laboratories, Ames, IA) included in each daily supplement. The MMN supplement contained one US RDA (17) for one-year old children of each of the nutrients that

were included, except for calcium and phosphorus, which were present at a level of approximately 0.3 RDA (Table 2). When children reached 12 months of age, they received 1.5 times the aforementioned doses of each supplement (i.e., ~21.3 g/d dry weight). The ingredients of each supplement were combined as dry agglomerates to enhance their ultimate dispersion in aqueous mixtures, and were stored in sealed, coded cans prior to labeling with the individual children's names and distribution to their respective homes. The composition and identity of the supplements were independently confirmed on several occasions by measuring the amounts of IgG and selected micronutrients in the mixtures.

Field workers visited each home daily (six days per week) and added 20 or 30 ml of clean water to the appropriate dose of dry supplement to make a pudding-like mixture, which was fed to the child by spoon. The field worker observed and recorded the amount consumed as 0%, 25%, 50%, 75%, or 100% of the assigned portion. On Saturdays, a dose of the dry supplement was left with the child caregiver for administration on Sunday, and on days when the child was not present in the home or was otherwise unavailable (~15% of days), the field worker prepared the day's dose and left it with the caregiver for administration shortly thereafter. In these latter cases, the amount consumed was recorded the following day.

Anthropometry - The children's anthropometric status was assessed at baseline and monthly thereafter in their own homes, using standard techniques (18). Measurements were taken by one of two anthropometrists who were periodically standardized against the field supervisor. Body weight was measured to the nearest 5 g, using a frequently standardized electronic balance (Tanita Model 1583; Tokyo, Japan). Recumbent length was measured to the nearest 0.1 cm, using a wooden length board (Shorr, Columbia, MD). Mid-upper arm

circumference (MUAC) was measured to the nearest 0.1 cm, using a fiberglass tape. Intra- and inter-observer measurement errors were assessed periodically by repeated measurement of a separate group of children, and the combined measurement error was ± 0.23 cm (coefficient of variation, CV = ± 0.3%) for length and ± 0.21 cm (CV = ± 1.4%) for MUAC.

Morbidity - Morbidity from common childhood illnesses was assessed by the same field worker who observed the daily administration of the supplements. The field worker questioned the child's primary caregiver systematically to elicit information on stool number and consistency, and the presence of specific symptoms of respiratory and other infections, using a modified version of a previously developed questionnaire (19). Diarrhea was defined as excretion of ≥3 loose or liquid bowel movements per day, and an episode of illness was considered to have ended when the stool pattern did not satisfy these criteria for at least two days. Diarrhea was also defined using a more demanding cut-off of ≥4 loose or liquid bowel movements per day to see if this affected the conclusions. An episode of diarrhea was designated as severe if any one of the following occurred on any day of the episode: 6 or more liquid or semi-liquid stools, fever, vomiting, blood or mucous in the stool, or dehydration. Diarrhea was defined as persistent if it lasted 14 days or more. Upper respiratory infection was defined as the presence of cough associated with purulent nasal discharge; while lower respiratory infection was defined as the presence of cough and age-specific increased respiratory rate (>50/min for infants <12 mo of age and >40/min for older children), as per recommendations of the World Health Organization (20).

Dietary assessment – Dietary intakes were assessed at baseline and every three months thereafter by means of direct weighing of all foods and beverages consumed during daytime

observations, including test-weighing of breast milk intake, and recall of any foods consumed at night. Breast milk consumption during the 10-hour daytime observations was extrapolated to 24 h, by multiplying the daytime intakes by a correction factor determined during 24-hour observations completed in a sub-sample of 16 mother-infant pairs. The macronutrient contents of breast milk were imputed, using values published by the World Health Organization (21). Energy and nutrient intakes from complementary foods were calculated by using the food composition table published by the Institute of Nutrition of Central America and Panama (22) and additional data for the zinc (23) and phytate (24) contents of foods.

Biochemical assessment - A sample of venous blood was obtained at baseline and at the end of the period of supplementation for measurement of hemoglobin and serum ferritin, retinol, and vitamin E, and plasma zinc, iron, and copper. The samples were generally obtained in the morning, but were not standardized in relation to recent meals or breast feeding episodes. When the children were acutely ill with diarrhea or fever on the day of scheduled blood drawing, the sample collection was postponed until the symptoms resolved. Hemoglobin was analyzed in the field using the HemoCue device, and results were corrected for altitude to determine the rates of anemia (25). The remainder of the blood samples was centrifuged and aliquoted in the field office prior to being transported to INCAP in cold boxes for subsequent storage at –17°C and shipment to the US on dry ice for analysis at UC Davis.

Serum ferritin was determined by enzyme immunoassay (Spectro Ferritin, Ramco Laboratories, Inc., Houston, TX); and retinol and vitamin E were measured by reverse-phase high pressure liquid chromatography (26), using a Shimadzu Class VP HPLC (Shimadzu, Columbia, MD) equipped with a photo-diode array detector. Aliquots of plasma for trace element analysis

were stored frozen in mineral-free, colorless plastic vials. For trace element analyses, 200 μL of the plasma was combined with 2.2 ml of 1N Ultrex II ultrapure HNO_3 (J T Baker Inc, Phillipsburg, NJ), allowed to stand overnight at 4°C, and then centrifuged. The supernatants were analyzed for trace element concentrations, using a Tracescan inductively-coupled-plasma atomic-emission spectrometer (ICP-AES; Thermo Jarrell Ash; Spectrocell Inc, Oreland, PA). All samples from the same child were measured in a single analytic run, and compared with appropriate ultra-pure acid blanks, pooled plasma samples and ICP standards (QC-21 Spex, Fisher Scientific; Springfield, NJ). All materials and procedures were checked to rule out possible zinc contamination.

Socio-economic status - Socio-economic status (SES) was assessed at the beginning of the study by parental interview and observation of the home. Information was collected on household composition; parental age, education and occupation; housing quality; and selected material possessions. The housing quality score was calculated as the sum of the floor, wall, and roof quality scores, each expressed on an ordinal scale of 0 to 1. The possessions score was the number of the following items owned by the family: radio, television, refrigerator, wardrobe, bed, iron, automobile, and bicycle.

Sample size - The required sample size was estimated for the primary outcomes of linear growth and weight gain during the planned eight-month period of supplementation. Using longitudinal data from previous studies of similarly aged children in Guatemala (27), a sample size of 43 per group was estimated to be sufficient to permit detection of a 1.4 cm difference in linear growth and a 0.50 kg difference in weight gain among treatment groups, using a two-tailed ANOVA, considering a probability of 0.05 and power of 0.80. These detectable differences are

less than half of the originally expected shortfall in growth of Guatemalan children compared with international reference data during this age interval. The desired enrollment was set at a minimum of 54 children per group to allow for an estimated 25% rate of attrition.

Statistical analysis – Descriptive statistics (mean, standard deviation, range, skewness) were calculated for all outcome variables. Cubic polynomial interpolation was used to estimate weight, length, and arm circumference at exact monthly intervals from the start of supplementation. Outcome variables were transformed, if necessary, to create approximately normal distributions with homogeneity of variance among groups. Outcomes of treatment were compared by using analysis of covariance, which included presence or absence of MMN and BSC as main effects and the following covariates: child demographics (sex and age at enrollment), baseline anthropometry (weight and length), baseline morbidity rates (prevalence of diarrhea, fever, and upper respiratory infection during the month prior to initiation of supplementation), baseline iron and zinc status (initial serum ferritin and plasma zinc concentrations), breastfeeding practice, maternal factors (age, height, hemoglobin concentration, and level of education), household SES variables (housing quality and possessions scores), and, where appropriate, the baseline value of the outcome variable. Interaction terms included two- and three-factor interactions involving MMN and BSC with initial weight and length, baseline diarrhea rate, initial iron and zinc status, breastfeeding practice, and baseline value of the outcome variable, where appropriate. Non-significant ($p>0.10$) interactions and main effects ($p>0.05$) were removed in a hierarchical fashion, with the exception that the intervention variables (i.e., treatment groups) were forced into the model. Morbidity variables were weighted by number of days of observation; children with less than 60 days of morbidity observations were

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not included in these analyses.

RESULTS

Enrollment and completion

A total of 315 children from nine communities were potentially eligible for the study and were included in the initial period of iron supplementation, as shown in the profile of study subjects **(Figure 1)**. Of these, 56 were not included in the intervention trial because the child's mother began to work or moved to another area (18%); the child still had low hemoglobin despite two months of iron supplementation (13%); the parents refused blood collection (12%) or dietary observations (11%); the child surpassed the age limit for enrollment (5%); or the parents declined to participate without providing a specific reason (41%). Thus, a total of 259 children were allocated to treatment group, of whom 249 (96.1%) completed ≥60 d of observation, 184 (71.0%) completed ≥180 d of observation, and 132 (51.0%) finished the full 8 months of observation. There were no significant differences in the number of dropouts by treatment group at any time point. The growth and morbidity data are presented for those children who completed 8 months of observation to be consistent with final biochemical results, which were obtained after eight months of supplementation. We also analyzed the growth increments for those children remaining in the study after 2, 4 and 6 months of supplementation, and we found that the conclusions drawn from the group-wise comparisons at earlier time points did not differ from those drawn from the children who completed all eight months of observation. Likewise, we found no differences between the children who completed eight months of observation and those who remained in the study for less time with regard to supplement intake, prevalence or

incidence of morbidity, and breastfeeding status.

Baseline characteristics -- Baseline anthropometric status, rates of morbidity from common infections during the month prior to initiation of treatment, rates of breast-feeding, and indicators of socio-economic status did not differ significantly by treatment group (Table 3). The children's mean age at the time of initiation of supplementation was 6.2 ± 0.3 months. 83% of the infants were breastfeeding at baseline, and more than two-thirds were still breastfeeding at 12 months of age. Mean daily total energy intakes at baseline ranged from 87 to 89 kcal/kg body weight (0.36-0.37 MJ/kg BW) in the different treatment groups, of which an estimated 58% ± 37% was provided by breast milk. The mean dietary intakes of iron and zinc were less than 2 mg/day, and the mean intakes of vitamin A ranged from 507 to 590 μg RE/d. There were no significant differences in baseline nutrient intakes by treatment group. Mean phytate intakes ranged from 28 to 60 mg, and were lower in the MMN+BSC group ($p<0.05$), although mean phytate:zinc molar ratios were low in all groups and did not differ by study group.

Mean baseline hemoglobin levels ranged from 11.4 to 11.6 g/dL in the different treatment groups. Mean serum ferritin levels ranged from 46.8 to 62.1 μg/L, and <4% of infants had low serum ferritin values (<12 μg/L). Mean serum retinol levels ranged from 23.1 to 25.4 μg/dL initially, and 34.6% of children had serum retinol values <20 μg/dL. Mean plasma zinc concentrations ranged from 64.8 to 70.7μg/dL, and more than half of the children had plasma zinc values <65 μg/dL. There were no significant differences among treatment groups at baseline for any of the micronutrients measured.

Consumption of supplements -- According to the field workers' observations at the time of their daily home visits, at least some portion of the supplements (i.e., ≥25% of the assigned

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amount) was consumed on >98% of study days, and this did not differ by treatment group ($p = 0.16$). On days when at least some of the supplement was consumed, the average amount consumed was ~80% of the assigned dose (Table 4). The children who received supplements containing BSC consumed ~12% less of the amount offered than those who received mixtures containing WPC (Table 4).

Growth -- Overall, the children gained approximately 8 cm during the eight-month period of observation (from approximately 6 to 14 months of age), which is ~75% of expected linear growth of North American children in this age range (16). The study subjects gained about 1.8 kg, which is ~63% of expected weight gain. There were no significant differences in growth increments (including change in MUAC) by treatment group during the period of supplementation (Figure 2), controlling for initial anthropometric status, sex and age at beginning of supplementation. Controlling for additional covariates, such as feeding practices, maternal characteristics, socioeconomic variables, and initial pre-supplementation morbidity rates did not affect the conclusions of these group-wise comparisons. Because of the high attrition rate during the course of the study, we also analyzed growth increments from baseline to 2 months, baseline to 4 months, and baseline to 6 months. In all cases, there were no significant treatment group-related differences in any of the growth indicators.

Predictors of growth – Because the type of supplementation did not affect the children's growth, we explored other factors that might have influenced length and weight increments in the subset of children who remained in the study for eight months. Initial infant weight was positively associated and initial length was negatively associated with subsequent linear growth (Table 5). Maternal height and level of education were positively associated with changes in

both infant length and weight; and continued breast feeding at 12 months was negatively associated with both growth indicators, even after controlling for household socioeconomic status. Growth was unrelated to initial plasma zinc or serum ferritin concentration, and there were no associations between morbidity prevalence during the supplementation period and growth and no significant interactions between treatment group and any of the other variables considered.

Morbidity -- All children who received supplementation for at least 60 days were included in the analyses of morbidity. Table 6 shows the mean prevalence and incidence of specific types of morbidity during the period of supplementation by treatment group. The mean prevalence of diarrhea ranged from 10.4 to 13.5%, and did not differ significantly by treatment group, either before or after controlling for sex, age at initiation of supplementation, initial diarrhea rates during the pre-supplementation observation period, breast-feeding practices, maternal characteristics, initial plasma zinc and serum ferritin concentrations, and socio-economic variables. Likewise, there were no significant differences in the incidence of all diarrhea combined, or in the prevalence or incidence of severe or persistent diarrhea by treatment group. The diarrhea rates displayed in the table are based on the definition of $\geq$3 liquid or semi-liquid stools per day. Results of group-wise comparisons did not change when a cutoff of $\geq$4 liquid or semi-liquid stools per day was used instead. There were also no significant differences by treatment group in the prevalence or incidence of respiratory infections or other symptoms of morbidity.

Predictors of morbidity status -- Factors that were associated with diarrhea and respiratory infections are summarized in Table 7. Both prevalence and incidence of diarrhea

were positively associated with initial, pre-supplementation rates of diarrhea. The prevalence of diarrhea was significantly lower for girls, taller children, and children with more educated mothers, and the incidence of diarrhea was negatively associated with age at enrollment and maternal education. The incidence of severe diarrhea was negatively associated with maternal education and positively associated with pre-supplementation rates of diarrhea and fever (data not shown). The prevalence of upper respiratory infections was associated only with the initial prevalence of URI. The incidence of LRI was negatively associated with being female, initial weight, and maternal education, and positively associated with age at enrollment.

Micronutrient status – The overall mean concentrations of both hemoglobin and serum ferritin declined during the period of supplementation (Table 8). Controlling for initial hemoglobin and other co-variates, the hemoglobin levels fell slightly less in the groups of children who received supplements that contained MMN ($p=0.054$), and a smaller proportion of the children who received MMN supplements were anemic at the end of the trial ($p<0.05$). Mean serum ferritin values declined in all groups, but this fall was significantly less in group MMN+WPC ($p=0.024$). None of the other markers of micronutrient status changed significantly in relation to treatment group, after controlling for initial micronutrient status and other covariates. In all four groups, the proportion of children with low plasma zinc and serum retinol concentrations tended to be lower at the completion of the study than at the beginning.

Predictors of micronutrient status -- Contrary to the growth and morbidity outcomes, with just one exception, final micronutrient status was not associated with any baseline maternal or child characteristics. The exception was the change in serum ferritin, which was positively affected by MMN supplements, although only among children with low initial plasma zinc

concentration (Table 9).

DISCUSSION

Dietary supplementation of Guatemalan infants with cereal-based preparations that contained bovine serum concentrate (BSC), multiple micronutrients (MMN), or both during periods of two to eight months did not affect their growth velocity or rates of infection compared with those of infants who received the same preparations containing added whey protein concentrate (WPC) only. On the other hand, the final hemoglobin levels were greater and rates of anemia were reduced among infants who received the MMN-containing supplements. Children who received MMN + WPC also had greater final serum ferritin concentrations, although this was not true for the group that received MMN + BSC. None of the other biochemical indicators of micronutrient status differed by treatment group.

These conclusions are bolstered by several aspects of the study design, including the randomized allocation to treatment group, the masking of group assignment, the direct observation of consumption of supplements on most study days and high rates of compliance, and the careful supervision of data collectors and other quality assurance procedures. On the other hand, the high rate of study abandonment, especially between months six and eight of the intervention, reduced the initially planned sample size, hence the desired statistical power of the analyses. Nevertheless, the dropout rate did not differ by study group, so this should not have biased the results; and all analyses were completed on an intention-to-treat basis, regardless of the individual subject's duration of participation in the study. Although the major results are presented in detail only for the subset of children who remained in the study for eight months (to

assure consistency in the presentation of the different sets of results), the interpretation of the group-wise comparisons did not differ when shorter periods of observation (hence, larger numbers of children) were considered. The treatment groups were similar at baseline, the dropout rates did not differ by study group, and those who left the study early did not differ significantly from those who remained with regard to their baseline characteristics. Thus, it is reasonable to attribute any final group-wise differences to the treatments that were provided.

The study subjects were moderately stunted at baseline, and the rate of low height-for-age (Z-score <-2) increased more than two-fold from ~22% to ~50% during the eight-month period of observation. Contrary to our original hypotheses, the children's growth was not affected by consumption of either BSC or MMN. Previous studies found that BSC increased dietary intakes and rates of weight gain of domesticated farm animals, possibly because the IgG contained in the preparation reduced the incidence of infections (13, 14). We are not aware of any relevant, prior studies in humans of BSC and growth. There are several possible explanations for the differences in the results of the present study in humans compared with the earlier findings in farm animals. Firstly, the earlier studies were conducted in weaned animals, whereas most of the children in the present study were breastfed. It is conceivable that the protective effect of BSC may occur only in individuals who are not already receiving the infection-reducing benefits provided by breast milk. Secondly, it is possible that the dose employed in the present study was not sufficient to protect against infections. The dose of BSC offered in the present study provided ~3 to 4.5 g of protein, or ~20% of the children's total dietary protein. Studies in weaned piglets similarly used amounts of plasma protein that were ~25% of the animals' dietary protein, but weanling pigs typically receive about three times more total protein, so their total

intake of plasma proteins would also be greater. Thus, it might be necessary to use a higher dose of BSC to achieve the same results as has been observed in farm animals. Thirdly, the specific antibodies present in the BSC may not have been appropriate to protect against the range of infections that occur most commonly in humans. Finally, there may be a different, as yet unexplained, mechanism for the growth-promoting effect observed in farm animals, which is not operative in humans

A number of previous studies have found increased growth among children who were supplemented either with single micronutrients, such as zinc (7), or with multiple micronutrients (8-10). With regard to zinc, the children's low dietary intakes and baseline plasma zinc concentrations suggest that they indeed may have been zinc-depleted at baseline. However, the results of previous zinc intervention trials suggest that zinc supplementation increases children's growth only when the baseline mean height-for-age of the study subjects is <-1.5 SD compared with international reference means (7), which is just about the initial mean height-for-age of the children in the present study. Thus, the current study subjects may not have been sufficiently stunted to manifest a response to supplemental zinc.

Fewer results are available from previous growth studies of infants who received MMN supplementation, and the findings are inconsistent. Positive effects on growth were observed among infants <12 months of age -- but not among older children -- in a recent study in Mexico, in which supplements were provided for one year (8). MMN supplementation spurred increased growth among Vietnamese children 6-24months of age who were stunted at baseline, but not among those who were non-stunted children (10). Among Tanzanian school children 6-11 years of age, a MMN-fortified drink produced increased rates of both linear growth and weight gain

(9). By contrast, there was no growth impact of MMN supplementation in a recent trial in Peru (11) or in a multi-center study recently completed in Indonesia, Vietnam, Peru, and South Africa (12). Differences across these different sets of studies may relate to the age of the study subjects, their underlying nutritional status, the duration of supplementation, the composition of the supplements, or their mode of delivery (e.g., with or without meals). In the studies from Mexico, Vietnam, and Tanzanian the MMN supplement was given in the form of an aqueous solution. By contrast, in the present study and in the trial in Peru the MMN supplement was provided as a cereal-based dietary supplement, which included several food ingredients. Thus, it is possible that the form of the supplement may have contributed to the observed differences among these studies.

Based on the observed variability in the children's growth velocity and the available sample size at each time point, we could have detected with a power of 0.80 group-wise differences in growth of 0.9 cm after six months of supplementation (or 0.5 cm if combining groups and examining only main effects of BSC or MMN) and 1.2 cm after 8 months (0.7 cm if examining only main effects). The actual shortfalls in the children's average growth rates were 1.9 cm for the period from 6-12 months of age and 2.4 cm for the period 6-14 months, compared with North American children of the same age ranges. Thus, it should have been possible to detect growth differences of this magnitude if the supplements had been able to mitigate the children's growth failure.

The factors that predicted the children's linear growth in the present study were their initial weight, as has been noted by other investigators (6, 28), maternal height, which also has been observed previously (29), and maternal education, as was seen in an earlier study in

Guatemala (6) and elsewhere (30). Initial length was negatively associated with linear growth, although this might reflect regression to the mean. Continued breastfeeding at 12 months was negatively associated with growth, even after controlling for household socioeconomic status. Other investigators have likewise noted this negative association (31), although it was not possible to rule out completely other potentially confounding factors or reverse causality.

Surprisingly, the prevalences of diarrhea and fever were not associated with the children's growth increments, a finding that differs from multiple earlier studies (32), including two that were conducted more than 30 years ago in rural areas of Guatemala (33, 34). However, since then, several researchers have suggested that the potentially adverse effects of diarrhea on children's growth can be mitigated by adequate energy intake (35, 36) and by breast feeding (36, 37). The baseline dietary data suggest that the present study subjects had adequate energy intakes, and the vast majority were breastfed throughout the course of the study. Thus, they may have been more resistant to the growth-limiting effects of diarrhea. Impaired intestinal mucosal function, as measured by altered permeability to sugar probes, is another biological factor that has been closely associated with stunted infant growth of African infants (38).. Intestinal mucosal permeability was not examined in the present study, but earlier research in the same study communities found high rates of abnormal intestinal mucosal function (39). If altered intestinal function were, indeed, an important determinant of children's growth in the study community, it would appear that neither BSC nor MMN supplementation was able to ameliorate this condition.

Although growth was the primary outcome in this study, we also examined the effect of the dietary supplement on morbidity rates. Although neither form of supplementation (BSC or MMN) affected morbidity outcomes, the current sample size would only permit detection of

fairly large differences in morbidity rates. The prevalence of diarrhea was less than expected and lower than reported in a previous study in a rural Guatemalan community in which daily supplementation with 10 mg zinc reduced the rates of diarrhea (40). While the benefit of zinc supplementation on reducing morbidity has been demonstrated in a considerable number of studies (41), the effect of multiple micronutrient supplements on morbidity outcomes is still unclear. The few results available to date have been disappointing (11,12).

Analysis of the infants' dietary data indicated low intake of several micronutrients, especially iron and zinc. By contrast, mean vitamin A intake appeared adequate because of consumption of breast milk and vitamin A fortified-foods, such as sugar, which contributed nearly 50% of the total vitamin A intake. Despite the low iron intakes, the infants had adequate iron status at baseline, presumably because they had received supplemental iron for 1-2 months prior to initiation of the intervention trial. Thus, we were able to examine the effect of the supplements in preventing reemergence of iron deficiency. Although serum ferritin concentrations declined in all groups, the decrease in hemoglobin was less among those children who received MMN and the reduction in serum ferritin was less in group MMN + WPC.

The lack of response of plasma zinc concentration was unexpected because most previous studies found significant increases following zinc supplementation (7). On the other hand, studies of MMN supplements have not always detected changes in plasma zinc concentration, possibly because some other component of the supplement interfered with zinc absorption or metabolism. The supplements that were distributed in the current study provided one RDA each of vitamins A, B_1, B_2, B_3, B_6, B_{12}, C, and D, folic acid, iron, zinc, iodine, and selenium and approximately 0.3 RDA of calcium and phosphorus. Several of the minerals, such as iron and

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calcium, may have limited the response to zinc (11, 42, 43).

In conclusion, the results of our study show that supplementation with a cereal-based pudding containing BSC and/or MMN six days per week during a 6-8 month period was not effective in preventing growth stunting of young children in a peri-urban setting in Guatemala, although MMNs did reduce the rates of anemia. Nutritional interventions to improve infant and child nutritional status and growth may need to start earlier in the life cycle (perhaps during pregnancy), and micronutrients may need to be provided in other chemical forms or delivery vehicles to be more effective.

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Figure Legends

Figure 1 – Profile of study subjects

Figure 2 – Mean (SD) length, weight and mid-upper arm circumference (MUAC), by treatment group (WPC = whey protein concentrate, BSC = bovine serum concentrate, MMN = multiple micronutrients). No significant differences by treatment group.

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Table 1 - Composition of dry supplements, by treatment group (amount of ingredient, in grams, per daily dose of supplement, infants 6-11 mo*)

Ingredient	Treatment group			
	WPC	MMN + WPC	BSC	MMN+ BSC
Maize flour	2.9	2.5	2.9	2.5
Maltodextrin (corn syrup solids)	1.6	1.6	1.6	1.6
Sugar	3.8	3.8	3.8	3.8
Lecithin, flavoring agents	0.9	0.9	0.9	0.9
Bovine serum concentrate	0	0	3.1	3.1
Whey protein concentrate	5.0	5.0	1.9	1.9
Micronutrient mix	0	0.4	0	0.4

Supplements prepared as semi-solid "pudding" by adding ~30 ml of water

* Children ≥12 mo received 1.5 times this dose

Table 2 - Composition of micronutrient mix provided to MMN groups (amount of ingredient per daily dose of supplement, infants 6-11 mo*)

Nutrient (compound)	Unit	Amount
Vitamin A (retinyl palmitate)	μg RE	400
Vitamin B_1 (thiamin mononitrate)	mg	0.4
Vitamin B_2 (riboflavin)	mg	0.5
Vitamin B_3 (niacinamide)	mg	6
Vitamin B_6 (pyridoxine HCl)	mg	0.6
Vitamin B_{12} (cyanocobalamin)	μg	0.5
Vitamin C (ascorbic acid)	mg	35
Vitamin D_3 (cholecalciferol)	IU	400
Folic acid	μg	35
Iron (as electrolytic iron)	mg	10
Zinc (as zinc sulfate)	mg	5
Iodine (as potassium iodide)	μg	50
Selenium (as sodium selenite)	μg	15

Calcium (as calcium phosphate)	mg	50
Phosphorus (as calcium phosphate)	mg	40

* Children ≥12 mo received 1.5 times this dose

Table 3 - Baseline characteristics of subjects included in at least one outcome assessment, by treatment group (mean ± SD or %)

	Group				
Variable	**WPC** (N = 62)	**MMN + WPC** (N = 64)	**BSC** (N = 63)	**MMN + BSC** (N = 60)	**P-value**[1]
Male/Female	30/32	30/34	31/32	29/31	0.99
Age at start of supplementation (mo)	6.7 ± 0.4	6.8 ± 0.4	6.8 ± 0.4	6.7 ± 0.4	0.58
Infant anthropometry at start of supplementation					
Weight (kg)	6.94 ± 0.94	6.91 ± 0.78	6.97 ± 0.85	6.90 ± 0.77	0.96
Length (cm)	64.2 ± 2.5	64.0 ± 2.2	64.0 ± 2.3	64.1 ± 2.0	0.93
Arm circumference (cm)	13.7 ± 1.2	13.6 ± 1.0	13.9 ± 1.0	13.6 ± 0.9	0.35
Weight-for-age (z)	-1.02 ± 0.77	-1.09 ± 0.95	-1.15 ± 0.98	-0.99 ± 0.77	0.81
Length-for-age (z)	-1.51 ± 0.83	-1.45 ± 0.77	-1.62 ± 0.76	-1.53 ± 0.76	0.77
Weight-for-length (z)	0.22 ± 0.68	0.08 ± 0.91	0.19 ± 0.88	0.27 ± 0.73	0.68
Infant morbidity during the 30 days prior to supplementation					
Prevalence of diarrhea (≥3 liq. or semi-liq. stools) (%)	9.0 ± 13.7	16.9 ± 22.9	11.6 ± 19.6	13.6 ± 22.4	0.12 (K-W)

	Group				
Variable	**WPC** (N = 62)	**MMN + WPC** (N = 64)	**BSC** (N = 63)	**MMN + BSC** (N = 60)	**P-value**[1]
Prevalence of diarrhea (>3 liq. or semi-liq. stools) (%)	4.9 ± 8.1	11.1 ± 18.8	7.6 ± 15.7	7.8 ± 14.7	0.30 (K-W)
Prevalence of fever (%)	9.4 ± 11.1	11.2 ± 12.0	11.5 ± 13.1	11.4 ± 13.1	0.77 (K-W)
Prevalence of upper respiratory infection (%)	8.9 ± 14.0	5.9 ± 11.2	7.5 ± 15.5	7.9 ± 13.4	0.59 (K-W)
Prevalence of lower respiratory infection (%)	0.1 ± 0.8	0.0 ± 0.0	0.6 ± 5.0	0.1 ± 0.6	0.79 (K-W)
Maternal nutrition and household socioeconomic variables					
Maternal age (years)	24.3 ± 5.7	25.3 ± 6.0	25.5 ± 6.0	26.3 ± 6.6	0.38
Maternal education (years)	3.8 ± 3.1	4.1 ± 2.8	4.3 ± 2.9	4.4 ± 3.2	0.73
Maternal height (cm)	148.7 ± 6.1	149.4 ± 6.0	149.9 ± 6.6	149.1 ± 5.0	0.74
Maternal hemoglobin (g/dL)	13.6 ± 1.3	13.2 ± 1.3	13.5 ± 1.1	13.4 ± 1.2	0.27
Housing quality score	0.92 ± 0.23	0.96 ± 0.24	0.95 ± 0.24	0.93 ± 0.26	0.82
Possessions score	4.2 ± 1.3	4.3 ± 1.6	4.5 ± 1.4	4.0 ± 1.6	0.33

	Group				
Variable	**WPC** (N = 62)	**MMN + WPC** (N = 64)	**BSC** (N = 63)	**MMN + BSC** (N = 60)	**P-value**[1]
Infant baseline daily nutrient intake					
Total energy intake (Kcal/kg)	87.1 ± 18.3	89.0 ± 27.0	87.3 ± 21.8	86.6 ± 19.5	0.93
Energy intake from breast milk (Kcal/kg)	53.1 ± 31.3	48.2 ±33.0	51.1 ± 30.8	50.3 ± 36.3	0.87
Protein intake (g/kg)	1.9 ± 1.0	2.0 ± 1.3	1.9 ± 0.9	1.9 ± 0.9	0.86
Iron intake (mg)	1.3 ± 1.4	1.6 ± 2.4	1.2 ± 1.2	1.0 ± 1.0	0.19
Zinc intake (mg)	1.7 ± 1.0	1.8 ± 1.2	1.8 ± 1.1	1.7 ± 1.0	0.89
Retinol intake (μg RE)	535.3 ±219.7	551.0 ±276.3	590.3 ±279.8	507.3 ±187.6	0.28
Total phytate intake (mg)	44.7 ± 69.7	61.8 ± 81.7	49.5 ± 70.2	27.6 ±37.1	0.04
Phytate: zinc molar ratio	2.5 ± 3.6	3.3 ± 4.0	2.6 ± 4.6	1.7 ± 2.0	0.13
Infant baseline biochemistry variables					
Hemoglobin (g/dL) (n=259)	11.6 ± 0.7	11.6 ± 0.6	11.6 ± 0.6	11.4 ± 0.6	0.46
Serum ferritin (μg/L) (n=132)	51.0 ± 49.4	46.8 ± 44.3	53.1 ± 40.3	62.1 ± 50.2)	0.48
% with low serum ferritin (<12 μg/L; n= 132)	0.0	5.6	3.1	6.7	0.50
Serum retinol (μg/dL)	23.1 ± 6.3	24.2 ± 10.8	25.4 ± 7.5	25.2 ± 9.9	0.64

(n=146)					
% with low serum retinol (<20 μg/dL; n= 146)	32.4	40.0	22.2	41.9	0.26
Vitamin E (mg/dL) (n=146)	0.43 ± 0.21	0.50 ± 0.26	0.46 ± 0.27	0.42 ± 0.23	0.48
% with low serum vit E (<11.6 μmol/L; n= 146)	67.6	55.0	63.9	60.5	0.71
Plasma zinc (μg/dL) (n=149)	70.7 ± 20.0	66.2 ± 15.3	69.5 ± 19.5	64.8 ± 18.9	0.48
% with low plasma zinc (<65 μg/dL; n= 149)	45.7	53.7	48.6	55.0	0.84
Plasma copper (μg/dL) (n=149)	136 ± 39	137 ± 33	133 ± 39	134 ± 36	0.95

[1] Significance of group-wise comparisons (ANOVA for continuous variables, Kruskal-Wallis for categorical variables)

Table 4. Amount of supplement consumed (g/d), by age and treatment group[1]

Age	Treatment group			
	WPC	WPC + MMN	BSC	BSC + MMN
<12 mo	12.2 ± 2.1 [a]	12.2 ± 2.2 [a]	10.7 ± 2.4 [b]	10.6 ± 2.6 [b]
≥12 mo	19.1 ± 3.3 [c]	17.8 ± 3.5 [c]	16.6 ± 4.5 [d]	16.1 ± 3.8 [d]

[1] Means are from >98% of days when at least some of the supplement was consumed.

Values with different superscripts are significantly different, $p<0.0001$ (3-way repeated measures ANOVA with main effects BSC treatment, MMN treatment, age and their interactions)

Table 5. Factors associated with growth of Guatemalan children from ~6 to ~14 months of age

Linear growth (cm/8 mo) (N=116)		
Variable	**Coefficient**	**P-value**
• Initial weight (kg)	0.50	0.02
• Initial length (cm)	- 0.24	0.006
• Maternal height (cm)	0.07	0.002
• Continued breastfeeding 0= non BF at 1 year; 1= BF at 1 year	-1.08	<0.0001
• Maternal education (years)	0.09	0.04
R^2 = 0.29		
Weight gain (kg/8 mo) (N=116)		
• Maternal height (cm)	0.03	0.004
• Continued breastfeeding 0= non BF at 1 year; 1= BF at 1 year	-0.67	<0.0001
Maternal education (years)	0.05	0.008
Initial length (BSC*MMN) 0 0	-0.16	0.001
Initial length (BSC*MMN) 1 1	0.16	0.01
R^2 = 0.45		

Table 6– Prevalence and incidence of morbidity during period of supplementation, by treatment group (mean ± SD) [1]

Variable	Treatment group				P-value ANOVA[2]
	WPC (n=57)	WPC + MMN (n=57)	BSC (n=54)	BSC + MMN (n=51)	
Prevalence of diarrhea (%)	10.2 ± 8.4	13.5 ± 13.2	10.4 ± 9.6	11.9 ± 13.0	0.98
Incidence of diarrhea (episodes per 100 d at risk)	3.6 ± 2.6	5.1 ± 4.8	3.8 ± 3.0	3.9 ± 4.1	0.76
Incidence of severe diarrhea (episodes per 100 d at risk)	1.9 ± 2.0	2.7 ± 3.0	1.9 ± 1.8	2.1 ± 2.5	0.96
Incidence of persistent diarrhea (episodes per 100 d at risk)	0.13 ± 0.34	0.19 ± 0.58	0.03 ± 0.19	0.15 ± 0.40	0.19[3]
Prevalence of fever (%)	9.4 ± 7.3	9.0 ± 6.2	10.5 ± 7.8	8.8 ± 6.8	0.86
Incidence of fever (episodes per 100 d at risk)	3.9 ± 2.9	3.6 ± 2.2	3.9 ± 2.7	3.6 ± 2.5	0.78
Prevalence of URI (%)	7.1 ± 10.2	6.0 ± 8.3	7.1 ± 9.6	6.9 ± 9.5	0.96
Incidence of URI (episodes per 100 d at risk)	1.4 ± 1.6	1.3 ± 1.7	1.4 ± 1.6	1.6 ± 2.3	0.53
Prevalence of LRI (%)	1.2 ± 3.4	1.1 ± 2.7	1.2 ± 2.7	1.4 ± 2.9	0.85
Incidence of LRI (episodes per 100 d at risk)	0.6 ± 1.6	0.5 ± 1.0	0.5 ± 0.9	0.7 ± 1.4	0.95

[1] Analyses include children with at least 60 days of observation after initiation of supplementation. Diarrhea defined as ≥3 liquid or semi-liquid stools per d.

[2] ANOVA, controlling for pre-treatment morbidity rates, as explained in text. Logarithm of morbidity rate was used in all analyses, except incidence of persistent diarrhea.

[3] Logistic regression was used for this variable. (Because of low incidence of persistent diarrhea, logarithmic transformation was not appropriate.)

Table 7 -- Factors associated with morbidity status of Guatemalan children from ~6 to ~14 months of age

Prevalence of diarrhea per 100 days (log + 3) (N= 222)		
Variable	**Coefficient**	**P-value**
• Sex (0=M; 1=F)	-0.17	0.04
• Initial length (cm)	- 0.04	0.02
• Maternal education (years)	-0.06	<0.0001
• Initial diarrhea	1.55	<0.0001
R^2 = 0.33		
Incidence of diarrhea per 100 days at risk (log +1) (N=216)		
• Age at enrollment (mos)	- 0.22	0.02
• Maternal education (years)	-0.04	0.003
• Initial diarrhea	1.40	<0.0001
R^2 = 0.23		
Prevalence of upper respiratory infections per 100 days (log + 1) (N=192)		
• Initial URI	2.44	0.0002
R^2 = 0.0.09		
Incidence of lower respiratory infections per 100 days (ln (incidence + 1)) (N=215)		
• Sex (0=M; 1=F)	-0.17	0.01
• Initial weight (kg)	- 0.09	0.02
• Maternal education (years)	-0.02	0.03

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• Age at enrollment (months)	0.17	<0.0001
R^2 = 0.08		

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Table 9 – Change in geometric mean serum ferritin concentration in children who did or did not receive MMN supplementation, by initial plasma zinc concentration

		No MMN supplementation	MMN supplementation
Initial plasma zinc status	Low	-39.8 ± 38.4 [a]	-28.2 ± 29.2 [b]
	Normal	-36.3 ± 41.6 [ab]	-36.1 ± 51.7 [ab]

Analysis of covariance conducted on change in log (serum ferritin), controlling for initial log ferritin and supplementation with BSC. P for interaction = 0.028. Means with different superscripts are significantly different from each other, p=0.056.

Figure 1

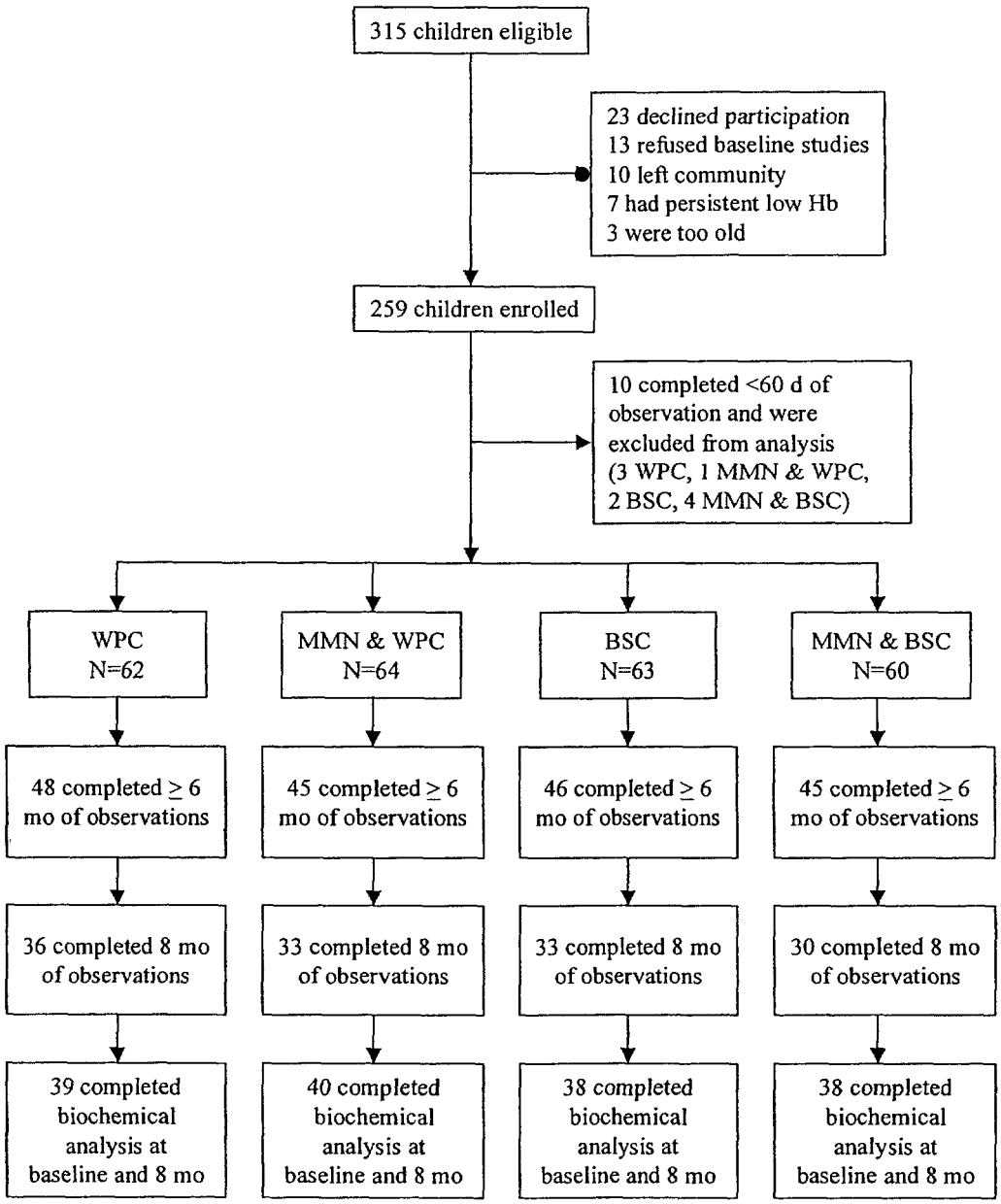
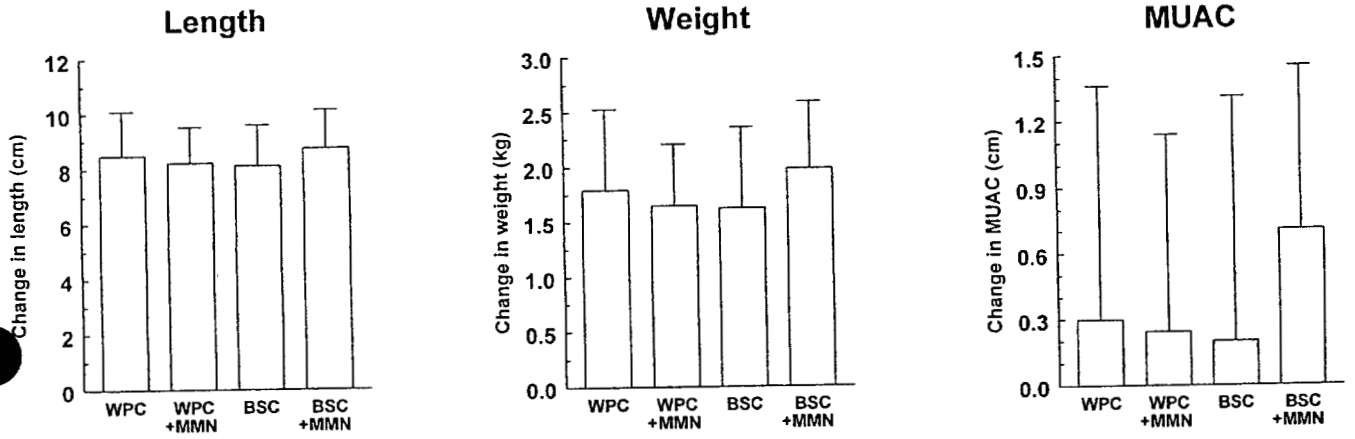

315 children eligible
23 declined participation
13 refused baseline studies
10 left community
7 had persistent low Hb
3 were too old
259 children enrolled
10 completed <60 d of observation and were excluded from analysis (3 WPC, 1 MMN & WPC, 2 BSC, 4 MMN & BSC)
WPC N=62
MMN & WPC N=64
BSC N=63
MMN & BSC N=60
48 completed ≥ 6 mo of observations
45 completed ≥ 6 mo of observations
46 completed ≥ 6 mo of observations
45 completed ≥ 6 mo of observations
36 completed 8 mo of observations
33 completed 8 mo of observations
33 completed 8 mo of observations
30 completed 8 mo of observations
39 completed biochemical analysis at baseline and 8 mo
40 completed biochemical analysis at baseline and 8 mo
38 completed biochemical analysis at baseline and 8 mo
38 completed biochemical analysis at baseline and 8 mo

Figure 2


Length
Change in length (cm)
12
10
8
6
4
2
0
WPC
WPC +MMN
BSC
BSC +MMN
Weight
Change in weight (kg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
WPC
WPC +MMN
BSC
BSC +MMN
MUAC
Change in MUAC (cm)
1.5
1.2
0.9
0.6
0.3
0.0
WPC
WPC +MMN
BSC
BSC +MMN

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Title: The effect of a bovine serum immunoglobulin fraction (bIg) on blood lipid indices in mildly hypercholesterolemic subjects

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ABSTRACT

Background: Hypercholesterolemia is one of the major risk factors for cardiovascular disease (CVD). Interestingly, the consumption of milk and yogurt has been found to lower cholesterol. The IgG fraction of milk, a serum protein, is a potential candidate responsible for the cholesterol-lowering effect.

Objective: We examined the effectiveness of oral supplementation of a serum immunoglobulin fraction (bIg) derived from USDA-approved bovine plasma on lipid indices in hypercholesterolemic humans (N = 52).

Design: Participants included men and women (25-70 y) with moderate hypercholesterolemia (210-270 mg/dL) not receiving cholesterol-lowering medication. Entry required participants to have two baseline total cholesterol (TC) readings not differing by more than 10%. Treatment consisted of participants receiving in a double blind, placebo controlled, randomized manner, 5g of bIg. Food frequency questionnaires were used to examine energy and macronutrient intakes at baseline and 6W.

Results: MANOVA and post-hoc evaluation, covaried for minor, yet significant total energy intake changes, showed no between group differences for mean (± SEM) TC. However, significant within group effects for bIg were observed vs. baseline (mean ± SEM; 244.5 ± 3.8 mg/dL) at 3W (230.9 ± 3.7; $P < 0.05$) and 6W (230.4 ± 5.1 mg/dL; $P < 0.05$) Corresponding placebo values for TC are baseline (238.0 ± 3.0 mg/dL), 3W (236.9 ± 4.5 mg/dL) and 6W (234.2 ± 3.6 mg/dL). An examination of change data (95% CI) within the bIg group at 3W (95% CI, -21.6, -5.8 mg/dL; $P < 0.01$) and 6W (95% CI, -23.6, -4.6 mg/dL; $P < 0.01$) were also significant and statistically different vs. the placebo condition ($P < 0.05$). This reduction in TC was largely due to reductions in the LDL-C at 3W (95% CI, -16.7, -1.5 mg/dL; $P < 0.05$) and 6W (95% CI, -

17.6, -3.8 mg/dL; $P < 0.05$) No significant changes were observed for HLD-C, VLDL-C, triglycerides, CRP or glucose. Furthermore, no significant changes were observed in other blood chemistry markers associated with hepatorenal or cardiovascular function.

Conclusion: Consumption of bovine IgG appears to positively modulate major lipid indices associated with CVD.

Key words: Cholesterol; cardiovascular disease; LDL, lipids, bovine IgG, oral supplementation

INTRODUCTION

Hypercholesterolemia is one of the major risk factors for cardiovascular disease (CVD). According to National Cholesterol Education Program (NCEP) guidelines, weight reduction and dietary management are the preferred alternatives for lowering cholesterol and heart disease risk despite the challenges they pose for most individuals (1). An "alternative" to lowering cholesterol, baring lifestyle changes, is the use of medication such as statin drugs. Though effective, some individuals experience negative sequealea that may contraindicate their use (2, 3). Therefore, the continued investigation of alternative solutions is well warranted. One such area for investigation is constituents found in milk.

The consumption of milk and yogurt has been found to have cholesterol-lowering effects (4-7); yet, the components of milk with cholesterol-lowering effects have not been adequately identified (8). Interestingly, the protein fraction of milk may be a likely candidate for inquiry because both skim milk and yogurt have been found to lower cholesterol when compared to whole milk (3, 4). Of the major proteins found in milk, those found in whey protein present an interesting avenue for investigation as casein is actually thought to be *hypercholesterolemic* when compared to other sources of protein (9, 10). Moreover, two studies have reported that the administration of 90 g of dried skim milk from hyper-immunized cows lowers cholesterol in human subjects with mildly elevated cholesterol concentration (11, 12). The primary difference between the control skim milk and the skim milk from hyper-immunized cows (immune milk) is the immunoglobulin protein content (IgG), where participants consumed ~ 500 mg BIg per day when ingesting immune milk. Whereas milk and whey contain very low concentrations of IgG (<1% and 3 to 4% respectively, immunoglobulin comprises at least 25% of the protein content of serum. This is an important consideration as the intake of 90 g skim milk solids may be

impractical for consumers; whereas the purification of IgG from bovine serum produces both a higher concentration and yield so that smaller quantities can be ingested. Serum protein also contains other factors that could alter cholesterol absorption. These include antibodies reactive with cholesterol (13) and lipoproteins, which might interfere with cholesterol absorption (14). Recently, in and 8-week un-reported pilot study involving 22 mildly hypercholesterolemic subjects, 5 g per day of bIg was found to be acceptable and effective in reducing total cholesterol (TC). The objective of this study is to examine the effects of bIg supplementation on blood lipids in a larger cohort of hypercholesterolemic individuals in a formalized clinical trial.

MATERIALS AND METHODS

Study Population

In this study, we examined 52 men and women aged 25-70 years who volunteered for this investigation conducted at The Cooper Institute Center for Human Performance and Nutrition Research (Dallas, TX USA). All participants were hypercholesterolemic with borderline to undesirable elevated total cholesterol ranging between 210-270 mg/dL (x-y mmol/L) and not receiving cholesterol-lowering medication or dietary supplements such as niacin. We recruited individuals from the surrounding Dallas/Ft. Worth community by radio, television and newspaper advertisements. Before study initiation, all participants took part in a formalized screening process inclusive of a phone screening and two unsupplemented baseline cholesterol screenings (see below). Following the initial phone screening, participants signed an informed written consent approved by The Cooper Institute Review Board.

Inclusion Criteria and Pretrial Screening

Inclusion criteria for this study necessitated that all participants have a total cholesterol concentration between 210 and 270 mg/dL. We chose total cholesterol as our criterion measure because pilot data suggested that this was the most prominent lipid index affected by bIg ingestion. We initially screened 500 potential participants by phone, with 250 qualifying to initiate the study. Following this initial phone screening, participants were asked to a complete medical history and food frequency questionnaire via an online Internet data collections system (Vitalink, Bellevue, WA, USA). Participants who did not have Internet access were mailed questionnaires. After completing these forms, participants became eligible to enter the blood cholesterol-screening portion of our study protocol. The time that elapsed between the phone screening and the first blood sample was less than two weeks.

Overall, 90 participants remained eligible to participate following all screening procedures; yet, 10 of these participants withdrew prior to randomization. In addition, 24 subjects failed to complete all visits and/or all necessary paper work and forms signifying successful trial completion. Following a successful pre-trial screening procedure, participants donated fasting baseline blood samples on two occasions, separated by 7-10 d, prior to supplement distribution. Successful entry into the trial at this point required that each baseline total cholesterol concentration not differ by more than 10%. If the variance was greater than 10%, a third baseline visit, again separated by 7-10 d, was used to define whether the participant was eligible for trial entry.

During the trial, all participants agreed to maintain their current diet and exercise habits during the trial and not initiate new vitamin or mineral supplements different from what they were taking 6W prior to the study. All of these parameters were examined during the trial

through the use of online and mail back questionnaires. We excluded pregnant or lactating women from participation. Postmenopausal women both on and off hormone replacement therapy (HRT) were accepted into the trial; however, we asked those on HRT to remain on their current medication and dosage schedule and notify us if the regimen was changed. Participants currently on standard medical therapy (for conditions such as hypertension, arthritis or other chronic diseases) were allowed to enter the study if they agreed to remain on their current therapy during the trial.

Participants were excluded from the trial if their BMI was <18.5 or >30, they had recently donated blood (<3 months) or failed to agree not to donate blood during the trial. Also excluded were participants with elevated blood pressure, total cholesterol, LDL-cholesterol or fasting plasma glucose requiring immediate drug therapy according to national guidelines (JNC VI, Adult Treatment Panel II, and American Diabetes Association). Lastly, we excluded those individuals who planned to move from the area during the trial, smoked, consumed alcoholic beverages exceeding an average of 3 drinks per day or consumed coffee in excess of 3 cups per day. If a participant was unsure of their cholesterol concentration prior to baseline screening, but thought that it was high, we informed these participants of their baseline cholesterol concentration so that they could make an informed decision whether to enter the trial or seek medical assistance via drug therapy through their family physician. We advised those participants with LDL-C greater than 160 mg/dL of the risks associated with LDL-C concentrations of this nature, as well as the recommendation for seeing their family physician. However, subjects choosing to continue participation in the study were allowed to do so.

Treatment and Assessment Indices

Each participant was randomly assigned, in a double-blind manner, to consume five treatment or placebo capsules, containing approximately 500 mg of the appropriate protein source, twice a day (n = 10) for 6W. The participants were verbally instructed to take them with a meal. The treatment consisted of participants consuming 5 g of the bIg fraction daily, which provided 2.4 g of bovine IgG. The source of bIg utilized in this study is a immunoglobulin-enriched protein fraction obtained through the partial removal of fibrinogen, lipids, and serum albumen from edible bovine plasma. The bIg-fraction contained 88.6% total protein and 48.4% bovine IgG, determined by radial immunodiffusion assay. The plasma is collected from USDA-inspected and approved beef cattle less than 30 months of age. Hydrolyzed gelatin was used as the source of protein for the control group. The control capsules were approximately 490 mg in weight and the 10 capsules provided the same level of protein as the bIg fraction . All bottles and capsules are distributed in identical bottles and colored capsules. We also used a randomized number sequence for treatment distribution so that in case of side effects, we could break the code of that particular individual, rather than sacrifice the integrity of the entire treatment group. One of our investigators was in charge of this aspect of the study (*aj*) and had no contact with any of the study participants at anytime, nor was involved in the data analysis.

Upon successful entry into the trial, participants were given 3W worth of treatment and asked to return at 3W for an interim measurement. Following the 3W visit, participants were given new treatment bottles with enough capsules to finish the trial. During each visit, all participants returned their pill bottles and a pill count was undertaken to examine compliance. Blood assessments were obtained in a fasting condition (>12 hours) for a variety of parameters inclusive of a blood lipid profile, blood glucose concentration, muscle, kidney and hepatorenal

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indices (Chem-16) and C-Reactive protein (CRP). Each blood collection consisted of obtaining approximately 50 mL of blood that was divided into one serum separator vacutainer (10 mL) and four K3 EDTA tubes (≈ 40 mL). All samples were subsequently spun within 3 minutes of venous collection in a cold centrifuge at 1200 rpm for 12 minutes. Separated plasma and red blood cells were divided into four cryovials and placed in -80°C freezer. Blood lipid profiles, fasting blood glucose concentration, and muscle and hepatorenal indices were analyzed at a commercial laboratory, (Lab Corp, Dallas, TX USA).

Statistical Analysis

The primary outcome variables for this trial are total cholesterol (TC) and LDL-C. Secondary outcome variables included lipid sub-fractions inclusive of VLDL-C, HDL-C and triglycerides. As tertiary measures, we also examined CRP and blood glucose. Given that this was a study using a "free living" population, and recognizing the fact that alterations in body mass and energy intake can affect cholesterol concentrations, we also weighed each participant before (baseline) and after (6W) the intervention. Each participant was asked to complete a food frequency questionnaire so that we could examine total energy intake, as well as macronutrient partitioning. If any significant changes were observed for body mass, total energy consumption or nutrient partitioning, these variables were included as covariates in our analysis. In order to examine participants for any potential side effects, we examined an additional blood chemistry panel for 12 variables (CHEM-12) associated with alterations in hepatorenal or cardiovascular function.

We examined all baseline TC concentrations for normal distribution characteristics. Subsequently, we examined mean and mean change data for all variables using repeated

measures MANOVA that was covaried according to our observations regarding body mass and total energy change. Therefore, these data were used as covariates for our statistical analysis. Though age and gender may also affect the statistical analysis of cholesterol data, we observed no statistical interaction for these variables. Thus, they were not included in our analysis as covariates. All variables showing a statistically significant effect were subsequently analyzed using a Tukey-Kramer post-hoc assessment for all within and between pair wise comparisons. All statistical analyses were performed using JMP statistical software V5.0.1.2 (SAS, Cary, NC, USA). Statistical significance refers to a value of $P < 0.05$. To gain further appreciation for any statistically differing variables, we have also shown the precision of our estimates of outcome statistics for all change data as 95% confidence limits (95% CI), which define the 95% likely range of the true effect in the population from which we drew our sample.

RESULTS

Study Population Demographic and Compliance. We found no between group treatment or gender differences for age or body mass in our study population. The mean (SEM; 95% CI) age for our participants was 51 (1.6; 95% CI, 47.9, 54.2). Baseline body mass was 176.3 (4.5; 95% CI, 167.3, 185.3). However, we did observe a modest change in body mass for all pooled participants during the treatment period 6W ($P < 0.01$). In general, within treatment group analyses showed that treatment group generally gained weight (95% CI, 3.8, 5.7), while the placebo group tended to lose weight (95% CI, -2.9, -0.9). Subsequently, body mass change was used as a covariate. Throughout the study, each group remained ~75% compliant to the study protocol. The overwhelming reason for lack of compliance cited was the number of pills the

subjects were asked to consume each day. (Was there anything else to report on acceptability, i.e. QOL?)

Energy Partitioning and Macronutrient Analysis. A description of our study populations macronutrient energy partitioning is presented in Table 1. Though no between group effects was noted, we did observe a modest change in total energy consumption within each treatment group ($P < 0.02$) that was not distinct to any nutrient inclusive of carbohydrates, protein, total fat and associated subfractions of saturated, polyunsaturated, or monounsaturated fat, alcohol, dietary cholesterol, or dietary fiber. While several of these variables showed a trend for significance, they did not reach statistical significance. Because all of these variables are "collinear" in some fashion to total energy consumption, we used change in total energy consumption as a covariant in our analysis.

Total Cholesterol. Our analysis of TC demonstrated no between group statistical differences for any data collection point (Table 2). However, we did observe a significant within group effect, whereby the group supplemented with bIg showed significant reductions from baseline in mean total cholesterol at 3W ($P < 0.05$) and 6W ($P < 0.05$), as well as change data at 3W ($P < 0.01$, 95% CI, -21.6, -5.8) and 6W ($P < 0.01$, 95% CI, -23.6, -4.6). We have detailed the individual response for each person during each person in Figures 1a. and 1b.

LDL Cholesterol. Our analysis of LDL-C demonstrated no between group statistical differences for any data collection point. Like TC, we observed a significant within group statistical effect,whereby the t group showed significant reductions from baseline in mean LDL-C at 3W ($P < 0.05$) and 6W ($P < 0.05$; Table 1). Examination of the change data for LDL-C supports these findings at 3W (95% CI, -16.7, 1.5) and 6W ($P < 0.5$, 95% CI, -17.6, -3.8), though significance regarding change data is not fully realized until 6W.

Secondary Lipid Parameters and Tertiary Outcomes. An examination of our data revealed no significant between or within mean or change data for HLD-C, VLDL-C, triglycerides, CRP or glucose. Furthermore, no significant changes were observed in other blood chemistry markers associated with hepatorenal or cardiovascular function.

DISCUSSION

In this trial, we observed a 6% reduction in TC in participants with hypercholesterolemia with bIg supplementation. Furthermore, the fraction of cholesterol most responsive to change was LDL-C which was reduced 7%. Though very minor reductions in HDL-C may account for part of our observation, the reduction in HDL-C was not statistically significant, nor did it affect the TC/HDL ratio. This reduction in TC and LDL-C is an encouraging finding as the effect was observed despite the anticipated improvements in both diet and lower cholesterol concentrations in both of the experimental groups. . Currently, we do not know the mechanism of action whereby supplementation with bIg reduces total and LDL cholesterol. However, several previous research findings that stimulated our interest in conducting the study may elucidate how this reduction takes place. The previous findings include: 1) Previous studies of the hypocholesterolemic effect of milk or fermented milk; 2) The lipogenic effects of endotoxin and cytokines; and, 3) Factors in plasma which may affect cholesterol absorption. The effects of bIg supplementation may be a result of one or more or a combination of these factors.

, as well as the effects of endotoxin and cytokines on cholesterol metabolism and the potential for plasma apolipoproteins to affect cholesterol absorption (14, 17). Thus, bIg may be a result of one or more of a combination of these factors.

The potential hypocholesterolemic effect of milk and fermented milk products was initially proposed from a study involving the Maasai people of Africa, whose normal diet is atherogenic, consisting of large amounts of meat, milk and blood serum (18). In this report, the consumption of up to 8 liters of milk per day resulted in lower TC, despite weight gain in the participants. Several other clinical studies on milk and fermented milk consumption appear to confirm these findings (4, 6, 8, 11, 19-23). Moreover, a recent study comparing soy protein with or without isoflavones to milk protein reported an unexpected LDL-C reduction for those consuming 42 g milk protein per day (24). Still, the mediating factors promoting a reduction in cholesterol remains elusive, though immunoglobulin G (11), magnesium (25), riboflavin (25), orotic acid (25), increased bacterial activity (26), and an unknown inhibitory factor (18) all have been proposed as potential mediators. The major constituents of milk that can be eliminated as potential cholesterol-lowering mediators given their hypercholesterolemic nature include saturated fat, lactose, calcium, and casein (10). Complicating comparisons with our study is the observation that the globulin protein concentrations used in previous studies involving milk products is usually unknown.

In our trial, we used a high bIg concentration product, which showed a pronounced lipid lowering effect. This lipid lowering finding is in agreement with previous observations using an immune milk product (12). The two previous studies using skim milk from immunized cows (11)(Insert Sharpe et al., citation) were conducted in individuals with much higher cholesterol concentrations, , 6.86 mmol/L and 7.26 mmol/L, respectively, than the TC at baseline in the current study, 6.33 mmol/L. Despite the lower baseline TC in the present study, supplementation with 5 g of the bIg fraction resulted in a similar reduction in total cholesterol concentration when compared to the supplementation of 90 g skim milk solids administered in the previous study of

immune milk. Additional studies will be necessary to determine the optimum level of supplementation of bIg relative to differing TC or LDL-C concentrations.

The effects of bIg supplementation reported in this study may point to a different means of modulating circulating cholesterol than previous dietary approaches such as the use of phytosterols, soluble fiber or soy protein, which affect the absorption of cholesterol. Data on the effects of feeding plasma protein components on cholesterol metabolism are scarce. The only previously reported animal study to evaluate the effects of plasma protein components on cholesterol absorption or metabolism found no differences in serum or liver cholesterol concentrations between animals fed soy protein and plasma protein, whereas significant increases in liver cholesterol concentration were reported for casein (15). Bovine IgG is not absorbed in healthy adults so the effects are most likely luminal. A luminal effect is possible since the gut is an important contributor to circulating cholesterol and the gut is second only to the liver in the body's production of cholesterol.(27, 28). Luminal factors may affect the production of cholesterol in the gut. Endotoxin, an pro-inflammatory molecule with a presence in the lumen, has been shown to be both lipogenic and proposed as a contributor to atherosclerosis and cardiovascular disease (29).The lipogenic effects have been found to be mediated through increased 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) activity and reduced cholesterol 7-alpha-hydroxylase activity (30-33). Interestingly, immunoglobulin and other plasma proteins possess an endotoxin binding capacity which when administered orally in sufficient quantity has the potential to bind endotoxin in the lumen and subsequently affect inflammatory cytokine production in the intestine (34). Helping to substantiate this premise is the finding that the oral administration of an IgA-IgG fraction of human plasma affects inflammatory cytokine production (35). Additionally, bovine plasma

contains relatively high concentrations of HDL and LDL (36). HDL, in particular, is known to bind endotoxin, which may also indirectly reduce the stimulation of cholesterol synthesis mediated by inflammatory cytokines (37, 38). Both HDL and LDL have also been shown to directly inhibit cholesterol synthesis in vitro (39).

Antibodies and other plasma proteins may also be involved in reducing cholesterol absorption. Antibodies to cholesterol have been found in normal human and animal plasma (40, 41). The antibodies may directly bind cholesterol in the lumen and hinder absorption. In addition, apolipoprotein A-I obtained from sheep plasma has been shown to reduce the brush-border uptake of cholesterol through inhibition of a scavenger-type receptor (14, 17).

While there have been many studies evaluating the effects of dietary protein sources on cholesterol concentrations, few studies have evaluated specific animal protein components and no clinical studies regarding the effects of plasma protein fractions on cholesterol metabolism have been conducted. This is the first report of the effects of the administration of a bIg-fraction on blood lipids in mildly hypercholesterolemic subjects. Though our results are encouraging, a factor that may have limited us from finding a more pronounced effect was treatment compliance. This was probably due to the nature of treatment requiring participants to ingest ten large capsules per day, a factor necessary to ensure blinding. Thus, a different means of administration of bIg, such as a beverage or a bar, should be considered for future studies. Nonetheless, this study demonstrates that globulin protein supplementation reduces total and LDL cholesterol in a population with mild hypercholesterolemia. In view of the size of the hypercholesterolemia problem in industrialized countries, these results should encourage further studies of the effects of bIg supplementation on cholesterol metabolism in humans.

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Figures

Figure 1: Data represent changes in individual total cholesterol concentration. Panel A represents individual data collected at 3 weeks. Panel B represents individual data collected at 6 weeks (i.e, study completion).

Table 1. Data represent nutrient intake for all study participants in the treatment and placebo groups before (baseline) and after (Week 6) treatment.

	Placebo				Treatment			
Nutrient Intake	**Baseline**		**Week 6**		**Baseline**		**Week 6**	
Total Energy								
(Kcal • d^{-1})	1820.1	(130.0)	1537.9	(165.8)	1871.9	(162.1)	1622.3	(92.0)
Protein								
(G • d^{-1})	84.4	(12.2)	62.2	(8.0)	77.7	(8.1)	70.5	(9.3)
Carbohydrates								
(G • d^{-1})	235.6	(28.7)	195.3	(26.2)	223.8	(21.7)	199.7	(19.0)
Total Fat								
(G • d^{-1})	74.8	(10.8)	56.9	(7.6)	70.7	(7.6)	67.2	(9.9)
Saturated Fat								
(G • d^{-1})	25.0	(4.0)	18.6	(2.5)	23.2	(2.5)	21.6	(3.3)
MUFA								
(G • d^{-1})	28.4	(4.2)	21.5	(2.9)	26.8	(2.9)	25.9	(3.8)
PUFA								
(G • d^{-1})	15.0	(2.0)	12.1	(1.7)	15.1	(1.7)	14.6	(2.0)
Diet Cholesterol								
(G • d^{-1})	273.4	(51.4)	193.2	(27.4)	264.3	(37.1)	221.7	(31.0)
Alcohol								
(G • d^{-1})	8.1	(2.4)	5.9	(1.5)	10.5	(5.4)	4.9	(1.4)
Fiber								
(G • d^{-1})	20.1	(2.7)	16.1	(2.5)	18.1	(1.7)	16.4	(1.5)

All data presented as means (± SEM). *Level of confidence significant at $P < 0.05$.

MUFA; Monounhydrated fatty acid

PUFA; Polyunsaturated fatty acid

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TABLE 2: Lipid parameters in treated (bIg) and control groups. Data are mean ± SEM for visit data, in addition to (95% CI) for change data.

	Treatment (n = 26)	Control (n = 26)
Total cholesterol, mmol/L		
Baseline	6.33 ± 0.10	6.16 ± 0.08
3Wk	5.98 ± 0.10 *	6.14 ± 0.12
6Wk	5.97 ± 0.13 *	6.07± 0.09
3Wk change vs. baseline	-0.35 ± 0.10 (-0.56, -0.15) **, †	-0.03 ± 0.09 (-0.22, 0.17)
6Wk change vs. baseline	-0.37 ± 0.12 (-0.61, -0.12) **, †	-0.10 ± 0.08 (-0.27, 0.07)
LDL cholesterol, mmol/L		
Baseline	4.12 ± 0.11	3.95 ± 0.09
3Wk	3.92 ± 0.12 *	4.00 ± 0.12
6Wk	3.84 ± 0.12 *	3.83 ± 0.12
3Wk change vs. baseline	-0.20 ± 0.11 (-0.43, 0.04)	0.05 ± 0.08 (-0.11, 0.20)
6Wk change vs. baseline	-0.28 ± 0.09 (-0.46, -0.10) *	-0.13 ± 0.09 (-0.32, 0.05)
HDL cholesterol, mmol/L		
Baseline	1.35 ± 0.05	1.49 ± 0.04
3Wk	1.28 ± 0.05	1.47 ± 0.05
6Wk	1.29 ± 0.06	1.49 ± 0.05
3Wk change vs. baseline	-0.07 ± 0.04 (-0.15, 0.00)	-0.03 ± 0.03 (-0.08, 0.03)
6Wk change vs. baseline	-0.06 ± 0.03 (-0.12, 0.00)	± 0.03 (-0.06, 0.07)
VLDL cholesterol, mmol/L		
Baseline	0.87 ± 0.09	0.71 ± 0.06
3Wk	0.78 ± 0.07	0.67 ± 0.06
6Wk	0.83 ± 0.09	0.72 ± 0.05
3Wk change vs. baseline	-0.09 ± 0.07 (-0.23, 0.06)	-0.05 ± 0.05 (-0.16, 0.06)
6Wk change vs. baseline	-0.03 ± 0.05 (-0.14, 0.08)	0.02 ± 0.03 (-0.05, 0.10)
Triglycerides, mmol/L		
Baseline	1.84 ± 0.17	1.51 ± 0.12
3Wk	1.68 ± 0.16	1.43 ± 0.13
6Wk	1.80 ± 0.19	1.67 ± 0.16
3Wk change vs. baseline	-0.16 ± 0.13 (-0.44, 0.11)	-0.08 ± 0.10 (-0.29, 0.14)
6Wk change vs. baseline	-0.04 ± 0.11 (-0.27, 0.18)	0.16 ± 0.14 (-0.12, 0.44)

* Represents significant within group treatment difference ($P < 0.05$) vs. baseline

** Represents significant within group treatment difference ($P < 0.01$) vs. baseline

† Represents significant difference between treatment and control ($P < 0.05$)

Figure 1a: 3-week change data

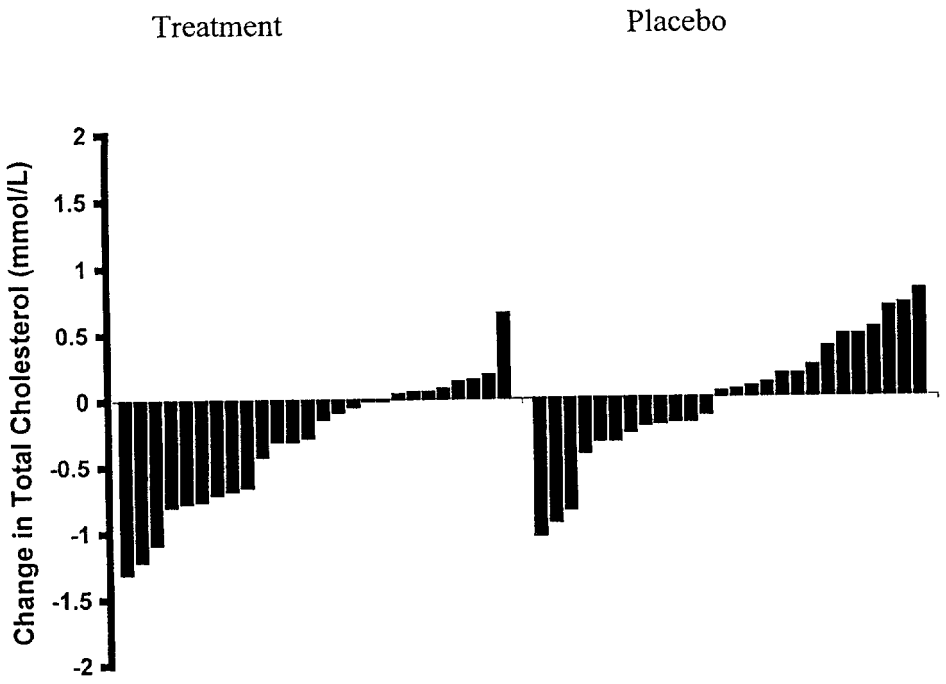
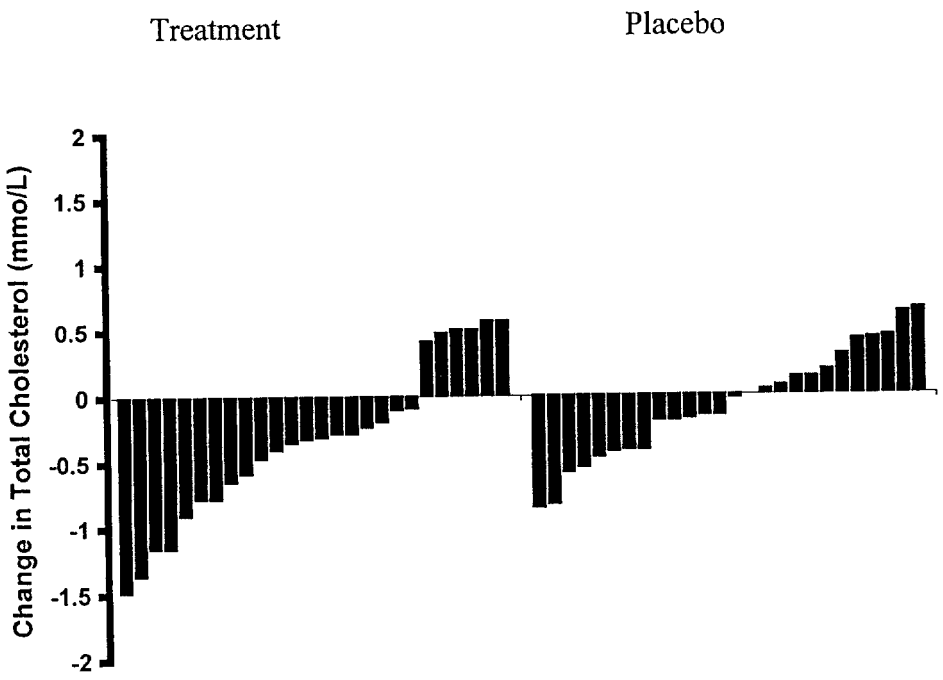


Figure 1b: 6-week change data



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Bovine Immunoglobulin Feeding Trial

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RATIONALE:

Recently the technology has become available to isolate active immunoglobulins from animal sources which are specific to the pathogens which commonly result in infections in humans, e.g. Escherichia coli and rotavirus (1). Exogenous immunoglobulins may help to replace gastrointestinal secretory immunity in immunocompromised hosts . Thus, use of animal-derived immunoglobulins has potential application in such conditions as AIDS or in formula-fed infants deprived of breastmilk immunoglobulins.

Studies in animals (1,2) and human infants and children (3-5) indicate that the addition of immunoglobulins to feedings are well tolerated and may offer protection against pathogens. Immunoglobulins given orally appear to retain their biological activity as they pass through the GI tract (3,6). Although SIgA is the predominant immunoglobulin in human milk and appears to be a more efficient immunologic agent than serum IgG, the latter may be equally effective if present in sufficient quantity (7).

A unique bovine immunoglobulin (BIgG) concentrate was developed by Dr. Murray Drew, AMPC, Inc. from bovine plasma (Table 1). This BIgG concentrate had been effective in conferring immune protection in piglets (1). Although bovine immunoglobulins are present in the food supply, the metabolic fate and immune consequences of BIgG had not been previously tested in humans. For the concentrate to function in gastrointestinal immune defense, it must be resistant to digestion in the upper small intestine. Because of the risk of allergic response, BIgG must also be resistant to be intact absorption across the intestinal tract (8). Therefore, preliminary work was needed before embarking on clinical trials in adult patients with AIDS or a safety and efficacy trial in infants.

OBJECTIVES:

The objectives of the current trial were to test the short term safety and digestibility of orally-ingested BIgG concentrate in healthy adults.

The specific aims were as follows:

1) to assess the postprandial plasma amino acid response to 10g BIgG in 284 g pudding versus unsupplemented pudding;
2) to determine whether intact BIgG could be detected, postprandially, in human plasma;
3) to assess the subject acceptance and gastrointestinal tolerance to BIgG over a two week supplementation period: and
4) to assess BIgG activity in feces collected for 48hrs towards the end of the BIgG supplementation period.

METHODS

A pilot trial was conducted to determine the optimal vehicle for oral delivery of BIgG. We elected not to use capsules/tablets due to the large volume of the 10 g supplement and the desire to present the supplement in a format which would lend itself to clinical trial. Although several liquid bases were tried, solubility was poor in milk-based beverages, juices, carbonated soft drinks, fruit juices and applesauce. Mixing was superior in semi-solids, e.g. ice cream, yogurt or pudding. A taste panel determined that the distinct aftertaste of the concentrate was masked most effectively in pudding.

SUBJECTS

Healthy adult volunteers were recruited by word-of-mouth from the staff and student populations of McMaster University. Volunteers were excluded if any of the following criteria applied: gastrointestinal problems, milk/ lactose intolerance, renal failure, diabetes, or other major health concerns. Subjects were provided with information about the study and written consent was obtained. The study was approved by the McMaster University Human Subject Review Committee. Subjects were financially compensated for their time upon completion of the study.

A sample size of at least 10 was determined to be needed to detect a 15% difference in plasma amino acids with BIgG feeding, based on an intraindividual, inter assay c.v. of plasma amino acids of 13% in a previous study in Dr. Hanning's lab (9), and accepting the risk of an alpha error of $p < 0.05$ and a power of 0.80. Twelve subjects participated in the study.

PROTOCOL

Experiment One: A double-blind cross-over trial was conducted in which fasted subjects received a 284 g serving of commercial pudding (Magic Moments) which was either unsupplemented or supplemented with 10 g BIgG in a random order on two consecutive days. The pudding provided ~ 7 g protein and the plasma concentrate a minimum of 8.7 g protein and 6.6 - 6.8 g amino acids (see composition, Appendix 1). Subjects arrived in the outpatient lab on the morning of day one, fasted, and had a catheter inserted in the forearm by a registered nurse. A fasting heparinized blood sample, 2 mL, was taken at baseline. Subjects then consumed pudding "X" or "Y", as designated, with the time of completion = time 0. Subsequent blood samples were obtained at 15, 30, 45, 60, 90, 120 and 150 minutes. On day two, the same procedure was repeated with the alternate pudding.

Experiment Two: Subjects who had participated in Experiment One were provided with 142 g ready-to-eat servings of commercial puddings and vials containing 2.5 g of BIgG to be consumed twice daily for each day of the 14 day supplementation period. Subjects were instructed to mix the complete contents of one vial in one pudding and to consume two supplemented puddings each day for 14 consecutive days. Puddings were supplied in a variety of flavours of choice.

Consumption of the supplemented puddings was recorded by subjects daily and the empty BIgG vials were collected to verify compliance. Subjects also completed a daily diary to document their general health and any unusual symptoms related to tolerance and acceptability of the supplemented puddings.

Stool samples were collected on days 0 (before initial consumption of the BIgG), 7, 13 and 14. Poly-R 478 markers were used to identify 24 or 48 hour stool collection periods.

Biochemical Analyses:

Plasma

Plasma samples were placed on ice and centrifuged at -20 C within 15 minutes of sampling. The supernatant was stored at -70 C for subsequent amino acid analysis. Plasma amino acids were measured by HPLC using Beckman's system GOLD program. Data are presented for total amino acids and for leucine, the predominant amino acid in BIgG. To ensure that no bovine concentrate was absorbed intact, the 90 minute blood sample was analyzed for the presence of BIgG by ELISA assay (Appendix 1). The coefficient of variation for the assay was 3.5% for replicate samples, 4.0% between days and 5.5% between plates.

Stool:

Individual stool samples from each time period were pooled and homogenized in a measured amount of distilled water. For the BIgG ELISA, 1 g pooled stool was mixed with 5 mL cold TBS and centrifuged at 4 C, 3500 rpm X 15 minutes. 100 uL supernatant was added to 100 uL of reagent diluent and the assay performed as outlined in Appendix A. To verify recovery of BIgG, duplicate pooled stool samples were mixed with 2.5 mL TBS and 2.5 mL TBS spiked with bovine protein concentrate (50 mg/10mL TBS) and processed as above.

Pudding:

To confirm the activity of BIgG in pudding samples, supplemented pudding was analyzed as per stool samples.

Data Analysis:

Differences in plasma amino acid responses to unsupplemented and supplemented puddings were assessed by ANOVA for repeated measures. Area under the postprandial plasma amino acid curve was calculated from baseline (time 0) until plasma amino acids returned to baseline values. Areas in response to supplemented and unsupplemented puddings were compared by Student's paired t test. Results are presented as $X \pm SD$ unless otherwise indicated.

RESULTS:

Twelve healthy subjects participated in the trial. There were six males age 19 - 25 years (mean = 23.3 years) and six females age 22 - 42 years (mean = 29.3). All were normal weight. Body mass index (BMI) values for males and females ranged from 22.0 - 30.2 and 19.0 - 22.3, respectively, with mean values of 24.1 and 20.1.

Experiment 1:

The postprandial (0 to 150 minute) responses for total plasma amino acids and leucine in response to eating unsupplemented pudding and pudding supplemented with BIgG are shown in Table 2 in Figs 1 and 2. In response to unsupplemented versus supplemented pudding, total plasma amino acids were lower at 90 and 120 minutes, $p<0.05$ and leucine was lower at 60 and 120 minutes, $p<0.01$. It must be noted that baseline (fasting) levels on the unsupplemented and supplemented study days tended to be different for of total amino acids (970.5 vs1061.5 umol/L, NS) and leucine (54.8 vs 58.1 umol/L, NS) and could potentially have contributed to the postprandial differences. However, when data were expressed as the percentage change from baseline, the plasma response to unsupplemented versus supplemented pudding remained significantly lower for total amino acids at 90 and 120 minutes ($p<0.05$) and for leucine at 60 minutes ($p<0.05$), 90 and 120 minutes ($p<0.01$) (Fig. 3 and 4). Plasma amino acids had, on average, returned to fasting levels by 90 minutes with pudding alone, but remained above the fasting baseline until 120 - 150 minutes with the BIgG-supplemented pudding.

Using baseline values as 0, the areas under the postprandial curves for total plasma amino acids were not significantly different on paired t testing for unsupplemented versus supplemented pudding (584 $\pm$ 6491 versus 15267 $\pm$ 5691). For leucine, the area under the curve was significantly lower when subjects received the pudding without versus with BigG (-707.7 $\pm$ 372.6 vs 364.9 $\pm$ 342.7, X $\pm$ SEM, $p < 0.02$)). It should be noted that because of hemolized or missing blood samples for some of the time periods in two subjects in experiment one, data for area under the plasma amino acid curve are presented for n=10.

Values for plasma bovine IgG in response to pudding without IgG and supplemented with bovine IgG are shown for each subject in Figure 5. There was no detectable bovine IgG in subject plasma.

Experiment 2:

All 12 subjects completed the trial. Although some reported taking two vials of concentrate at one time if they missed a "feed", complete consumption of the total number of vials was achieved by all subjects by the end of the trial. Moreover, a twice daily ingestion pattern was followed on the days of stool collection. This was verified by subject records and empty vial count.

Unusual symptoms reported in the individual health diaries and the number of subjects reporting the symptoms were as follows:

Symptom	Number of Subjects
fatigue	2
sore throat	1
softened stools	1
increased urination	3
stomach cramps	3
headache	2
nausea	1
constipation	1
irritability	1

These symptoms occurred infrequently to the affected subject throughout the two weeks.

Subjects were asked to comment on the supplemented pudding at the end of the trial. Several subjects reported that they disliked consuming the volume of pudding given and had taken the concentrate in the least amount of pudding possible. A few subjects reported an aftertaste to the BIgG, but reported that they got used to it over time. Six would have preferred a pill format to a pudding supplement. The general consensus was that two weeks of pudding consumption was monotonous.

Figure 6 depicts a typical ELISA for stool IgG of subject A on day 0, before receiving any BIgG. The spiked stool sample (A 0 + IgG) and the day 14 sample (A14) both show the presence of IgG. This suggests that IgG activity is retained with passage through the GI tract.

DISCUSSION:

The results of this study support the short term safety of oral bovine immunoglobulin concentrate (BigG). The concentrate was well tolerated as a single (10g) bolus and during prolonged (2 week) daily ingestion. No absorbed intact BigG was detected in plasma in these adult subjects, though intact absorption might occur in infants and young children (8).

It appears that some of the protein in BIgG is digested in the adult GI tract, resulting in a heightened plasma total amino acid and leucine response one to two hours after consumption. The total areas under the postprandial amino acid response curves were higher when BigG was consumed , however, responses were highly variable and the areas under the postprandial total amino acid curve were not significantly different. It was not possible to express the plasma amino acid response in relation to the quantities of protein ingested due to the variability of the responses. It must be noted that BIgG contains proteins, other than immunoglobulins, which would be liable to digestion, such as transferrin, growth factors and peptides. Thus the rise in plasma amino acids may be due to these smaller proteins and not to the larger immunoglobulins.

This suggestion is supported by the observation of intact BIgG activity in faecal samples from subjects consuming BIgG concentrate where such activity was absent at baseline.

The BIgG activity in stool samples did not reflect quantitative recovery of the dose of supplement ingested. This is not likely due to the assay conditions, since stool samples which were spiked before processing showed recovery. In piglets, Drew et al also found reduced immunoglobulin activity after passage through the GI tract, though activity was retained in the small intestine (where its protective benefits would be advantageous) (personal communication).

The lack of activity is unlikely to have been due to poor compliance. Subject records and vial counts verified consumption and comments such as "I hate pudding" and "Only two days to go" shared between the 12 subjects suggested that they were adhering to the protocol. The subjects were all graduate students or faculty researchers with an appreciation of the research process and importance of compliance.

Since some degradation appears to occur with passage through the GI tract, future research should consider congugating BIgG with a large moleculare weight tag, e.g. polyethylene-glycol (11).

There was a lack of any predominant or persistent side effect to the BIgG concentrate, suggesting that BIgG may have potential application in enteral feeds for humans. The distinct aftertaste, however, needs to be masked. A more palatable medium should be investigated in order to optimize BIgG use in subjects who may be less motivated than those in the current trial.

Texture is also affected by BIgG. Although Experiment one was designed to be double blind, the pudding into which the immunoglobulin had been stirred was recognizably thinner in consistency than the unsupplemented pudding. Although , the loss in blinding was unlikely to have affected the objective outcomes (plasma amino acid concentrations) in the current trial, the poor solubility of BIgG will be a challenge to those investigating a suitable food medium for supplementation.

In summary, this pilot study supports the short term safety and acceptability of BIgG in human adults. Since BIgG activity was detected in feces, it appears the the concentrate is, at least in part, resistant to digestion. Thus the potential benefits of BIgG in conferring GI immune protection merit further study. The effectiveness of BIgG may be enhanced by tagging it with a non-absorbable marker, such as polyethylene glycol, and by selecting a food vehicle with high taste and textural appeal.

ACKNOWLEDGEMENTS:

The investigators acknowledge, with gratitude, the superb assistance of Sharon Wong, BA, and Lorraine Moss BSc in the conduct of this research.

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PRODUCT : INFORMATION **AMPC**

Globulin Concentrate

DESCRIPTION: Globulin Concentrate is a selected bovine plasma protein produced under rigid USDA inspection (EST. 6899) using stainless steel equiment throughout processing. Globuin Concentrate has had all of the fibrin and most of the bovine serum albumin removed. The remaining globulin concentrate contains immunoglobulins, transferrin and other protective proteins and peptides and is an excellent protein source for infant forumlas.

TYPICAL ANALYSIS:	Protein	Min. 87.0%
	Carbohydrate	0%
	Fat	0%
	Moisture	Max 7.0%
	Ash	6.0%
	Standard Plate Count	<20,000
	Salmonella	Negative
	Coliforms	Negative
AMINO ACID COMPOSITION:	Alanine	2.82%
	Lysine	4.22%
	Aspartate + Asparagine	5.48%
	Methionine	0.88%
	Arginine	3.34%
	Phenylalanine	3.41%
	Cystine	1.64%
	Proline	4.00%
	Glycine	2.39%
	Serine	5.20%
	Glutamate + Glutamine	7.74%
	Threonine	4.67%
	Histidine	1.61%
	Tryptophan	0.39%
	Isoleucine	3.00%
	Tyrosine	4.10%
	Leucine	7.87%
	Valine	4.91%

PRODUCT CHARACTERISTICS: Off white in color; no aroma or flavour.

This information is presented for consideration in the belief that it is accurate and reliable; however, no warranty, either expressed or implied is made and no freedom from liability from patents, trademarks or other limitations should be inferred. Any data listed are averages only, and are not to be considered as guarantees expressed or implied, nor as a condition of sale.

AMPC, Inc.
2325 North Loop Drive, P.O. Box 645 • Ames, Iowa 50010 U.S.A.
Telephone (515) 296-7100 • Fax (515) 296-7110

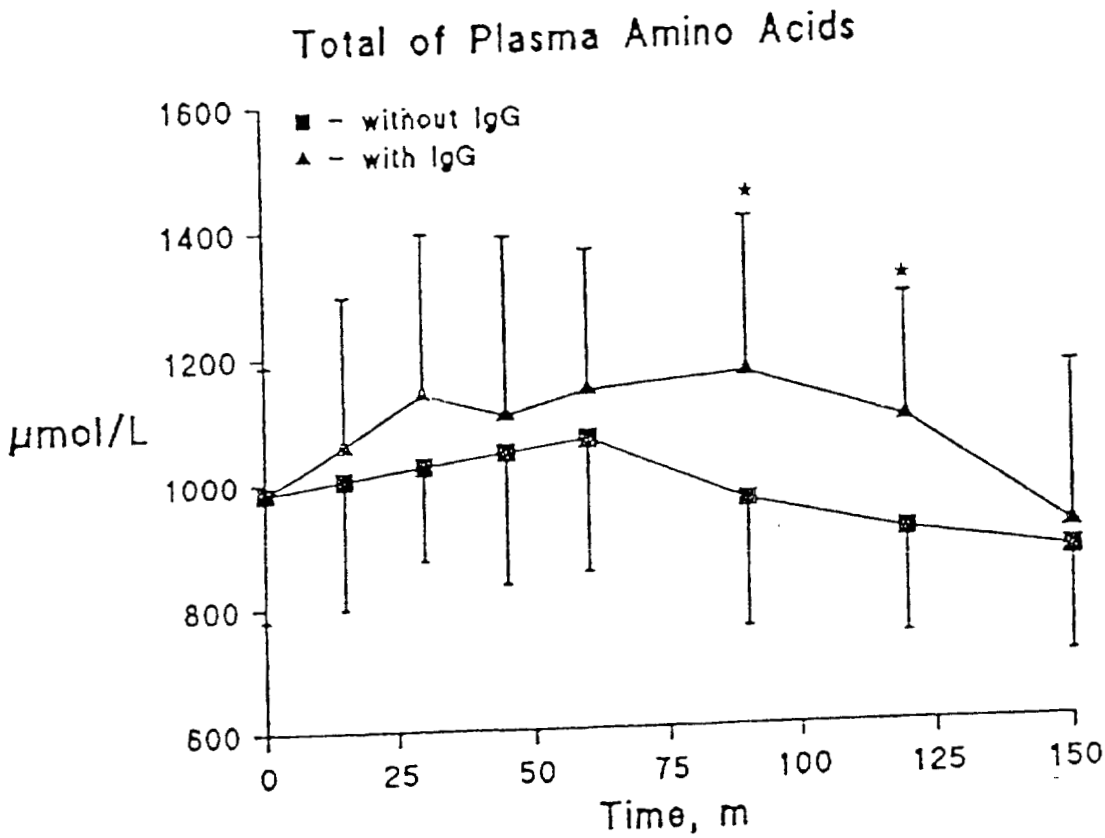
Table 2: Postprandial plasma amino acids (umol/L) in response to unsupplemented and BIgG - supplemented pudding.

TIME (minutes)	TOTAL AMINO ACIDS				LEUCINE			
	BIgG		BIgG		BIgG		BIgG	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
0	970.5	222.9	1061.5	202.5	54.8	15.9	58.1	16.6
15	1030.0	196.1	1102.9	187.4	58.2	15.8	62.5	17.3
30	1025.9	153.2	1160.2	183.0	56.5	13.0	65.4	15.8
45	1074.2	219.1	1149.0	205.5	57.8	15.9	64.0	16.0
60	1064.5	212.8	1186.1	191.7	53.5	10.7	65.0**	14.9
90	961.6	203.0	1260.0*	202.8	46.0	14.2	67.3	12.2
120	906.9	164.4	1203.0*	168.3	41.9	11.4	60.8**	10.3
150	855.9	176.5	951.7	240.2	40.6	13.5	46.3	16.3

* Different from unsupplemented. $p < 0.05$

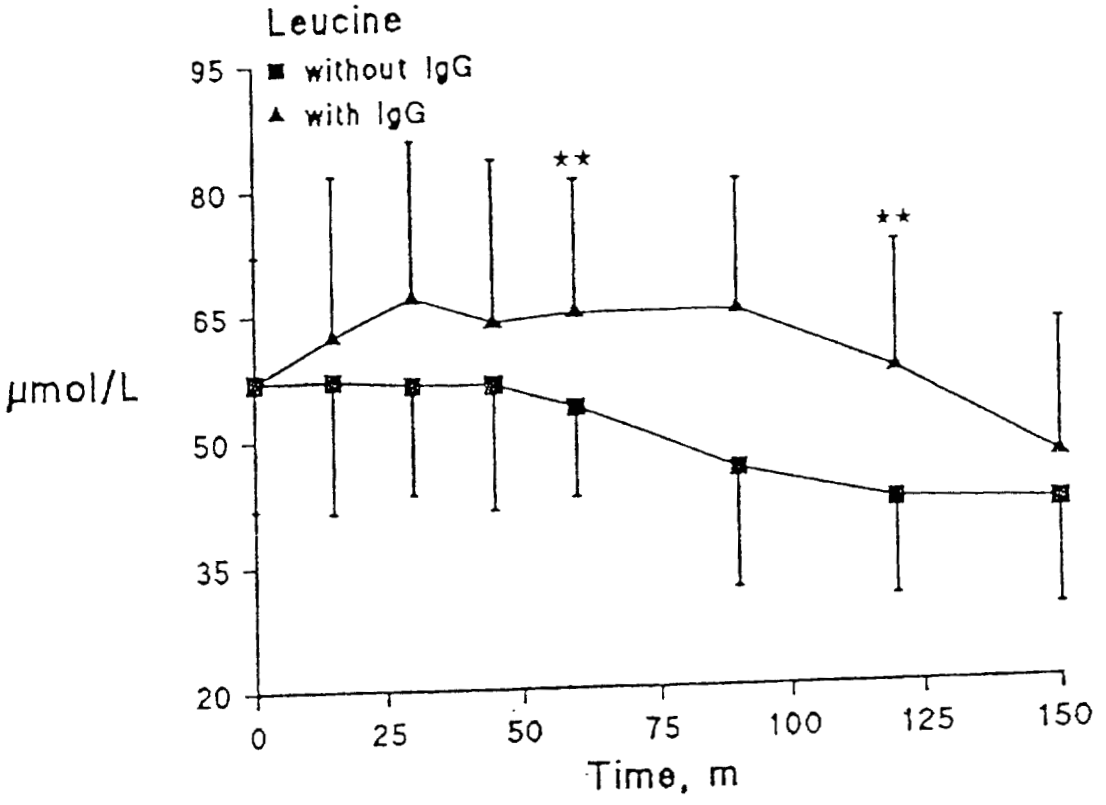
** Different from unsupplemented. $p < 0.01$

Fig. 1: Plasma total amino acid responses in fasted subjects (n = 12) on consecutive mornings after receiving, in a random order, pudding with and without bovine immunoglobulin concentrate (BigG) (X ± SD).



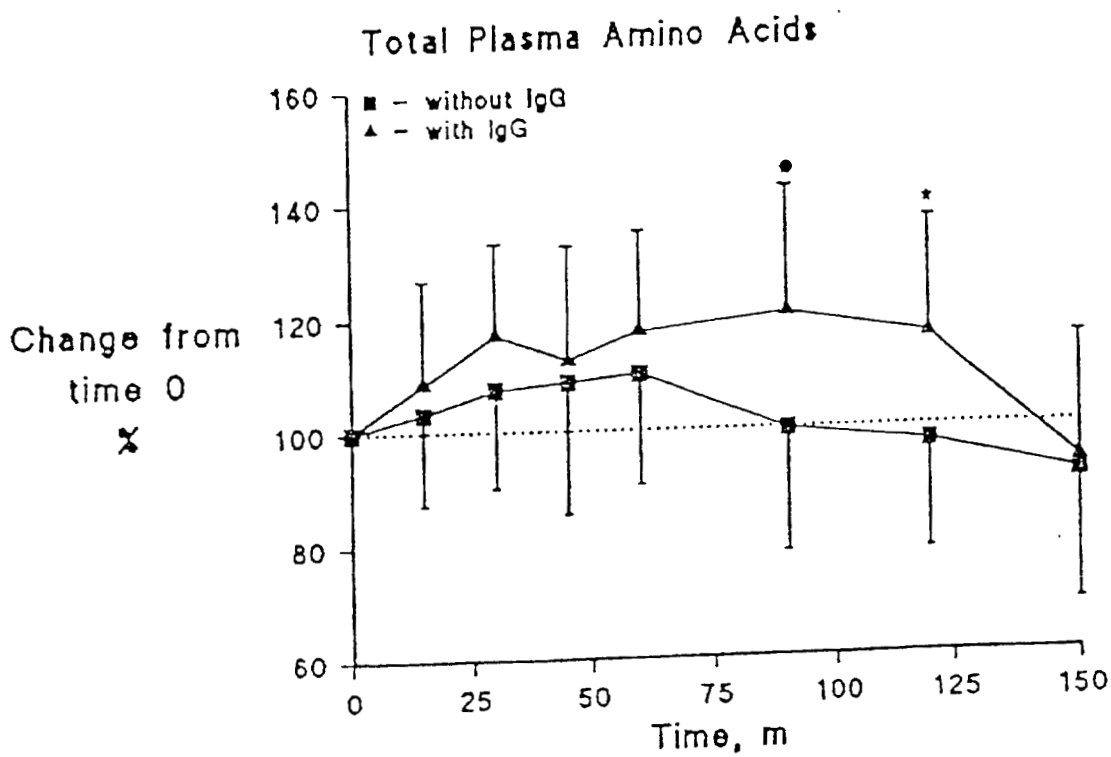
* = $p<0.05$

Fig. 2: Plasma leucine responses in fasted subjects (n = 12) on consecutive mornings after receiving, in a random order, pudding with and without bovine immunoglobulin concentrate (BigG) ($\bar{X} \pm SD$).



** = p<0.01

Fig. 3: The change in plasma total amino acids from fasting levels in response to pudding with and without bovine immunoglobulin concentrate (BigG) (n = 12 pairs, X ± SD).



* = p<0.05

Fig. 4: The change in plasma leucine from fasting levels in response to pudding with and without bovine immunoglobulin concentrate (BigG) (n = 12 pairs, X ± SD).

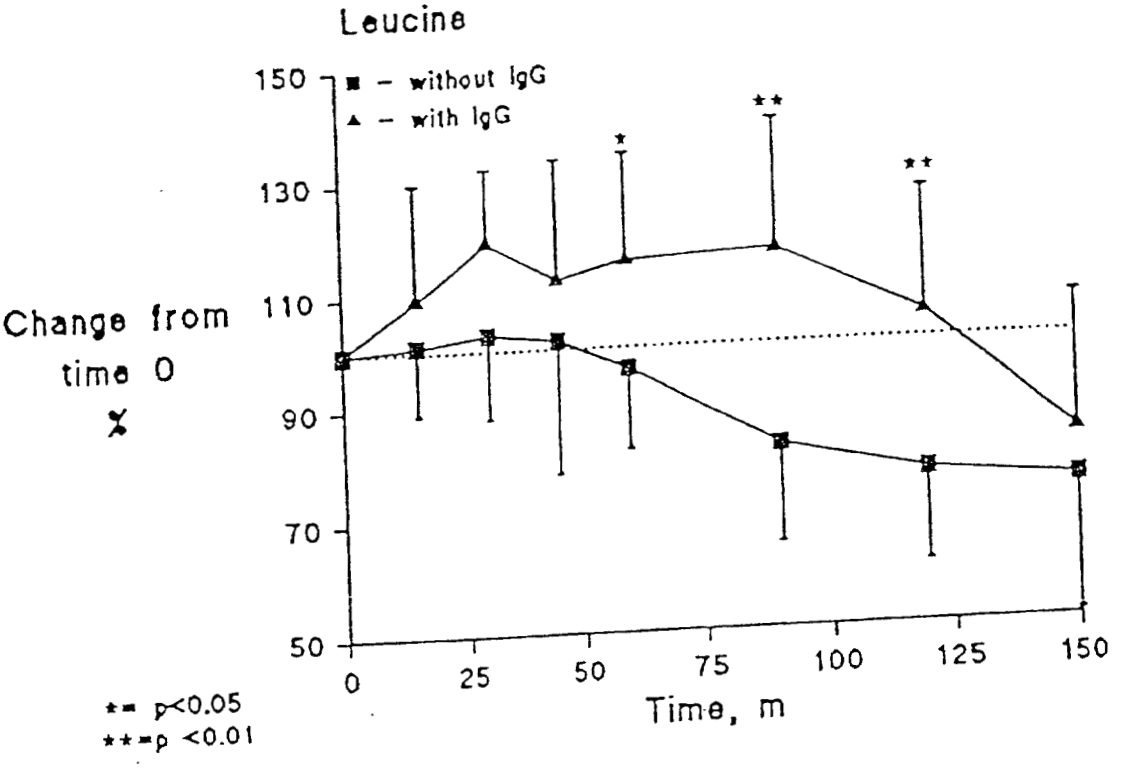


Fig 5: ELISA results for plasma BIgG 1.5 hours after ingesting pudding without or supplemented with BigG.

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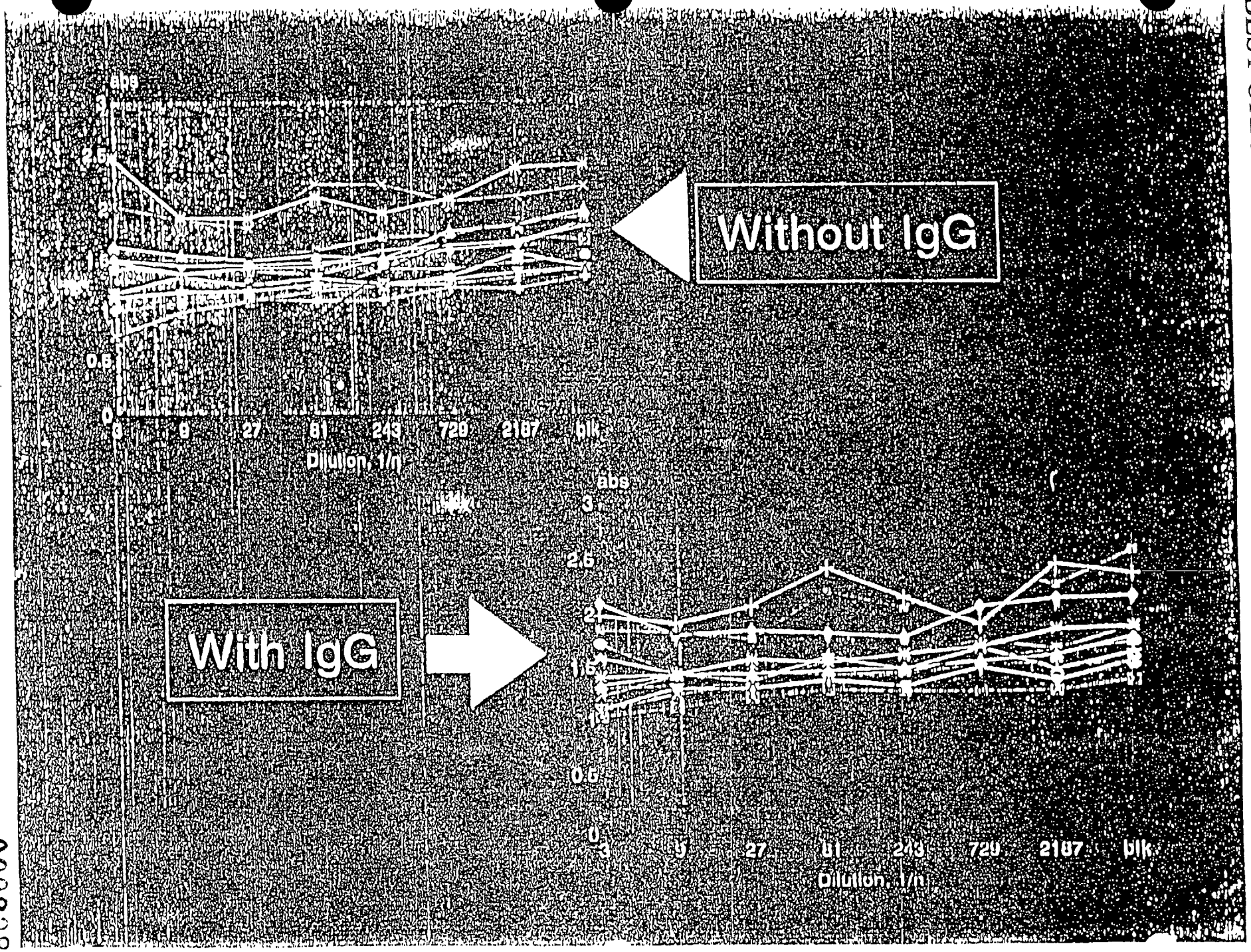

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Fig 6: ELISA assay of BIgG in pooled (24h) stool samples from a typical subject on day 0, before receiving BigG concentrate, day 0 sample spiked with BIgG (10 mg/g stool), and after 14 days of BIgG ingestion (10 g/d).

MSA-APC#5

4/9/93

Principal Investigator: APC

Sample: #5

File Name: 5-M44A1

Requisition #: AAA-054-93

Hydrolysis Type: Liquid-phase MSA 24 Hr/110 C

Modification: NONE

Weight of Sample Hydrolyzed: 10.45 mg

Initial Hydrolysate Dilution: 1 mL

Second Dilution: 5 uL:200 uL

Injection Loop Volume: 50 uL

Total Dilution Factor: 800::1

820B Bovine Plasma

Residue	Concentration nmol/Inj.	Amino acid M.W.	Micrograms of amino acid	Mole %	Weight %
Cys	2.580	103.14	0.266	3.53%	2.12%
Asx	6.827	115.09	0.786	9.35%	6.26%
Thr	4.947	101.11	0.500	6.78%	3.98%
Ser	6.026	87.08	0.525	8.26%	4.18%
Glx	8.455	129.12	1.092	11.58%	8.69%
Pro	4.133	97.12	0.401	5.66%	3.20%
Gly	3.938	57.06	0.225	5.40%	1.79%
Ala	5.072	71.08	0.361	6.95%	2.87%
Val	5.215	99.14	0.517	7.15%	4.12%
Met	1.073	131.21	0.141	1.47%	1.12%
Ile	1.998	166.7	0.333	2.74%	2.65%
Leu	6.565	166.7	1.094	8.99%	8.71%
Tyr	2.687	163.18	0.438	3.68%	3.49%
Phe	2.835	147.18	0.417	3.88%	3.32%
His	1.732	137.15	0.238	2.37%	1.89%
Lys	5.661	128.18	0.726	7.76%	5.78%
Trp	0.255	186.21	0.047	0.35%	0.38%
Arg	2.987	156.2	0.467	4.09%	3.71%

Total Nanomoles of Residue/Inj.: 72.986

Total Micrograms of Residue/Inj.: 8.307

Total amino acid weight % = 66.14%

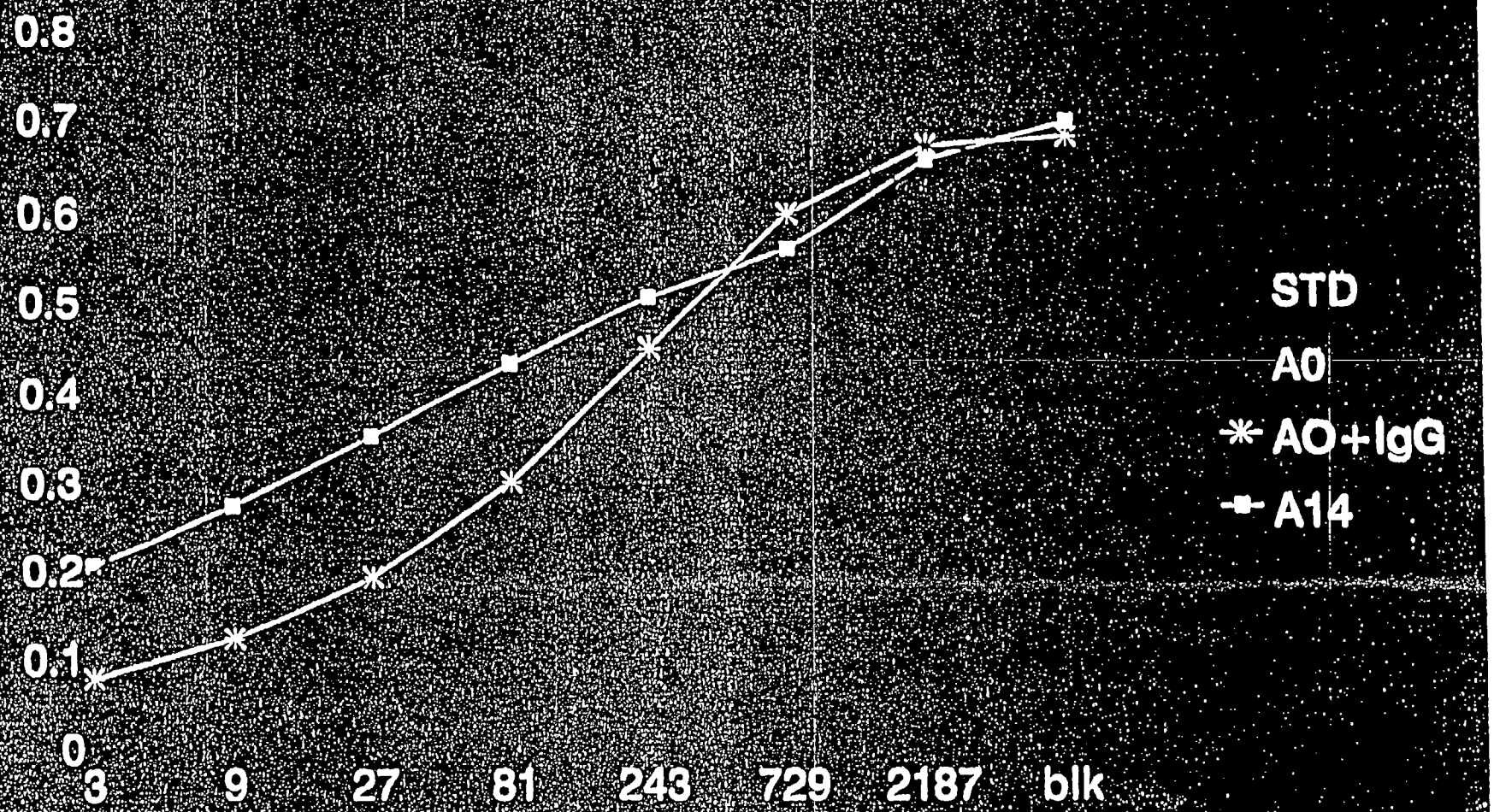
Expected NLE Recovery: 0.312 nmol

Experimental NLE Recovery: 0.3 nmol

Ratio: 1.040

The Short Term Safety and Digestibility of Bovine Immunoglibulin Concentrate in Healthy Adults

R.M. Hanning and M.D. Drew



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Chedoke-McMaster Hospitals

MEMORANDUM

DATE: September 29, 1994

TO: Dr. D. Rosenbloom - MUMC-1E4
Co-Chairman, Research Advisory Group
Dr. D. Offord - CH-A
Co-Chairman, Research Advisory Group

cc - R. Hanning
M. Drew

FROM: F. G. H. Baillie (5215)
Chairman, Medical Advisory Committee

(b) [redacted]

RE: "PILOT TRIAL OF DIGESTIBILITY OF BOVINE IMMUNOGLOBULIN CONCENTRATE IN ADULTS" - Project #94-49

This is to advise that the Board of Trustees, at its meeting on September 28, 1994, approved the above research project as recommended by the Medical Advisory Committee.

By means of a copy of this memo, investigators in this project should be aware that they are responsible for ensuring that evidence of consent is inserted in the patient's health record. In the case of invasive or otherwise risky research, the investigator might consider the advisability of keeping personal copies.

BEST ORIGINAL COPY

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(T) Section 108(b)(3) of Public Law 90–399 is amended by striking "section 201(w) as added by this Act" and inserting "section 201(v)". 21 USC 360b note.

(6) REGULATIONS.—On the date of enactment of this Act, the Secretary of Health and Human Services shall implement sections 571 and 573 of the Federal Food, Drug, and Cosmetic Act and subsequently publish implementing regulations. Not later than 12 months after the date of enactment of this Act, the Secretary shall issue proposed regulations to implement section 573 of the Federal Food, Drug, and Cosmetic Act (as added by this Act), and not later than 24 months after the date of enactment of this Act, the Secretary shall issue final regulations implementing section 573 of the Federal Food, Drug, and Cosmetic Act. Not later than 18 months after the date of enactment of this Act, the Secretary shall issue proposed regulations to implement section 572 of the Federal Food, Drug, and Cosmetic Act (as added by this Act), and not later than 36 months after the date of enactment of this Act, the Secretary shall issue final regulations implementing section 572 of the Federal Food, Drug, and Cosmetic Act. Not later than 30 months after the date of enactment of this Act, the Secretary shall issue proposed regulations to implement section 571 of the Federal Food, Drug, and Cosmetic Act (as added by this Act), and not later than 42 months after the date of enactment of this Act, the Secretary shall issue final regulations implementing section 571 of the Federal Food, Drug, and Cosmetic Act. These timeframes shall be extended by 12 months for each fiscal year, in which the funds authorized to be appropriated under subsection (i) are not in fact appropriated. Effective date. Deadlines. Publication. 21 USC 360ccc note.

(7) OFFICE.—The Secretary of Health and Human Services shall establish within the Center for Veterinary Medicine (of the Food and Drug Administration), an Office of Minor Use and Minor Species Animal Drug Development that reports directly to the Director of the Center for Veterinary Medicine. This office shall be responsible for overseeing the development and legal marketing of new animal drugs for minor uses and minor species. There is authorized to be appropriated to carry out this subsection $1,200,000 for fiscal year 2004 and such sums as may be necessary for each fiscal year thereafter. Government organization. 21 USC 393 note. Appropriation authorization.

(8) AUTHORIZATION OF APPROPRIATIONS.—There is authorized to be appropriated to carry out section 573(b) of the Federal Food, Drug, and Cosmetic Act (as added by this section) $1,000,000 for the fiscal year following publication of final implementing regulations, $2,000,000 for the subsequent fiscal year, and such sums as may be necessary for each fiscal year thereafter.

TITLE II—FOOD ALLERGEN LABELING AND CONSUMER PROTECTION

Food Allergen Labeling and Consumer Protection Act of 2004.

SEC. 201. SHORT TITLE. 21 USC 301 note.

This title may be cited as the "Food Allergen Labeling and Consumer Protection Act of 2004".

SEC. 202. FINDINGS. 21 USC 343 note.

Congress finds that—

(1) it is estimated that—

(A) approximately 2 percent of adults and about 5 percent of infants and young children in the United States suffer from food allergies; and

(B) each year, roughly 30,000 individuals require emergency room treatment and 150 individuals die because of allergic reactions to food;

(2)(A) eight major foods or food groups—milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans—account for 90 percent of food allergies;

(B) at present, there is no cure for food allergies; and

(C) a food allergic consumer must avoid the food to which the consumer is allergic;

(3)(A) in a review of the foods of randomly selected manufacturers of baked goods, ice cream, and candy in Minnesota and Wisconsin in 1999, the Food and Drug Administration found that 25 percent of sampled foods failed to list peanuts or eggs as ingredients on the food labels; and

(B) nationally, the number of recalls because of unlabeled allergens rose to 121 in 2000 from about 35 a decade earlier;

(4) a recent study shows that many parents of children with a food allergy were unable to correctly identify in each of several food labels the ingredients derived from major food allergens;

(5)(A) ingredients in foods must be listed by their "common or usual name";

(B) in some cases, the common or usual name of an ingredient may be unfamiliar to consumers, and many consumers may not realize the ingredient is derived from, or contains, a major food allergen; and

(C) in other cases, the ingredients may be declared as a class, including spices, flavorings, and certain colorings, or are exempt from the ingredient labeling requirements, such as incidental additives; and

(6)(A) celiac disease is an immune-mediated disease that causes damage to the gastrointestinal tract, central nervous system, and other organs;

(B) the current recommended treatment is avoidance of glutens in foods that are associated with celiac disease; and

(C) a multicenter, multiyear study estimated that the prevalence of celiac disease in the United States is 0.5 to 1 percent of the general population.

SEC. 203. FOOD LABELING; REQUIREMENT OF INFORMATION REGARDING ALLERGENIC SUBSTANCES.

(a) IN GENERAL.—Section 403 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 343) is amended by adding at the end the following:

"(w)(1) If it is not a raw agricultural commodity and it is, or it contains an ingredient that bears or contains, a major food allergen, unless either—

"(A) the word 'Contains', followed by the name of the food source from which the major food allergen is derived, is printed immediately after or is adjacent to the list of ingredients (in a type size no smaller than the type size used in the list of ingredients) required under subsections (g) and (i); or

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tissues.

Article 2.3.13.15.

When importing from a country or zone with a moderate BSE risk, *Veterinary Administrations* should require:

for *fresh meat* (bone-in or deboned) and *meat products* from cattle

the presentation of an *international veterinary certificate* attesting that:

1. the country or zone complies with the conditions in Article 2.3.13.6. to be considered as presenting a moderate BSE risk;

2. the feeding of ruminants with *meat-and-bone meal* and *greaves* derived from ruminants has been banned and the ban has been effectively enforced;

3. ante-mortem inspection is carried out on all bovines;

4. cattle from which the *fresh meat* or *meat products* destined for export originate were not subjected to a stunning process, prior to slaughter, with a device injecting compressed air or gas into the cranial cavity or to a pithing process;

5. the *fresh meat* and *meat products* destined for export do not contain the tissues listed in point 1 and point 2 of Article 2.3.13.18. nor mechanically separated meat from skull and vertebral column from cattle over 6 months of age, all of which have been completely removed in a manner to avoid contamination with these tissues.

Article 2.3.13.16.

When importing from a country or zone with a high BSE risk, *Veterinary Administrations* should require:

for *fresh meat* and *meat products* from cattle

the presentation of an *international veterinary certificate* attesting that:

1. the country or zone complies with the conditions in Article 2.3.13.7. to be considered as presenting a high BSE risk;

2. the *fresh meat* destined for export does not contain the tissues listed in point 2.3.13.18.1. of Article 2.3.13.18., all of which have been completely removed in a manner to avoid contamination with these tissues;

3. the *fresh meat* destined for export, if obtained from animals over 9 months of age, has been deboned and does not contain nervous and lymphatic tissues exposed during a deboning process, all of which have been completely removed in a manner to avoid contamination with these tissues;

4. the *meat products* destined for export are derived from deboned meat and do not contain the tissues listed in point 1 and point 2 of Article 2.3.13.18. nor nervous and lymphatic tissues exposed during a deboning process, nor mechanically separated meat from skull and vertebral column of bovine animals, all of which have been completely removed in a manner to avoid contamination with these tissues;

5. a system is in operation enabling the *fresh meat* and *meat products* destined for export to be traced back to the *establishments* from which they are derived;

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SILLIKER, Inc.
Iowa Laboratory
405 Eighth Avenue SE
Cedar Rapids, IA 52401-2153
319/ 366 3570 Fax 319/ 366 4018

CERTIFICATE OF ANALYSIS

COA No:	CDR-30338990-0
Supersedes:	None
COA Date	9/22/04
Page 1 of 3	

COPY TO:
Ms. Bonnie J. Humm
Director of Quality Assurance
Proliant Inc. / APC Inc.
2425 SE Oak Tree Court
Ankeny, IA 50021

ORIGINAL TO:
Mr. Chad Peterson
Product Release Coordinator
Proliant Inc. / APC Inc.
2425 SE Oak Tree Court
Ankeny, IA 50021

Received From:	Boone, IA
Received Date:	9/1/04
P.O.# / ID:	BB
Location of Test: (except where noted)	Cedar Rapids, IA

Analytical Results

Desc. 1:	LIMS Lab #–	**Desc. 4:**	BB40090101, BB40200101	**Laboratory ID:**	301789899
Desc. 2:	SKU #56001	**Desc. 5:**	BB40330101, BB40470101	**Condition Rec'd:**	NORMAL
Desc. 3:	ImmunoLin Composite	**Desc. 6:**	BB40610101, BB40860101	**Temp Rec'd (°C):**	23

Sample Comment: BB41420101, BB41810101, BB42240101, and BB42260101

Analyte	Result	Units	Method Reference	Test Date	Loc.
Arsenic	<0.22	mg/100g	AOAC 984.27	9/7/04	CHG
ICP Sample Prep - Microwave	Microwave	-	AOAC 984.27	9/7/04	CHG
Lead	<0.11	mg/100g	AOAC 984.27	9/7/04	CHG
Mercury	0.11	ppm	EPA 7471	9/8/04	MID
Organochlorine Pesticide Screen			CDFA Method	9/22/04	EMA
a, b, d - BHC	< 0.010	ppm			
Alachlor, (Lasso)	< 0.020	ppm			
Alert, (Pirate)	< 0.040	ppm			
Aldrin	< 0.010	ppm			
Benfluralin, (Balan, Benefin)	< 0.020	ppm			
BHC, (Benzahex)	< 0.010	ppm			
Bifenox, (Modown)	< 0.050	ppm			
Bifenthrin	< 0.040	ppm			
Bromacil	< 0.040	ppm			
Captafol	< 0.040	ppm			
Captan	< 0.020	ppm			
Chlordane	< 0.250	ppm			
Chlorobenzilate, (Acaraben)	< 0.400	ppm			
Chlorthalonil, (Bravo)	< 0.010	ppm			
Cyanazine	< 0.100	ppm			
Cyfluthrin	< 0.050	ppm			
Cypermethrin	< 0.050	ppm			
Dacthal, (Chlorthal)	< 0.020	ppm			
DDD	< 0.020	ppm			
DDE	< 0.020	ppm			
DDT	< 0.020	ppm			
Dichlobenil, (Casoron)	< 0.030	ppm			
Dichlone, (Phygon)	< 0.050	ppm			
Dicloran, (Botran)	< 0.020	ppm			
Dicofol, (Kelthane)	< 0.050	ppm			
Dieldrin	< 0.010	ppm			
Dyrene, (Anilazine)	< 0.100	ppm			
Endosulfan alpha	< 0.010	ppm			

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The results of these tests relate only to the samples tested. This report shall not be reproduced except in full, without the written approval of the laboratory.

SILLIKER, Inc.
Iowa Laboratory
405 Eighth Avenue SE
Cedar Rapids, IA 52401-2153
319/ 366 3570 Fax 319/ 366 4018

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Supersedes:	None
COA Date	9/22/04
Page 2 of 3	

COPY TO:
Ms. Bonnie J. Humm
Director of Quality Assurance
Proliant Inc. / APC Inc.
2425 SE Oak Tree Court
Ankeny, IA 50021

ORIGINAL TO:
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Product Release Coordinator
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Desc. 3: ImmunoLin Composite **Desc. 6:** BB40610101, BB40860101 **Temp Rec'd (°C):** 23
Sample Comment: BB41420101, BB41810101, BB42240101, and BB42260101

Analyte	Result	Units	Method Reference	Test Date	Loc.
Endosulfan beta	< 0.010	ppm			
Endosulfan sulfate	< 0.010	ppm			
Endrin	< 0.010	ppm			
Esfenvalerate, (Asana)	< 0.020	ppm			
Ethalfiluralin, (Sonalan)	< 0.030	ppm			
Fluvalinate	< 0.050	ppm			
Folpet	< 0.050	ppm			
Heptachlor	< 0.010	ppm			
Heptachlor epoxide	< 0.020	ppm			
Iprodione, (Rovral)	< 0.050	ppm			
Lindane, (gamma-BHC)	< 0.010	ppm			
Linuron	< 0.020	ppm			
Methoxychlor	< 0.050	ppm			
Metribuzin	< 0.020	ppm			
Mirex	< 0.020	ppm			
Myclobutanil, (Rally)	< 0.050	ppm			
Nitrofen, (TOK)	< 0.050	ppm			
Oxadiazon, (Ronstar)	< 0.050	ppm			
Oxyfluorfen, (Goal)	< 0.040	ppm			
Pendamethalin	< 0.050	ppm			
Penthachloronitrobenzene (PCNB)	< 0.020	ppm			
Permethrin	< 0.100	ppm			
Perthane, (Ethylan)	< 0.100	ppm			
Polychlorinated Biphenyls	< 0.250	ppm			
Profluralin, (Tolban)	< 0.020	ppm			
Pronamide, (Kerb, Propyzamide)	< 0.050	ppm			
Pyrethrins, (Total)	< 0.100	ppm			
Tetradifon, (Tedion)	< 0.020	ppm			
Toxaphene, (Attac, Strobane)	< 0.250	ppm			
Tridimefon, (Bayleton)	< 0.020	ppm			
Trifluralin, (Treflan)	< 0.020	ppm			
Vegadex, (Diethyldithiocarb. acid)	< 0.050	ppm			
Vinclozolin, (Ronlian)	< 0.020	ppm			
Sulfa Drug Screen			AOAC 993.32	9/13/04	BRD

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SILLIKER, Inc.
Iowa Laboratory
405 Eighth Avenue SE
Cedar Rapids, IA 52401-2153
319/ 366 3570 Fax 319/ 366 4018

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Sample Comment: BB41420101, BB41810101, BB42240101, and BB42260101

Analyte	Result	Units	Method Reference	Test Date	Loc.
Sulfadimethoxine	<0.01	ppm			
Sulfathiazole	<0.01	ppm			
Sulfamethazine	<0.01	ppm			
Sulfaquinoxaline	<0.01	ppm			
Tetracycline Screen			AOAC 967.39	9/13/04	BRD
Tetracycline	<0.10	ppm			
Chlortetracycline	<0.10	ppm			
Oxytetracycline	<0.10	ppm			

(b)

Jodene Jurgens, PhD. Laboratory Director

Noted Test Locations: CHG-Silliker, Inc. Illinois Laboratory, 1304 Halsted Street, Chicago Heights, IL 60411
Noted Test Locations: MID-Midwest Laboratories, Inc., 13611 B Street, Omaha, NE 68144-3693
Noted Test Locations: EMA-Environmental Micro Analyses, Inc., 40 N. East Street, Suite B, Woodland, CA 95776
Noted Test Locations: BRD-Barrow-Agee, 1555 Three Pl, Memphis, TN 38116

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[redacted]

[redacted]

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IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

Center for Designing Foods to
Improve Nutrition
1127 Human Nutrional Sciences Bldg
Ames, Iowa 50011-1120
515 294-8489
FAX 515 294-5390

April 30, 2005

Dr. Eric Weaver
Proliant Health Ingredients
2425 SE Oak Tree Court
Ankeny, IA 50021

Dear Dr. Weaver,

I have reviewed the materials related to your GRAS petition for the use of bovine serum globulin in human foods. I think that you have made a very strong case for its safety and appropriateness for GRAS status in accordance with the FDA's regulation 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). This is based upon the general recognition of safety through the quality and quantity of scientific evidence presented. This includes:

- Years of supplying spray-dried bovine globulin and other blood products for use in animal feeds and human food throughout the world without documented negative effects.
- Published and unpublished data that demonstrate no untoward effects (microbiological, heavy metals, or toxins) in animals related to the consumption of the bovine serum globulins. These studies were conducted with dosages appropriate to this application. Furthermore, these studies demonstrated several beneficial effects of bovine serum globulin consumption.
- Published and unpublished feeding studies with human adults and children have not observed any negative health risks with consumption of the bovine serum globulins. The studies were conducted with dosages appropriate to this application. Furthermore, these studies demonstrate potential beneficial effects.
- It is not likely that allergenic responses to these products would be a significant problem or risk to the general population.
- The risk of Bovine Spongiform Encephalopathy (BSE) contamination from animals used to produce the bovine serum globulin is negligible. First, there are no known cases of contamination in the animals used. Furthermore, scientific data suggests that it is highly unlikely that the blood tissue used to isolate the bovine serum globulin would harbor the infective agents of BSE, even in BSE-contaminated animals.
- USDA/FSIS permits animal blood as an ingredient in meat food products with restrictions only for labeling, and USDA/FSIS processes are in place and used to inspect and approve edible beef plasma.

I find the information contained in this application to be complete and accurate to the best of my knowledge. Therefore, based on the data presented and general scientific knowledge, I do not take exception to the use of bovine serum globulin in human food and support your GRAS status petition.

Sincerely,

(b)

Paul J. Flakoll, Ph.D.
Director, Center for Designing Foods to Improve Nutrition
Professor, Food Science and Human Nutrition

UNIVERSITY OF ILLINOIS
AT URBANA-CHAMPAIGN

Department of Animal Sciences

College of Agricultural, Consumer
and Environmental Sciences
132 Animal Sciences Laboratory
1207 West Gregory Drive
Urbana, IL 61801

May 6, 2005

To Whom It May Concern:

I have worked in the area of swine nutrition throughout my career, in the following positions:

- Manager of Swine Research, Moorman Manufacturing Company; Quincy, IL
  - This company manufactured livestock feeds, with a large national market share.
  - 5 years
- Associate Professor/Professor of Animal Science, University of Minnesota
  - Research and teaching responsibilities
  - 17 years
- President, Pettigrew Consulting International, LLC; Louisiana, MO
  - 4 years
- Faculty Excellence Professor of Animal Sciences, University of Illinois
  - Research, teaching and extension responsibilities
  - Since 2001

I have been active at national and international levels, and have given more than 300 invited talks in many parts of the world. I have worked with issues in swine nutrition at both theoretical and practical levels, and have formulated diets fed to many pigs in commercial production systems.

One of the most important developments in swine nutrition during my career has been the adoption of complex diets for newly weaned piglets. These diets contain highly digestible ingredients that are appropriate for the immature state of physiological development of the young pig. In addition, they contain two ingredients that appear critical for good health and growth performance of these young pigs:

- Milk products
- Spray-dried animal plasma

I would not formulate diets for pigs immediately after weaning without these ingredients, even though we do not fully understand their physiological mechanisms.

The swine industry has an enormous amount of experience with spray-dried animal plasma in pig diets, and it has been positive. The industry has adopted the product in spite of its high cost. Meta-analyses of a large number of experiments show increases in

growth rate on the order of 25% when spray-dried animal plasma is added to diets of pigs for a short time after weaning. There is an accumulating body of evidence that plasma in the diet offers some protection against disease. Some have suggested there is a compensatory reduction in growth rate when plasma is removed from the diet. but the industry has not judged that to be of significant concern. I am not aware of any detrimental effects of feeding plasma to pigs that have been encountered by the industry.

Therefore, based on the data presented and my general knowledge of the related science and the industry, I do not take exception to the use of bovine serum globulin in human food and I support the GRAS status petition.

I appreciate the opportunity to comment on this issue, and stand ready to provide further assistance if appropriate.

Sincerely,

(b)

James E. Pettigrew
Faculty Excellence Professor

N

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COPY
MEL

LG Laboratories

Nutritional Health | Innovative Research | Natural Products

November 18, 1999

Special Nutritionals (HFS-450)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street SW
Washington, DC 20204

In accordance with Section 403(r)(6), this is a letter of notification with regard to the marketing of EnteraHealth™ Intestinal Wellness, a dietary supplement containing NutraGammax™, a protein complex with immunoglobulin concentrate from bovine serum.

Manufacturer: LG Laboratories
2325 North Loop Drive
Ames, Iowa 50010

"Statements Made in Labeling."

"... actively support and maintain gastrointestinal health"
"... provides intestinal wellness support ..."
"... to help maintain gastrointestinal balance and support normal nutrient absorption"
"NutraGammax™ promotes normal gastrointestinal health by:

- Promoting Intestinal Function
- Increasing Intestinal Absorptive Surface Area
- Optimizing the Utilization of Nutrients ..."

LG Laboratories, Inc | Iowa State University Research Park | 2501 North Loop Drive | Suite 800 | Ames, IA 50010
Phone 515-296-6819 | Fax 515-296-6820 | www.lglabs.com

". . . to promote intestinal health in humans"
". . . provides GI and immune support while optimizing the uptake of nutrients"
". . . to help . . . promote and maintain intestinal health function"

- Promote GI balance
- Support normal digestive absorption for stable GI function
- Provide a supplemental source of antibodies for immune support

"To the best of my knowledge, the information contained in this notice is complete and accurate, and LG Laboratories has substantiation that the statements are truthful and not misleading."

(b)
[redacted]

Mel Vanden Berg
V.P. Quality Assurance

MVB/jsh

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Pages 000254-000262 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

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Pages 000293-000298 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Pages 000299-000307 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

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000307.001

Effects of Spray-Dried Plasma in the Diets of Pigs and Poultry

J. D. Quigley[1], J. M. Campbell, C. J. Hammer, J. D. Crenshaw and L. E. Russell
APC, Inc.
2425 SE Oak Tree Court
Ankeny, IA
USA

J. Polo
APC Europe
08400 Granollers
Spain

Date Prepared: February, 2004

[1] Correspondence: 2425 SE Oak Tree Court, Ankeny, IA 50021.

000308

Executive Summary

Spray-dried animal plasma (**SDAP**) is a product produced by careful collection, processing and spray-drying of blood collected from government inspected abattoirs. Methods used to collect and process SDAP result in a product that contains functional proteins, including immunoglobulin G (IgG), which can provide intestinal immunity when consumed orally.

Spray-dried animal plasma contains approximately 15% IgG, which is composed of antibodies with titers against economically important diseases. These antibodies are partially resistant to digestion and a significant proportion reach the intestine. Antibodies (IgA and IgG) are an important component of the intestinal immune system and research has shown that circulating IgG moves from the circulation into the lumen of the intestine to inhibit binding of pathogens. Research in calves, pigs, rodents and humans indicates that intestinal Ig (produced locally or transported into the intestine) are a key part of the overall intestinal immune response. In addition, SDAP contains other functional proteins, including growth factors such as insulin like growth factor 1 and transforming growth factor beta, which have been shown to improve cellular growth, particularly in animals following intestinal damage.

Spray-dried animal plasma is widely used in diets for young animals, particularly young pigs. It has been estimated that over 90% of pigs in the U.S. are fed SDAP in their weaning diets. Many veterinarians and nutritionists consider SDAP an essential ingredient to reduce weaning stress, improve intake, reduce the incidence and severity of diarrhea and replace antibiotics in the diet. The functional proteins in SDAP serve as a "first line of defense" against enteric pathogens and work with the immune system to reduce the detrimental effects of these common challenges.

Recent research with broilers and turkeys demonstrate that poultry also respond to the addition of SDAP to their diets. Significant improvements in both rate and efficiency of growth have been reported. In addition, in the face of a respiratory challenge (Pasteurella multocida) mortality was significantly lowered when serum proteins were added to the drinking water.

Reducing the reliance on sub-therapeutic antibiotics is a goal of animal agriculture. Many producers have identified the use of SDAP as a means of reducing the dependence on antibiotics without impairing health or growth of animals.

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Introduction

Blood and plasma proteins have long been recognized as high quality feed ingredients for farm animals, including swine, cattle, and poultry. Blood proteins are readily available and have an excellent amino acid profile and high digestibility. Indeed, plasma has been recommended for use in animal diets for hundreds of years – not only as a source of protein, but also as a source of immunological support. Prior to the development and widespread use of antibiotics in animal agriculture, proteins such as egg yolk, blood and colostrum were used as a source of immunologically active proteins to help animals recover from disease. Further, use of blood proteins in animal diets removes this by-product stream from environmental contamination and can increase the value of the animal to the farmer.

Traditionally, proteins were considered simply sources of amino acids for the animal. However, increasingly, scientists recognize that some proteins retain biological activity in the animal – these are called *functional proteins*. Functional proteins are recognized as having some function in the animal that elicits a physiological response. Spray-dried animal plasma (**SDAP**) is an ingredient widely used in animal feed applications. Several important functional proteins are retained in SDAP that can improve animal survival, health and performance of animals.

Method of collection

Blood proteins have traditionally been collected and heated to high temperatures in the manufacture of blood meal, which destroys the functional components. Conversely, spray-drying preserves the functional characteristics of the proteins, including biologically active peptides, such as albumin and IgG. In this process, blood is collected into stainless steel tanks, troughs or other devices containing an anticoagulant in abattoirs under government inspection. Blood is only collected from animals determined to be fit for slaughter for human consumption by government veterinary inspection. The blood is then transferred to stainless steel tanks, followed by centrifugation to separate plasma from the cellular fraction and chilled to 4-5°C. Chilled plasma is then transported by dedicated equipment to off-site dedicated facilities where it is spray-dried to produce a light brown, freely flowing powder that may be used in feed applications. Recently, the North American Spray-Dried Blood and Plasma Producers Association was formed to establish policies and Manufacturing Procedures to insure the manufacture of high quality and safe products (Russell, 2001).

Characteristics of Plasma

Functionality. The method of processing will influence the degree of biological activity that a protein will retain. Spray-drying plasma maintains the functionality of important proteins in plasma, including protease inhibitors, growth factors and immunoglobulins. Because plasma contains approximately 15% IgG, the functionality of this fraction is biologically meaningful to the animal consuming SDAP. Further fractionation can be used to increase IgG concentration to greater than 90%. Of course, the method of processing and preparation have important effects on the degree of functionality of the proteins.

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The IgG in plasma are maintained and are biologically active. The Ig titers reflect the combined immunological history of the animals from which blood was collected and typically reflect both the vaccination and pathogenic exposure history of the animal. Plasma is collected into large lots, therefore, wide swings in Ig titer are avoided. It is of interest that the immunological history of animals (swine and cattle) will change over time; therefore the Ig titers in plasma will change also. (Borg, et al., 2002) Spray-dried animal plasma typically contains a wide array of specific antibodies that can be measured by standard serology tests.

Much research has been done to determine whether Ig from one species of animal may provide immunological support in another species of animal. In the feed industry, bovine, porcine and mixed plasma are available. In several studies, improvements in animal performance have been reported in animals fed either swine or bovine plasma (Campbell et al., 2003; Quigley et al., 2003). Additionally, a tremendous number of published literature is available that documents the use of IgG (primarily bovine IgG or chicken IgY) as oral therapy in humans.

Use of plasma in animal diets

Spray-dried animal plasma has been included in the diets of commercially reared early-weaned pigs for at about 20 years. Today, the vast majority of all feeds fed to pigs immediately after weaning (pig starters) in the U.S. contain SDAP at the rate of 2 to 10% of the formulation. Most research has reported improved intake, animal growth, feed efficiency and animal health in animals (including pigs and calves) when animals were fed SDAP (Gatnau et al., 2000; Morrill et al., 1995; Kats et al., 1994; Quigley and Bernard, 1996; Quigley et al., 2002, 2003; Van der Peet-Schwering, 1995, 1997). Others have compared SDAP to antimicrobials in diets of pigs (Coffey and Cromwell, 1995; Conde et al., 2000; Torrallardona et al., 2002, 2003) and calves (Quigley and Drew, 2000). We have summarized 47 research trials that used plasma in diets of pigs immediately after weaning – the average improvement in body weight gain, feed intake and feed efficiency were 34, 23 and 9%, respectively. Pigs are very susceptible to anorexia immediately after weaning, therefore, a highly palatable ingredient could make a tremendous difference in animal performance (Ermer et. al., 1994). The availability of SDAP in weanling pig diets allowed growers to reduce the age at weaning, and although the overall cost of the weaning ration increased with the inclusion of SDAP, overall return to the producer was improved.

Pigs fed SDAP under low antigenic conditions (excellent hygiene and low exposure to enteric pathogens) showed little improvement in performance when plasma was fed. On the other hand, when pigs were raised in a more "conventional" environment with typical exposure to enteric pathogens (indicated in the study by greater incidence of scours), animals fed plasma grew much faster than those fed the control ration. Coffey and Cromwell (1995) confirmed this finding when they fed pigs diets without or with plasma in "low antigenic" or "high antigenic" conditions. Pigs exposed to greater environmental stressors responded more dramatically to the inclusion of SDAP in the diet. These findings suggest that components in SDAP reduced effects of environmental stress. Likewise, similar observations have been reported in broilers (Campbell et al., 2003) and turkeys (Campbell et al., 2004a).

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Plasma reduces the effect of enteric challenge

Numerous studies have been conducted in many classes of animals that indicate that plasma reduces mortality and morbidity associated with enteric challenge.

Pig challenge studies. Studies have evaluated the use of SDAP in the diet of pigs challenged with enteric pathogens, particularly *Escherichia coli* and rotavirus, which are economically important pathogens to the swine industry. While a thorough review of all of these studies is outside the scope of this presentation, several trials are particularly instructive. Researchers in the U.S. reported that the administration of water-soluble SDAP reduced the effects of oral challenge with *E. coli* (Borg et al., 1999). Italian researchers (Bosi et al., 2001) challenged pigs with *E. coli R88 0.148* at weaning. Pigs were fed for 15 days and intake, gain, feed efficiency and mortality were determined. Feeding SDAP reduced mortality, improved growth and efficiency following oral challenge.

A follow-up study by the same researchers (Bosi, et al., 2004) also reported improved performance in pigs fed 6% SDAP or antibiotic (250 mg/kg colistin + 500 mg/kg amoxycycline) in the diet and challenged with *E. coli* (10^{10} CFU *E. coli* K88 on d 3 after weaning). These researchers also measured the amount of specific K88 antibody in both the plasma and saliva of the pigs on d 15 after challenge. Interestingly, production of K88 specific *E. coli* antibodies in plasma and saliva were reduced when either the antibiotic or SDAP was included in the diet. These data suggest that animals were not exposed to the challenge organism, and therefore, did not produce antibody.

Plasma reduces the effect of respiratory challenge

Spray-dried plasmas action beyond enteric challenge was recently observed by Campbell et. al, (2004b). In this study, 5-week old turkeys were challenged with 3.0×10^8 cfu of *Pasteurella multocida* Type III by swabbing the tonsils. Two weeks (14 d) following the challenge, administering water-soluble serum resulted in reduced mortality associated with the respiratory challenge. Survival of control poults was 65%; however, survival was 95% for poults consuming drinking water containing 1.3% Innavax (water-soluble serum).

Effects of plasma in lactation feeds

Recently there has been an interest in the addition of SDAP to sow lactation feed. Following parturition feed intake is typically depressed especially when environmental temperature is high (Spencer et al., 2003). In a series of trials by APC, Inc., (reported in Feedstuffs, Volume 73 (40) by staff editor, Tim Lundeen, 2001), dietary SDAP (0.25%) improved feed intake (4.7 kg/d vs 5.0 kg/d), reduced lactating sow weight loss (-23.8 kg vs –21.2 kg), improved number of pigs weaned per litter (9.2 to 9.5) and improved litter weight at weaning (46.6 kg vs 48.3 kg). In addition, reduced days to breeding after weaning was noted (8.7 d to 6.9 d). More recently (2004), in a large trial involving 894 sows, feeding 0.5% SDAP resulted in a significant increase in lactation feed intake (adjusted to 18 d lactation period), especially in PI and PII sows (77.8 kg

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vs 87.5 kg and 90.6 kg vs 100.0 kg; for PI and PII respectively). Improved litter weaning weight, reduced sow weight loss and reduced days to breeding following weaning are consistent with increased lactation feed intake (Patience et al., 1995).

Effect of plasma in broiler feeds

Recently a number of researchers have reported that rate and efficiency of growth of broiler chicks are improved when SDAP is included in the feed (Yi et al., 2001; Campbell et al., 2003). In addition, skinless, boneless breast meat yield has been increased when SDAP is fed (Campbell et al., 2003, unpublished data). Currently work is underway to establish minimal dietary inclusion and optimal time periods to feed SDAP in poultry diets.

Other effects of SDAP

Recent research suggests that the functional proteins in SDAP may play important roles in other biological functions of the animal.

Researchers in Spain fed weanling pigs (21 days age weaning) diets containing 0 or 7% SDAP and measured concentrations of caecal microflora following challenge with *E. coli* (Torradellona et al., 2002). Counts of Clostridium were reduced from 1.56 to 0 log CFU/g of caecal contents when pigs were fed SDAP. Further, concentrations of Lactobacilli were increased with SDAP in the diet (8.83 vs. 8.01 log CFU/g).

Modification of bone mass in pigs fed SDAP after weaning has also been reported (Jiang et al., 2000), as well as a significant reduction in intestinal weight in rats (Moreto, 2001, unpublished data) and pigs (Jiang et al., 2000). Jiang et al. (2000) also reported reduced urea N concentrations when pigs were fed SDAP postweaning, and suggested that changes in intestinal mass and cellularity was related to changes in circulating urea N concentrations. Plasma or purified globulin fed to pigs has been shown to reduce the concentration of circulating IgG in pigs (Campbell et al., 2000, unpublished data). Bosi et al., (2004) reported that the inclusion of SDAP protein or antibiotics reduced concentrations of IL-8, TNF-α and IFN-γ in the jejunum in pigs challenged with *E. coli.*

When taken collectively, these data suggest that dietary SDAP may serve as a "first line of defense" against enteric pathogens, including viruses and bacteria. This "passive enteric immunity" reduces stimulation of the immune system under modern commercial animal production. The animal's immune system is not over-stimulated, and as a result, pro-inflammatory cytokines secretion by macrophages is reduced. Cytokines secreted by macrophages affect many tissues and are responsible for liberating nutrients for use in supporting an immune response. Recent reports (Touchette et al., 2002) indicate that feeding SDAP reduced secretion of TNF-α, IL-6, and IL-1-β. When the immune system is not overstimulated, more nutrients are available for tissue accretion. According to many researchers, SDAP may serve an antimicrobial role in the intestine (Jiang, et al., 2000), thereby reducing the overall enteric challenge.

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Constituents in SDAP have also been reported to improve repair of damaged intestinal epithelial tissue in vitro (Rhoads et al., 2000) and in humans (Lembcke et al., 1997). Reports of improved intestinal integrity in pigs challenged with enteric pathogens (Torrallardona et al., 2002; van Dijk, 2001a,b) are also consistent with these observations. It is not yet clear which component(s) are responsible for the observed improvements in regrowth of damaged tissue.

Research continues to elucidate an important role for the functional proteins in SDAP in animal agriculture and potentially, in human nutrition and health. The urgent need to reduce the overuse of antibiotics in diets of animals demands that viable alternatives be found. Numerous published research trials have documented the value of SDAP in reducing the effects of experimental and on-farm enteric challenges.

Summary

Use of SDAP is well-accepted in animal agriculture. The value of functional proteins, including IgG, to support enteric health, reduce the effects of pathogenic challenge and reduce morbidity and mortality are well documented in both scientific and popular literature. Although not reviewed in this paper, the use of functional proteins (especially IgG) to improve human health is also well documented.

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American Society for Nutritional Sciences
American Association of Anatomists

American Association of Veterinary Anatomists
American Federation for Medical Research
American Society for Clinical Nutrition
The Biomedical Engineering Society
The Microcirculatory Society
North American Society for Biorheology
Society for Experimental Biology and Medicine
Society for International Nutrition Research

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371.1

EFFECT OF SUPPLEMENTATION WITH MULTIPLE MICRONUTRIENTS (MMN) AND/OR BOVINE SERUM CONCENTRATE (BSC) ON THE GROWTH OF LOW-INCOME, PERI-URBAN GUATEMALAN INFANTS AND YOUNG CHILDREN. KH Brown, MC Santizo, F Bezin, B Torún. Pgm in International Nutrition & Dept. of Nutrition, Univ of California, Davis, CA 95616 and Institute of Nutrition of Central America and Panama, Guatemala City, Guatemala.

We conducted a randomized, double-masked, community-based intervention trial to assess the effect of supplementation with MMN and/or spray-dried BSC on the growth, morbidity and micronutrient status of 259 Guatemalan children ~6-14 mo of age from low-income, peri-urban communities. Infants received supplemental iron from ~4-6 mo and were then randomized to one of the following treatment groups: 1) MMN and whey protein concentrate (WPC), 2) BSC only, 3) MMN & BSC, or 4) WPC only, all administered under supervision 6 d/wk. Groups were similar at baseline for age, sex, anthropometrics, morbidity from 5-6 mo, and biochemical indicators of MN status. Consumption of supplements averaged ~80% overall. There were no significant differences by treatment group in change in length, weight, or arm circumference among 171 children available during ≥7 mo of observation, even after controlling for initial age, sex, anthropometric status, feeding practices, maternal height and indicators of household socio-economic status (SES). Significant predictors of children's growth were baseline status, maternal height, infant feeding practices, and/or household SES.

Support provided by American Protein Corporation.

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EFFECT OF SUPPLEMENTATION WITH MULTIPLE MICRONUTRIENTS (MMN) AND/OR BOVINE SERUM CONCENTRATE (BSC) ON SERUM RETINOL (R) AND α-TOCOPHEROL (TL) CONCENTRATIONS OF LOW-INCOME, PERI-URBAN GUATEMALAN INFANTS AND YOUNG CHILDREN. [1]MJ Haskell, [2]MC Santizo, [2]F Bezin, [2]B Torun, [1]JM Peerson, and [1]KH Brown. [1]Pgm. in Int'l Nutrition & Dept. of Nutrition, Univ. of California, Davis, CA 95616 and [2]Institute of Nutrition of Central America and Panama, Guatemala City, Guatemala.

We conducted a randomized, double-masked, community-based intervention trial to assess the effect of supplementation with MMN and/or spray-dried BSC on growth, morbidity, and micronutrient status of 259 Guatemalan children 6-14 mo of age from a low-income, peri-urban community. Infants received either 1) MMN and whey protein concentrate (WPC), 2) BSC only, 3) MMN & BSC, or 4) WPC only, all administered under supervision 6d/wk. Serum concentrations of R and TL were measured before and after the 8-month supplementation period. Preliminary data for 55 children indicate that mean initial serum R and TL concentrations were 0.80 ± 0.24 µmol/L and 11.4 ± 6.0 µmol/L, respectively. Initially, 41.2% of children had serum R concentrations <0.70 µmol/L (20 µg/dL), 12.6% had concentrations <0.52 µmol/L (15 µg/dL), and 30.9% had serum TL concentrations <1.6 µmol/L (0.5 mg/dL). There was no difference in change in serum R concentration across treatment groups. SES was the only significant predictor ($p<0.001$) of initial serum R concentrations when SES, growth, and prior morbidity were included in the regression model.

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United States Department of Agriculture
Food Safety and Inspection Service
Office of Policy, Program Development and Evaluation
Washington, D.C. 20250/3700

AUG 17 2001

Mr. Mel Vanden Berg
Proliant, Inc.
2325 North Loop Drive
Ames, IA 50010

Dear Mr. Vanden Berg:

I am responding to your letter of July 5, 2001, concerning two beef blood plasma protein products that your company will be producing. The products consist of a high-globulin fraction extracted from beef plasma. You have requested comments concerning the proposed label for the product that will be produced under Food Safety and Inspection Service (FSIS) inspection.

During our meeting on June 27, 2001, we stated that FSIS regulations currently do not permit the use of synthetic antioxidants in products derived from fresh livestock blood. Consequently, you decided to produce two separate products. The high-globulin product containing antioxidants will be manufactured under FDA jurisdiction and will be clearly labeled as a dietary supplement. The second product, which does not contain antioxidants, will be produced under FSIS inspection and will bear the U.S. Department of Agriculture inspection legend.

The Federal meat inspection regulations (9 CFR, Section 317.8 (b) (7) (ii)) require that ingredients of livestock origin be designated on the label by names that include the species and tissue from which the ingredients are derived. Consistent with these regulations, you have proposed to name the product as "Globulin from Beef Plasma." As such, we do not have any objection to the proposed label. However, please be advised that you must submit an "Application for Approval of Labels, Marking or Device (Form 7234-1)" to the Labeling and Consumer Protection Staff to obtain formal approval of the label.

If you have any additional questions, please contact Mr. Bill Jones or me at Area Code (202) 205-0279.

Sincerely,

(b)

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

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ImmunoLin®

Immunoglobulin Protein Isolate*

Ingredients: Immunoglobulin Protein Isolate

Distributed by:
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Ankeny, IA 50021 USA

Net weight: 25kg (55.1lb)

Lot no._____

*Bovine Sourced

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HEALTH INGREDIENTS

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Submission End

DEPARTMENT OF HEALTH AND HUMAN SERVICES

FD

MEMORANDUM OF TELEPHONE CONVERSATION

Date: July 13, 2005

Time: 10:30AM-11:00AM

Location: Conference Room 2077
5100 Paint Branch Parkway
College Park, MD 20740

Participants:
Proliant Health Ingredients

Mel Van den Berg
Betty Humm

FDA

Rudolph Harris	HFS-255
Jeremy Mihalov	HFS-255
Timothy Twaroski	HFS-255
Felicia Williams	HFS-255

Subject: Spray-Dried Bovine Globulin

The meeting was requested by Mel Van den Berg to consult with FDA regarding the use of Spray-Dried Bovine Globulin in finished products. We presented the visitor with an overview of GRAS and answered general questions regarding the submission of a GRAS notice.

Your notice, in its present form, does not provide a sufficient basis for a determination that the subject use of spray-dried bovine globulin in food is GRAS. We need clarification on several points mentioned in your notice: 1) Provide an explanation of the consumption of bovine globulin for its intended use. Provide an overall estimated daily intake (EDI) of bovine globulin from proposed uses and probable consumption; 2) clarify the intended use (i.e. food categories and specific use levels); 3) clarify basis for GRAS determination by (a) scientific method or (b) history of use; 4) a significant number of published animal data is presented in the notice; however, the notice weighs heavily on the unpublished human data; 5) the above considerations should reflect whether the use levels and EDI are for IgG or the globulin concentrate; 6) we cannot equate the percentage globulin mentioned in the published literature to an exposure; 7) elaborate on the fact that plasma proteins are deficient in methionine, is this a safety concern; 8) use as a dietary supplement is not handled by the Office of Food Additive Safety; however, if Proliant Health Ingredients wishes to pursue the use of spray-dried bovine globulin as a

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dietary supplement the Office of Nutritional Products, Labeling and Dietary Supplements should be contacted and 9) we believe the GRAS panel could benefit by having someone with scientific training and experience in evaluating food safety.

The meeting concluded by informing Mr. Mel Van den Berg and Ms. Betty Humm that comments to our inquiries should be addressed within 10 working days. If Proliant Health Ingredients is unable to provide comments within this time frame they can send a withdrawal letter to FDA or the FDA will issue a no basis letter.

Felicia B. Williams, Ph.D.

(b)

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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

September 28, 2005

FD

Ms. Bonnie Humm
Proliant Health Ingredients
2425 SE Oak Tree Court
Ankeny, Iowa 50021

Re: GRAS Notice No. GRN 000169

Dear Ms. Humm:

The Food and Drug Administration (FDA) is responding to the notice, dated May 16, 2005, that Proliant Health Ingredients submitted in accordance with the agency's proposed regulation, 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). The notice was filed on May 24, 2005, and designated as GRN No. 000169.

The subject of the notice is bovine globulin. The notice informs FDA of the view of Proliant Health Ingredients that bovine globulin is GRAS, through history of use.

In a letter dated July 19, 2005, you asked that FDA cease evaluating Proliant Health Ingredients' notice in light of the technical requirements discussed during our July 13, 2005 telephone conversation with the understanding that Proliant Health Ingredients may, in the future, submit another GRAS notification on bovine globulin. Accordingly, we ceased our evaluation of GRN No. 000169 effective, July 19, 2005, the date that we received your letter.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36 (c) (1), are available for public review and copying on the homepage of the Office of Food Additive Safety (on the Internet at http://www/cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,

(b)

Antonia Mattia, Ph.D.
Director
Division of Biotechnology
and GRAS Notice Review
Center for Food Safety
and Applied Nutrition

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[redacted]

DEPARTMENT OF HEALTH AND HUMAN SERVICES

September 28, 2005

Ms. Bonnie Humm
Proliant Health Ingredients
2425 Southeast Oak Tree Court
Ankeny, Iowa 50021

Re: GRAS Notice No. GRN 000169

Dear Ms. Humm:

The Food and Drug Administration (FDA) is responding to the notice, dated May 16, 2005, that Proliant Health Ingredients submitted in accordance with the agency's proposed regulation, 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). The notice was filed on May 24, 2005, and designated as GRN No. 000169.

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Sincerely,

Antonia Mattia, Ph.D.
Director
Division of Biotechnology
and GRAS Notice Review
Center for Food Safety
and Applied Nutrition

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DEPARTMENT OF HEALTH AND HUMAN SERVICES

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

FD

November 17, 2005

Ms. Bonnie Humm
Proliant Health Ingredients
2425 SE Oak Tree Court
Ankeny, Iowa 50021

Re: GRAS Notice No. GRN 000169

Dear Ms. Humm:

I am enclosing a corrected copy of the letter dated September 28, 2005, from Dr. Antonia Mattia responding to GRAS Notice No. 000169. We discovered that there was an error in the text. The phrase "through history of use" in the second line of the second paragraph is being replaced by the phrase "through scientific procedures." We corrected this error on the enclosed letter, November 17, 2005, which will be made available on our Internet site during our routine update for December. We regret any inconvenience this error may have caused.

If you have any questions, please contact me at 301-436-1258.

Sincerely yours,

(b) [redacted]

Felicia B. Binion Williams, Ph.D.
Division of Biotechnology
and GRAS Notice Review
Center for Food Safety
and Applied Nutrition

(b) [redacted]

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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

Ms. Bonnie Humm
Proliant Health Ingredients
2425 SE Oak Tree Court
Ankeny, Iowa 50021

Re: GRAS Notice No. GRN 000169

Dear Ms. Humm:

The Food and Drug Administration (FDA) is responding to the notice, dated May 16, 2005, that Proliant Health Ingredients submitted in accordance with the agency's proposed regulation, 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received the notice on May 23, 2005, filed it on May 24, 2005, and designated it as GRN No. 000169.

The subject of the notice is bovine globulin. The notice informs FDA of the view of Proliant Health Ingredients that bovine globulin is GRAS, through scientific procedures.

In a letter dated July 19, 2005, you asked that FDA cease evaluating Proliant Health Ingredients' notice in light of the technical requirements discussed during our July 13, 2005, telephone conversation with the understanding that Proliant Health Ingredients may, in the future, submit another GRAS notification on bovine globulin. Accordingly, we ceased our evaluation of GRN No. 000169 effective, July 19, 2005, the date that we received your letter.

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Sincerely,

Antonia Mattia, Ph.D.
Director
Division of Biotechnology
and GRAS Notice Review
Center for Food Safety
and Applied Nutrition

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Reference List for Industry Submission, GRN 000169

Pages	*Author*	*Title*	*Publish Date*	*Source*	*BIB_Info*
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000149-000154	Lembcke, Jorge L.; Peerson, Janet M.; Brown, Kenneth, H.	Acceptability, Safety, and Digestibility of Spray-Dried Bovine Serum Added to Diets of Recovering Malnourished Children	October 1997	Journal of Pediatric Gastroenterology & Nutrition	Volume 25, Number 4, pp 381-384
000254-000262	Coffey, Richard D.; Cromwell, Gary L.	Use of spray-dried animal plasma in diets for weanling pigs	2001	Pig News and Information	Volume 22, Number 2, pgs 39N-48N
000263-000264	Straw, Barbara E.; D'Allaire, Sylvie; Mengeling, William L.; Taylor, David, J.	Diseases of Swine	1999	Diseases of Swine	8th Edtion
000265-000275	Nelssen, J.L.; Dritz, S.S.; Tokach, M.D.; Goodband, R.D.	Nutritional Programs for Segregated Early Weaning	Sep 1996	The Compendium on continuing education for the practicing Veterinarian	Volume 18
000276-000285	Hansen, J. A. ; Nelssen, J.L.; Goodband, R.D.; Weeden, T.L.	Evaluation of Animal Protein Supplements in Diets of Early-Weaned Pigs	1993	Journal of Animal Science	Volume 71, Issue 7 pgs 1853-1862
000286-000292	Kats, L.J.; Nelssen, J.L.; Tokach, M.D.; Goodband, R.D.; Hansen, J.A.; Laurin, J.L.	The Effect of Spray-Dried Porcine Plasma on Growth Performance in the Early-Weaned Pig	1994	Jounal of Animal Science	Volume 72, pgs 2075-2081
000293-000298	Gomez, G.G.; Phillips, O.; Goforth, R.A.	Effect of Immunoglobulin Surce on Survival, Growth, and Hematological and Immunological Variables in Pigs	1998	Jounal Animal Science	Volume 76, pgs 1-7
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NA- Not applicable

Pages	Author	Title	Publish Date	Source	BIB_Info
000318-000321	Quigley, Jim	Calf Note #98 - BSE and bovine blood proteins	2004	Calf Notes.com	
000322-000327	Nash, G.S.; Macdermott, R.P.; Schloemann, S.; Bertovich, M.J.; O'Neal, J.; Porter, L.; Kulczycki, A.	Bovine IgG1, but not IgG2, binds to human B cells and inhibits antibody secretion	1990	Immunology	Volume 69, pgs 361-366
000328-000332	Copelan, E.A.; Bechtel, T.P.; Klein, J.P.; Klein, J.L.; Tutschka, P.; Kapoor, N.; Featheringham, N.C.; Avalos, B.R.	Controlled trial of orally administered immunoglobulin following bone marrow transplantation	1994	Bone Marrow Transplantation	Volume 13, 87-91
000333-000336	Melamed, Isaac, Griffiths, Anne M.; Roifman, Chaim M.	Benefit of oral immune globulin therapy in patients with immunodeficiency and chronic diarrhea	September 1991	The Journal of Pediatrics	Volume 119, Number 3
000341-000343	NA	Food Safety and Inspection Service, USDA	January 1, 2001	Code fo Federal Regulations Title 9	Volume 22
000344-000354	Yoder, Ralph D.	US Patent & Trademark Office	December 4, 2003		

NA- Not applicable