FINAL REPORT
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TITLE OF PROJECTS
Separation and evaluation of supramammary lymph node proteins, Evaluation of supramammary lymph node powder *in vivo*, Effects of bovine supramammary lymph nodes on poultry performance and health.

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Duration of Project: 6 years

Abstract:
Many new dietary sources of protein, vitamins, complex minerals and bioactive compounds have been proposed as alternatives to traditional sources for nutritional supplements. The discovery of a new source could replace some additives and also serve as a modifier of metabolism. Supramammary lymph nodes (SMLN) are a component of the bovine udder lymphatic system. These nodes destroy foreign materials and provide lymphocytes to defend the udder and thus are a component of the natural defense system. By virtue of their location and function lymph nodes, a co-product of processing bovines for human use are a potential source of diverse proteins, peptides and other bioactive factors.

The protein containing supernatants from supramammary lymph nodes of dairy and beef udders were used as serum replacements for cell culture experiments. The inclusion of these sources of proteins, that can support the growth of cell lines in culture, could lead to a huge market for biomedical and veterinary products. The cost of fetal calf serum or bovine calf serum runs $36-72/100 mL. Additionally, the cost of peptides and factors that have therapeutic uses for humans and animals can run hundreds to thousands of dollars per milligram or microgram. The recovery of these valuable proteins and factors from supramammary lymph nodes could provide

a quick, less invasive, yet value added product for the processing and rendering industries.

Our laboratory group tested the efficacy of these supernatant proteins on the growth of important cell lines used in biomedical and veterinary testing for diagnosis of diseases and for production of valuable products (e.g., monoclonal antibodies, hormones, growth factors, etc.). Furthermore, this project identified separation technology for recovery of different components of the extract by solubility in concentrated salt solutions, size and charge. Proteins were recovered based upon the characteristics just described, the activities were assessed with cells in culture that lead to identification of proteins by critical analysis.

A preliminary feeding trial was conducted for chick growth and feed conversion data. Substitution of protein in a standard corn-soy chick starter diet with freeze-dried SMLN powder at 1%. The substitution was done such that protein concentration and amino acid composition of the diet was unaltered from the standard formulation. The immediate objective of that study was to determine if any addition of the SMLN in the diet would have any negative effects on the growth and performance of young chicks. It was envisaged that substitution of protein in the diet with the SMLN would allow for a supplementation that could potentially result in good growth and productivity of the chicks and improved gut health. The final feeding trial tested the efficacy of SMLN substituted for protein in chick starter diets at 0, 1.5% and 3% from hatch to six weeks of age.

The *in vitro* cell proliferation study (Duffy *et al.,* 2008) determined the heat inactivated preparation of lymph node extract stimulated cells to proliferate, however the preliminary and final feed trials with SMLN protein did not stimulate chick growth.

Introduction:

The bovine supramammary lymph node (SMLN) located on the dorso-caudal surface of the udder (Bradley *et al*., 2001) is discarded during meat processing of cows. This protein-rich lymphoid organ could be recovered during processing when the udder is removed. The apparent source of bioactive proteins in the lymph node led to the idea of substituting a lymph node extract for serum in cell culture media. In order to do this, the lymph node must have the appropriate growth factors to support growth of a variety of cell lines.

Lacasse *et al.* (1996) reported mitogenic activity of bovine mammary gland lymph on bovine mammary epithelial cells (MAC-T) and mammary fibroblasts. PGE2, which has been shown to stimulate proliferation of rodent mammary epithelial cells, was high in bovine lymph, and when more PGE2 was added to the lymph mitogenic activity increased (Lacasse *et al*., 1996). A study also using MAC-T cells tested the mitogenic effects of mammary tissue extracts from prepubertal heifers raised to high- or low-rate of gain (Berry *et al*., 2003). The mammary tissue extracts from high-fed heifers stimulated less mitogenic activity compared to the mammary tissue extracts from low-fed heifers. Berry *et al*. (2003) also extracted tissue from ovariectomized (OVX) heifers and found there were no differences in proliferative responses of MAC-T between OVX or control mammary tissue extracts. Weber *et al*. (1999) showed that mammary extracts from prepubertal heifers stimulated mammary epithelial cell proliferation much more than heifer serum or IGF-I alone. The results from these studies collectively show that the bovine mammary gland contains growth factors for mammary epithelium. IGF-I as well as TGF-α are produced in the mammary gland and stimulate DNA synthesis of mammary epithelial cells (Weber *et al*., 1999). IGF-II is also found in the mammary tissue of ewe lambs. In order to assess how much influence IGF-I has on cell proliferation, recombinant human insulin like growth factor binding protein-3 (rhIGFBP-3), which would bind to IGF-I, was added

to serum and extracts, and it ultimately inhibited one third of the DNA synthesis produced by serum or extracts (Weber *et al*., 1999). Clearly, IGF-I is an important growth factor for mammary epithelial cells. Weber *et al*. (2000) also tested mammary tissue extracts on bovine primary mammary epithelial cells and investigated whether or not exogenous growth hormone (GH) or feeding level influenced mitogenic activity. Five percent mammary extract stimulated [^{3}H] thymidine incorporation into DNA of more cells than 10% fetal bovine serum or 100 ng/ml of IGF-I. In concurrence with Berry *et al*. (2003) high-fed compared to low-fed heifer mammary extracts inhibited [^{3}H] thymidine incorporation into cultured mammary epithelial cells. On the other hand, when GH was administered, high-fed compared to low-fed heifers had an increased [^{3}H] thymidine incorporation into mammary epithelial cells in response to mammary tissue extracts. Growth hormone treated high-fed heifers also had increased tissue IGF-I levels (Weber *et al*., 2000).

From the above, it is apparent that the mammary gland contains various mitogenic factors. The SMLN is one component of the entire udder, but it concentrated and diverse number of proteins makes it an appealing source for mitogenic factors. Our current work has shown that a heat-inactivated preparation of the extract can support growth of epithelial cells and antibody producing hybridoma cells. Other work involving the same preparation and various growth factors has shown support of equine chondrocyte growth in culture. We can obtain an extract with a high protein content made of numerous proteins, as seen with electrophoretic separation of protein bands. The protein concentration is almost as great as that found for most bovine sera sources, and the total protein composition revealed by electrophoresis shows that the extract has more apparent proteins that serum. The positive mitogenic effects of the extract, along with the variety of proteins therein, makes the extract an attractive source of biologically active proteins for various uses in culture, for dietary supplementations, and as possible therapeutics.

Materials and Methods:

Lymph node preparations for all studies

SMLN of both beef and dairy cows were obtained from Brown Packing (Gaffney, SC). The lymph nodes were trimmed of fat with scissors and were processed through a Hobart meat grinder until moderately homogenous. The homogenate was placed in freezer bags at -80° C for at least 48 hours. Frozen lymph homogenate was then crushed into small pieces using pestle and mortar and lyophilized for approximately seven days in a VirTis freeze dryer (SP Industries Inc., Warminster, PA, USA). The dried homogenate was further ground into a fine powder using a small food processor. The final SMLN powder was sent to Clemson University's Agricultural Service Laboratory for final content analysis prior to beginning each project.

The initial project entitled "Separation and evaluation of supramammary lymph node proteins" including the following:

1. The first objective involved separation of proteins based upon salt solubility, charge and size. This is a sequence approach that allowed for improved resolution of proteins so that the resulting fractionations would yield near pure samples of proteins. The salt solubility involved stepwise increases in the ammonium sulfate concentration in solution. The salt content of the solutions were 20, 40, 60 and 80%. The original lymph node extract preparation at 27 mg/ml was be subjected to sequential salt cuts in order to stepwise precipitate proteins in solution at each ammonium sulfate percentage.

2. Candidate salt fractions demonstrating bioactivity were then subjected to chromatofocusing through a pH gradient of 6.0 – 9.0. The chromatofocusing column was equilibrated at pH 9.0 prior to addition of selected salt fractions. After addition of the fractions onto the column, a drop wise addition of elution buffer at pH 6.0 was slowly run through the column. Two milliliter fractions off the column were collected in order to catch proteins as they detached at their respective isoelectric points. Collected fractions were pHed to confirm isoelectic points of fractionated proteins.
3. The remaining fractions with bioactivity were then subjected to size separation with a Sephacryl S-100 column. This column separated and resolved proteins in the molecular weight range of 1000 – 100,000 Daltons. Once the extract was processed through ammonium sulfate fractionation and chromatofocusing, each one was "clean enough" to run on the column. The candidate fractions were loaded onto a pre-packed column and eluted with buffer under low to moderate pressure in order to separate proteins by molecular size.

The second project entitled "Evaluation of supramammary lymph node powder *in vivo"* included the following:
1. As the salts precipitated out of proteins, samples of the remaining soluble proteins were recovered and subjected to dialysis against phosphate buffered saline. Each dialyzed sample was prepared for analysis in bioassays of cultured cells. As various bioactivity was observed or eliminated with the salting-out process, decisions were made about evaluation of the possible protein candidates in the salt fractions. All chromatofocused fractions were adjusted to neutral pH and the fractions evaluated for activity in bioassays. The Sephacryl S-100 fraction collection process was used to capture 2 ml fractions that was evaluated in bioassays for detectable activity.
2. Cells (MDA-MB-435, MAC-T and IC6) were used in a CyQuant cell proliferation assay to detect mitogenic activity of the various factions. Mitogenic activity was assessed by examining the proliferation potential of cells in culture for 2-4 days. In this period of time, cells that were sensitive to the actions of protein ligands exhibited a burst of cell division resulting in numerous cell copies in culture. The CyQuant assay involved a DNA stain fluorescent dye that directly stains the total DNA of the cells in a given culture container. Within minutes (2-5) of staining the cells, the fluorescence was detected and read with a multi-well plate reader set for 485 nm excitation and 520 nm emission. Fluorescence intensity was directly indicative of cell numbers.

The third project entitled "Effects of bovine supramammary lymph nodes on poultry performance and health" included the following:
This project was repeated as Phase 1 with 1 and 2% total protein. Phase 2 added 0, 1.5 and 3% of SMLN in replacement of protein in chick starter diet.
1. Chicks were obtained from a local commerical hatchery and housed in battery brooder units in the PEC building or in floor pens at the Morgan Poultry Farm. The chicks were identified, weighed, and brooded with supplemental heat and provided feed and water *ad libitum*. The chick diets were formulated and mixed at the Morgan Poultry Farm feed mill. The lymph node supplement was mixed into diets at a rate of 1 and 2% (Phase 1) and 0, 1.5% and 3% (Phase 2) of the total protein. At termination of each experiment blood was collected to determine biochemical and immune responses.
2. Chicks were weighed each week to record body weights and feed was weighed in order to determine feed consumption. At weekly intervals and at the end of the study, chicks were bled

via the wing vein and then euthanized by CO_2 inhalation. Blood samples were sent for analysis and final body weights were collected.

Results:

Initial studies began with the optimization of SMLN collection, homogenization, mincing, freeze drying and recovery of the crude protein. Secondary studies determined protein concentrations and studies with MAC-T cells. These studies showed the crude SMLN extracts contained 50% of the protein concentration of bovine growth serum (BGS), however the growth of the cells was better in SMLN conditions. This initial work led to the manuscript Influence of supramammary lymph node extract on *in vitro* cell proliferation (Duffy *et al.,*2008). Please reference this paper for specific results.

These results led to the hypothesis that animal health and growth could be optimized by supplementing SMLN as a protein source into the diet. Phase 1 feeding trial included chick starter diets formulated for 1 and 2% SMLN basis for the study.

Table 1. Proximate analysis of finely ground and sifted SMLN

	Sample 1	Sample 2	Mean
Moisture,%	14.7	14.1	14.4
Dry matter, %	85.3	85.9	85.6
Crude protein, %	54.8	55.6	55.2
Soluble protein, %	31.2	31.2	31.2
Phosphorus, %	0.85	0.86	0.855
Calcium, %	0.14	0.04	0.09
Magnesium, %	0.05	0.05	0.05
Potassium, %	0.87	0.88	0.875
Sulphur, %	0.44	0.45	0.445
Iron, mg/kg	328	244	286
Zinc, mg/kg	59	53	56
Copper, mg/kg	5	4	4.5
Manganese, mg/kg	6	2	4

Table 2. Protein and amino acid analysis of finely ground and sifted SMLN

	Sample 1	Sample 2	Mean
Crude protein, %	56.02	55.72	55.87
Dry matter, %	93.97	94.18	94.08
Methionine	0.95	0.96	0.96
Cystine	0.72	0.72	0.72
Methionine + cystine	1.67	1.67	1.67
Lysine	3.95	3.96	3.96
Threonine	2.17	2.15	2.16
Arginine	3.80	3.79	3.80
Isoleucine	1.80	1.86	1.83
Leucine	3.73	3.75	3.74
Valine	2.49	2.56	2.53
Histidine	1.09	1.10	1.10
Phenylalanine	1.97	1.97	1.97
Glycine	4.51	4.48	4.50
Serine	2.42	2.35	2.39
Proline	2.96	3.01	2.99
Alanine	3.19	3.17	3.18
Aspartic acid	4.27	4.26	4.27
Glutamic acid	6.67	6.64	6.66
Total (w/o ammonia)	46.69	46.73	46.71
Ammonia	0.84	0.92	0.88
Total	47.53	47.65	47.59

Sixty-four chicks were divided into 8 cages of 8 chicks each. For one week, all chicks were fed the standard chick starter diet and provided water *ad lib*. At the end of the first week, half the cages were randomly assigned to receive the chick starter diet supplemented with SLNP at 1%. Chicks were monitored daily and provided feed and water *ad lib* for 3 weeks. At both 3- and 4-weeks of age body and feed weights were recorded. At the conclusion of the study at 4-weeks of age, all chicks were bled and euthanized. In addition to body weights the livers and spleens of each chick were weighed. Clotted blood was centrifuged to separate sera from red blood cells, and the sera were used for determinations of IgG and IgM concentrations.

At 3- and 4-weeks of age there were no differences in body weights of chicks fed either diet, and the body weight gains from 3- to 4-weeks of age were not different. Feed conversions were not different at the end of study. Also, there were no differences in relative liver and spleen weights or serum concentrations of IgG and IgM.

Table 3. Average body and serum parameters (mean ± SEM) for broilers chicks fed a standard corn-soy starter diet or one supplemented with 1% freeze-dried SMLN. The supplemented diet was adjusted on the basis of protein concentration and amino acid composition of the lymph node powder in order to be equivalent to the standard diet.

Parameter	Standard Diet	SMLN Diet	*P* value
3-Wk Body Wt (g)	799.44 ± 16.88	775.75 ± 16.88	0.3291
4-Wk Body Wt (g)	1364.25 ± 27.15	1332.25 ± 27.15	0.2941
3- to 4-Wk Body Wt. Gain (g)	564.56 ± 19.10	547.50 ± 19.10	0.5262
Feed Conversion (g/g)	1.23 ± 0.02	1.20 ± 0.02	0.3547
Rel Liver Wt (mg/100 g)	2.10 ± 0.11	2.17 ± 0.10	0.6185
Rel Spleen Wt (mg/100 g)	0.11 ± 8.37	0.11 ± 8.37	0.8816
Serum IgG (mg/ml)	18.15 ± 4.93	11.59 ± 4.93	0.3543
Serum IgM (mg/ml)	2.09 ± 0.21	1.65 ± 0.21	0.1586

Phase 2 feeding trial included chick starter diets formulated for 0, 1.5 and 3% SMLN basis for the study.

Table 4. Proximate analysis of finely ground and sifted SMLN

	Sample
Moisture, %	3.8
Dry matter, %	96.2
Crude protein, %	76.6
Fat, %	14.9
Phosphorus, %	1.29
Calcium, %	0.03
Magnesium, %	0.07
Potassium, %	1.47
Sulphur, %	0.65
Iron, ppn	198
Zinc, ppn	77
Copper, ppn	4

Eighteen chicks were divided into 3 cages of 6 chicks each. For one week, all chicks were fed the standard chick starter diet and provided water *ad lib*. At the end of the first week, cages were randomly assigned to receive the chick starter diet supplemented with SMLN at 0%, 1.5% and 3%. Chicks were monitored daily and provided feed and water *ad lib* for 6 weeks. The chicks were weighed at hatch and the end of each week during the study. Blood samples were collected from wing veins at the end of weeks three and weeks six. It was determined by statistical analysis there was not a significant change in body weight between the three groups now was there a significant change in blood analysis.

Graph 1. Average body weights for broiler chicks.

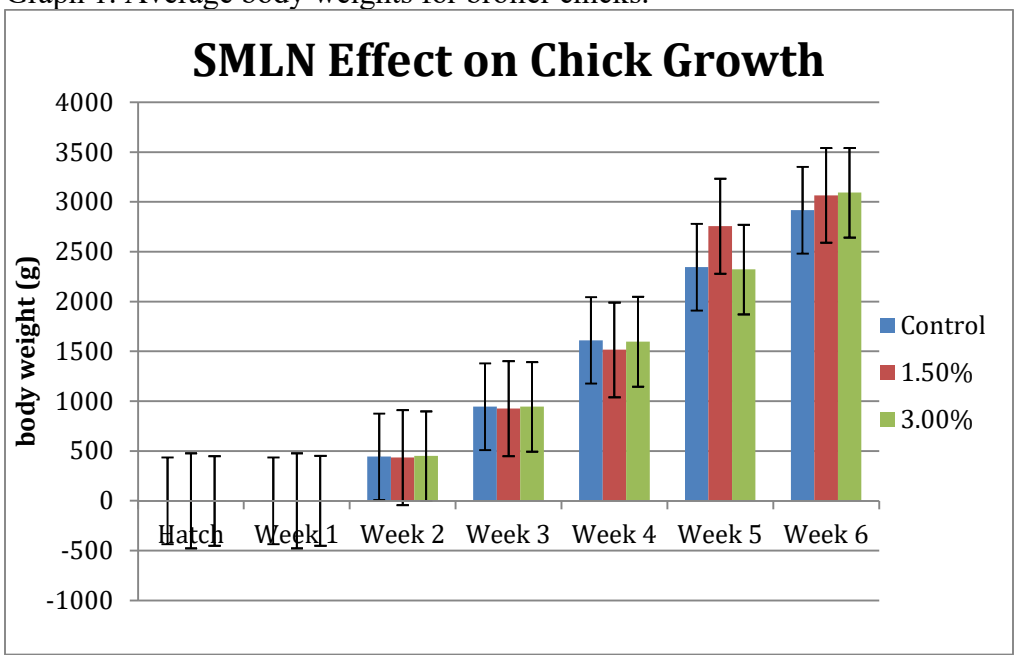

Table 5. Blood analysis for broiler chicks.

	week 3 Control	week 6 Control	week 3 1.5%	week 6 1.5%	week 3 3%	week 6 3%
Total Protein (g/dL)	2.80	3.03	2.33	2.97	2.50	2.57
Albumin (g/dL)	0.93	1.80	1.17	1.27	0.90	1.2
Globulin (g/dL)	1.87	1.23	1.17	1.7	1.60	1.37
Vet A/G Ratio	0.63	2.73	1	0.77	0.63	0.87
Glucose (mg/dL)	237.00	249.67	228.67	243	250.33	243
BUN (mg/dL)	1.5	1	1.66	1.67	2.00	1.33
Creatinine (mg/dL)	0.06	0.13	0.06	0.13	0.06	0.1
Calcium (mg/dL)	9.67	9.60	9.97	10.53	10.07	9.47
Total Bilirubin (mg/dL)	0.23	0.33	0.43	0.07	0.40	0.37
Direct Bilirubin (mg/dL)	0.00	0.00	0	0	0.00	0
Indir Bilirubin (mg/dL)	0.23	0.33	0.43	0.07	0.40	0.37
ALT (SGPT) (units/L)	5	5.00	5	5	5.00	5
AST (SGOT) (units/L)	418	331.00	249.67	329	227.00	383
GGTP (units/L)	14	20.33	15	19	13.00	15.67
Uric Acid (mg/dL)	9.07	8.33	7.7	10.1	7.73	5.03

Conclusions:

The supramammary lymph node is one component of the entire udder, but its concentrated and diverse number of proteins makes it an appealing source for growth factors (Lacasse et al., 1996). Our past work (Duffy et al., 2008) has shown that a heat-inactivated preparation of the extract can support growth of epithelial cells and antibody producing hybridoma cells. Other work involving the same preparation and various growth factors has shown support of equine chondrocyte growth in culture (Reed, 2007). We can obtain an extract with a high protein content and made-up of numerous proteins, as seen with electrophoretic separation of protein

bands (Duffy et al., 2008). The protein concentration is almost as great as that found for most bovine sera sources, and the total protein composition revealed by electrophoresis shows that the extract has more apparent proteins than serum. The positive growth effects of the extract, along with the variety of proteins therein, make the extract an attractive source of biologically active proteins for various uses in cell culture.

The SMLN appeared to be an acceptable source of protein for animal diets based upon its composition analyses. With our previous knowledge of the potent protein factors present in the lymph node extracts that supported growth of different types of cells in culture, we anticipated possible beneficial effects for the growth of young animals fed diets containing lymph node powder. Results from the two feed studies showed there was not a significant change in overall body weight or growth to warrant further evaluation. However, it is possible that SMLN can have positive growth effects in other species.

Due to the labor intensive practices to collect, grind, dry and evaluate SMLN it is not ideal to add for poultry diets. Provided a more streamlined approach for collection of bovine mammary lymph nodes is possible, serum replacement in culture using SMLN could lead to cost reduction of cell culture techniques.

Budget:

ACREC has contributed $120, 376 over a period of six years. During this time, the PI's and collaborators have submitted semi-annual reports and met with ACREC personnel to present research findings.

Clemson University has invested $321,176 over a period of six years in this project alone. During this time three master's degrees were completed with theses published, one manuscript was published in a peer-review journal and a patent licensed. The detailed budget justification of Clemson's investment is outlined below.

From the figures stated above, ACREC has received a very good return on investment from the $120,376 investment.

Budget justification of Clemson University's investment:

Salaries	**Total**
F&A costs- indirects (48%)	46,200
Faculty personnel	
Scott (3 yr)	31,500
Scott fringe (26%)	8,190
Maurice (2 yr)	15,600
Maurice fringe (26%)	4,056
Ellis (1 yr)	3,500
Ellis fringe (26%)	910
Walker (1 yr)	15,000
Walker fringe (26%)	3,900
Non-faculty personnel	
Owens (2 yr)	3,500
Owens fringe (28%)	980
Korn (1 yr)	1,750
Korn fringe (28%)	490
Graduate student personnel	

Two MS assistantships for 2 years (15K per semester)	120,000
One MS assistantship for 1.5 years (15K per semester)	45,000
Manuscript fees	600
Patent fees	20,000

TOTAL invested by Clemson University **$321, 176**

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