FINAL REPORT **Date:** 13 October 2015

Title: **Adding Value to Animal Protein Ingredients while Meeting the Needs of the Worldwide Aquaculture Industry**

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Project Start Date: 1 May 2009 **Effective Completion Date:** 30 September 2015

Objectives:

A) Overall Objectives

The overall objective of this project is to achieve a better characterization of the nutritive value of rendered animal protein ingredients currently produced in North America and add value to these ingredients by improving their nutritive value and suitability for various aquatic species cultured around the world.

B) Specific Objective (As per the Original Proposal)

1) Proceed with the fine characterization of the nutritional composition of different types of rendered animal protein ingredients with emphasis on minor nutrients and to contrast the composition of these ingredients with what is currently known about nutrient requirement of aquatic species cultured worldwide

2) Evaluate the potential of various relatively simple (cost-effective) processing techniques (air classification, incubation) to improve digestibility and availability of nutrients in rendered animal protein ingredients.

3) Develop and evaluate high digestible nutrient density (high protein, low ash) "low pollution" animal protein ingredients for use in high value feeds for marine "carnivorous" fish species (Atlantic salmon, white sea bass), derived from poultry, porcine or ruminant raw material (to respect different market requirements).

4) Develop and evaluate lower economical value of high ash animal proteins (e.g. bone fraction from air-classification of conventional meat and bone meal or poultry by-products meal) as cost-effective sources of phosphorus, micro-minerals, phospholipids, and cholesterol for warm water gastric aquatic species (Nile tilapia, shrimp) and agastric species (e.g. carps) species widely cultured in Asia and Latin America.

5) Explore the potential of various feed additives and simple processing techniques to improve the digestibility of phosphorus and other minerals in high ash animal proteins fed to agastic fish species (carps) widely cultured in Asia and of high nutrient density animal protein ingredients fed to marine carnivorous fish species.

6) Modify and validate an existing phosphorus digestibility model developed for salmonid fish (Hua and Bureau, 2006) to various fish species cultured around the world, notably those widely cultured in Asia and Latin America.

Summary of Project Results

1) Matching Funds

A proposal titled "Cost-Effective Processing Technologies to Improve the Value to Aquaculture Species of Products and By-Products from the Agricultural and Food Sectors in Ontario" was submitted on 1 March 2010 to the OMAFRA/UofG Research Program, to seek matching funds for the present FPRF project. In May 2010, we received news that our proposal was selected for partial matching funds by the "Bioeconomy – Industrial Uses" and "Product Development and Enhancement" Programs. A total for $ 50,000 (Canadian dollars) was awarded to the project for three years. As part of the conditions for obtaining funds from the OMAFRA/UofG Research Program, we have to expand our work to certain by-products of the grain and food processing industries.

Funding from the OMAFRA/UofG Research Program allows us to tap in into institutional resources (e.g. easier access to university infrastructures (research stations), use of service vehicles, analytical facilities, etc.). Combining the FPRF and OMAFRA/UofG Research projects is allowing us to develop a more comprehensive and solid research effort.

We were able to attract two visiting professors (Dr. Yuhong Yang and Dr. Lus Lopez) for the period of August 2010 to July 2011. They have played leading roles in the project over that period. Subsequently, Prof. Chungfan Cai from Soochow University joined our research team as visiting professor from January to December 2012 and also very meaningfully contributed to the project since her arrival. Finally, Prof. Chunfang Wang from Huazhong Agricultural University joined the research team in September 2014 and contributing to wrapping up this project, notably with a focus on the elemental and nutrient mass balance work.

2) Development of Phosphorus Digestibility Models for Different Fish Species

Between April 2009 and July 2010, Dr. Katheline Hua carried out a project aimed at constructing digestibility models to estimate digestibility of phosphorus in complete feeds for different fish species most widely cultured around the world, namely carp, tilapia, and salmonid species. The modeling effort resulted in two novel mathematical models to estimate the digestibility of P in carp and tilapia feeds formulated with a wide variety of ingredients. These models are simple and will be very useful tool for fish feed formulators around the world.

The results of this modeling effort suggest that very significant differences in P apparent digestibility exist between carp, tilapia, and salmon species. Carp species appear to have low ability to digest mineral P compounds of low solubility. They cannot effectively digest P bound in bone particles (digestibility was estimated to be nil), and their ability to digest dibasic calcium phosphates is lower (slightly below 40%) in comparison to tilapias and salmons (in the range of 56-64%). This difference is attributable to the absence of true stomach in carps. This observations stress the urgent need to work on improving P digestibility of rendered animal proteins, such as meat and bone meal and poultry by-products meal, for carp species in order to improve the nutritive and economical value of these ingredients for this very important sector of the aquaculture industry.

A scientific manuscript was submitted and accepted for publication in Aquaculture, a widely read scientific journal for the aquaculture nutrition community. The article was published late in 2010. The Specific Objective #6 of the proposal is therefore completed.

Reference:

Hua, K. and D.P. Bureau. 2010. Quantification of differences in digestibility of phosphorus among cyprinids, cichlids, and salmonids through a mathematical modelling approach. Aquaculture, 308: 152-158.

3) Partial Redefinition of Project Objectives and Workplan

In August 2010, we took an executive decision and created to two research teams to work on two separate (but highly complementary) objectives of the project which are addressing the Objective #1 to 5 of the original project proposal. This represents a simple realignment of tasks and evolution of objectives on the basis of resources and results obtained.

Objective #1: Characterization of the Nutritive Value of by-Products from the Animal Agriculture / Meat Processing Industry and Development of Novel Ingredients Using Cost-Effective Separation Techniques

Team members:

Dr. Lus Lopez, Visiting professor (Mexico) (July 2010-June 2011)
Mr. Jorge Castillo, Industry consultant
Mr. Kabir Chowdhury, PhD student (May 2009-Jan 2012) & PDF (Feb-Aug 2012)
Ms. Jamie Hooft, PhD student
Dr. Chunfang Wang, Visiting scientist

Objective 1.1 To characterize the chemical composition of numerous types of rendered animal protein ingredients (and batches thereof) and common fish feed ingredients with emphasis on trying to obtain more complete picture of their composition, contrasting the nutritional composition of these ingredients with what is currently known about nutrient requirement of aquatic species cultured worldwide and developing a feed ingredient composition database that integrates and makes sense of the existing information (sources of variations for different nutrients, etc.) and identifies the most significant gaps in our knowledge.

Progress:

The team has sought, compiled, and interpreted existing information on fine chemical composition of different rendered animal protein ingredients (fish meals, poultry by-products meals, meat and bone meals, feather meals, blood meals). It has initiated the development of a feed ingredient composition database that integrates and makes sense of the existing information (sources of variations for different nutrients, etc.). The team has identified significant gaps in our knowledge.

Seven ingredients of animal origin, namely 1) fish meal (herring meal), 2) low-ash poultry by product meal, 3) high-ash poultry by-product meal, 4) porcine meat meal, 5) feather meal, 6) meat and bone meal (mixed species), and 7) spray-dried porcine blood cell meal, were obtained from various suppliers in Ontario, Canada. Proximate, elemental and mineral analysis of these ingredients has been completed. Results from these analyses are presented in Tables 1 to 4.

All essential and non-essential amino acids were analyzed by two contracting laboratories (Toronto Hospital for Sick Children and Procter-Gamble/IAMS Company) employing state of the art techniques. It is noteworthy that the Procter-Gamble Laboratory carries out amino acid analyses on a daily basis for the needs of the IAMS Company, one of leading pet food manufacturers in the USA.

The essential and non-essential amino acid profiles of the seven ingredients tested are presented in Tables 3 and 4. Amongst relatively novel results is the estimation of the taurine and hydroxyproline content of animal protein ingredients tested. There is excellent evidence now that taurine is an essential nutrient for marine fish species and for early-life stages of many fish

species. Our results suggest that poultry by-products meal is an excellent source of taurine since it contains about 0.5% taurine, a level very similar to that of herring meal (Table 4). Meat and bone meal (0.1%), feather meal (0.03%) and blood meal (0.01% proved to be poorer sources of taurine. Hydroxyproline is another nutrient of interest to some aquaculture species nutritionists who believe this nutrient may have beneficial effects on Atlantic salmon. Poultry by-products meal and meat and bone meal proved excellent sources of hydroxyproline with levels of hydroxyproline (2.3-2.7%) that were almost as high as that of herring meal (2.7%).

The focus of this part of the project is now on trying to carry out a "mass balance" effort on the basis of the elemental analysis (C, N, P analysis) and analysis of individual chemical components (amino acids, etc.). In recent years, analysis of individual nutrients in feed ingredients is becoming increasingly common. However, no systematic efforts have been carried out to reconcile results of proximate analysis (which are based on elemental N analysis in case of crude protein) and results of analysis of individual nutrients. This is especially critical since results of analysis of individual nutrients (e.g. amino acids) are often highly variable, costly and very difficult to evaluate critically. We believe that the elemental mass balance could provide renderers, nutritionists and feed manufacturers with a simple and straightforward approach for critically evaluating the reliability of results of nutrient analysis of ingredients. It would also allow more sound analysis of results of different feeding trials with different ingredients since it would mean comparison of different nutrients on a “true nutrient” basis.

One of the initial goals of this mass balance effort was to determine for example, what is the proportion of total N of animal by-products is contributed by amino acids (essential vs. non-essential) or by non-protein compounds. In the past, assessment of the nutritive value of feed ingredients has been solely based on proximate analysis of these ingredients. All N is assumed to be as protein (amino acids) which is not necessarily a reasonable assumption. This may have skewed the results of the prediction of the nutritive value of ingredients.

Our results at this point in time suggest that NPN represent only a very small proportion (less than 0.2% with an average of 0.05%) of the dry matter content of rendered animal protein ingredients (Table 5). In our study, the NPN included were NH_3-N, DNA-N, and RNA-N. Through the mass balance calculation, we found that there is a gap between N from crude protein and N contributed by amino acid and these NPN (Table 6.1). For example, on average, about 13 % of the total N was missing N when trying to reconcile N content obtained by Kjedahl N analysis and the theoretical N content based on the analysis of individual amino acids and non-protein nitrogen (NPN) compounds (based on stoichiometry). The discrepancy varied from 3 to 25% of the total N. Similar results were found in C mass balance (Table 7) where about 11% of the total C was missing when estimating total C content of feed ingredients on the basis of analyzed individual amino acids, fat, carbohydrate, DNA and RNA.

In order to verify our previous results, we carried out a comprehensive survey of available crude protein and amino acid data for 81 animal protein ingredients.. The data in this survey were derived from analyses carried out by the Fish Nutrition Research Laboratory (analyzed values), and a variety of research papers published in recent months, as well as values found in several online database, such as Feedipedia, the Asian Aquaculture Feed Formulation Database (AAFFD), Evonik’s AminoDat.

We calculated the average missing N percentage for each ingredient category, and the values are as follow: fish meals 17%, fish protein concentrates 11%, fish solubles 26%, fish silages 6%, krill meals 13%, shrimp meals 14%, squid meals 10%, blood meals 20%, feather meals 20%, meat and bone meals 8%, poultry by-product meals 21%, and milk products 15% (Table 6.2).

The basis for the discrepancies for between measured and theoretical N content of these feed ingredients is not known and requires more investigation. However, our results highlight considerable discrepancies between elemental and individual nutrient both on a nitrogen and carbon basis. Based on the techniques used and our experience, elemental analysis are generally high robust (i.e. accurate and repeatable). Significant “shortcomings” may exist in individual nutrient analysis, notably amino acid analyses. Since amino acids are the basic units and the building blocks of protein and thus the basis of “true protein”, elemental N mass balance developed in our study provides an innovative way to "audit" the quality of analytical data on amino acid contents of feed ingredients and the gap in our knowledge.

We are planning to develop an online tool to allow renderers and feed manufacturers to upload proximate and amino acid analysis data for different batches of ingredients and obtain an estimate of the true protein content of their ingredient and highlight potential discrepancies between proximate and individual nutrient analyses.

It is clear from this exercise that considerable gap may exist between the true nutritive value of feed ingredients predicted on the basis of proximate analysis and that predicted from individual nutrient analyses. It is very important to address this gap in our knowledge and our team will attempt to close this gap in the future through targeted research efforts.

Objective 1.2 To determine the yield and chemical composition of different fractions of poultry by-products meals and meat and bone meals obtained through air-classification and other separation techniques.

Progress: Poultry by-products meal was air-classified at the rendering plants operated by Rothsay in Ontario in April 2011. Samples of the original (regular) poultry by-products meal, the low ash fraction (low ash poultry by-products meal) and the high ash fraction (poultry bone meal) separated by air-classification were collected and are currently being characterized and use in some assays.

Objective 1.3 To carry out digestibility trials with rainbow trout to determine the digestibility of nutrients in different fractions of meat and bone meals and poultry by-products meal obtained by air-classification.

Progress: To objective was abandoned for the time being due to heavy investment in order parts if the project.

Objective # 2: Strategies for the Improvement of the Phosphorus and Mineral Digestibility in Rendered Animal Protein Ingredients

Team members: Dr. Yang Yuhong, visiting professor (China) (Aug 2010-Jul 2011)
Dr. Chungfan Cai, visiting professor (China) (Jan-Dec 2012)
Miss Anne-Sophie Lemoine, visiting MSc student (France) (Jan-Jun 2013)
Mr. Patricio Saez, PhD student
Mr. Jorge Castillo, Industry consultant

Objective 2.1 Validation of an *in vitro* P Bio-Availability Assay

The project initially focused on setting up and validating a total phosphorus (P) determination assay based on published method. Some modifications were applied to the method to make it more reliable for rendered animal protein ingredients. The project then focused on adaptation and validation of a popular assay: the *in vitro* phosphorous bio-availability assay (PBA assay) developed by Sullivan et al. (1992). The PBA is a tool that we hoped would allow us to rapidly screen and/or predict the *in vivo* digestibility of P of feed ingredients developed in this project.

After a few months of working with the PBA assay, we determined that it is not suitable for estimating the bio-availability of P in low P ingredients, such as blood meal and feather meal. For higher ash ingredients, preliminary results indicated that grinding had a very significant ($p<0.05$) positive effect on the PBA values of (Table 8). This suggested that "particle size" has a significant effect on the estimate of P bioavailability obtained with the PBA assay. This particle size effect is not expected to be physiologically meaningful since grinding has been shown to have no effect on apparent digestibility of P in *in vivo* digestibility trials with salmonids (Lall,

1991). However, efforts should be invested in more precisely examining the effect of grinding on P bio-availability in different fish species.

We decided to carry out a very comprehensive study to characterize the effect of particle size on estimates of P bioavailability obtained with the PBA assay. This part of the project required close to 12 months of work by the team. It requires extensive sample preparation, as well as development, modification and evaluation of different experimental protocols. It also required characterization of particle size by image analysis, which was extremely time-consuming.

There seems to be a very strong effect of grinding (particle size) within same batch of ingredient (Table 9). However, across batches of similar ingredients, the effect of particle size is not consistent. In the case of poultry by-products meals (meals with P content < 3%), there does not appear to be any significant relationship between particle size and *in vitro* P bioavailability (Figure 1). Conversely, for poultry bone meal (meals with P content > 6%), a very strong negative association between particle size and *in vitro* P bioavailability is noted (Figure 2).

These inconsistencies and potentially significant "methodological artifact" appear to severely limit the value of the PBA assay to predict the bio-availability of P *in vivo*. However, the PBA assay can probably be used as a screening tool to determine the effectiveness of "treatment" within batches of the same ingredient (see Milestone #6 below). However, it is clear that when taken alone, *in vitro* P bioavailability estimates do not appear to mean much. More efforts should be invested in *in vivo* assessment of P bioavailability (through digestibility trials or growth trials) which is known to produce more reliable and meaningful results than *in vitro* assays. We are currently preparing a scientific manuscript on these novel and interesting results.

Objective 2.2 Lab-Bench Scale Experiments to Examine Effects of Different Treatments on Digestibility of P in Poultry By-Products Meal

A series of lab bench experiments (general approach is described in Figure 3) were carried out between January and November 2012 to explore the effects of different factors and reagents on the bio-availability of P in PBM. It was decided that the use a simple water solubilisation technique to determine the amount of soluble P that was freed up by the different treatments since we believe that this technique may more realistically estimate the digestible (available) P content of the PBM and meat and bone meals for agastric fish species (carps) than the PBA assay described and used in Objective 2.1.

This series of lab bench experiments used the same test ingredient, a relatively high ash poultry by-products meal (PBM) with approximately 2.6% phosphorus (as is basis) obtained from Rothsay in April 2011 (from a single production lot). We estimated that a very large proportion of the P content of this PBM is as bone-P (hydroxyapatite).

The first series of experiments examined the effect of different concentration of citric acids and of a chelator (EDTA). The effect of incubation period (time) and temperature were examined. The results suggest that the use of EDTA in combination with citric acid appeared to

be highly effective at solubilising P. A simple graphical analysis suggests that EDTA and citric acid do not appear to act in synergy but rather have additive effects (Figure 4). The use of combinations of citric acid (0 to 10%) and EDTA (0 to 9%) (expressed as % of total PBM weight, as is) resulted in an increase in the soluble P level from 0.25% to about 1.1%. This suggests that citric acid and EDTA combinations could potentially improve the availability of P in PBM to agastric fish species from less than 10% to more than 40%.

Another series of experiments compared different organic acids (or source thereof) under standardized conditions (3h at 50°C, 3.8% EDTA). These experiments suggest that citric, tartaric, oxalic formic acids were some the most efficient organic acids for freeing phosphorus (Figures 5 and 6). Lactic, malic and acetic acid had moderate effects while benzoic and sorbic acids did not prove to be effective. Corn steep liquor, which we had hope to use as a source of organic acid in our initial experimental plan, did not prove effective at solubilising phosphorus

Of all the compounds tested, citric acid and EDTA appears to have the greatest potential due to their reasonable cost, and GRAS (Generally Recognized As Safe) classification by the US Food and Drug Administration (FDA) and because they are registered feed ingredients in Canada (albeit with significant restrictions).

The solubilisation of P by citric acid and EDTA treatment appeared to be very rapid (less than 1 h) (Figure 7). Increasing temperature (20-70°C) only had a very small effect on the solubilisation of P, regardless of the type of organic acid used (Figure 7) and long incubation periods (up to 200 h incubation time) did not yield any improvement in P solubility (results not shown). A moisture level of at least 30-40% is required to obtain repeatable results when citric acid and EDTA are added as dry powders (Figure 6). This result was confirmed by a kinetic study of six (6) selected low molecular weight organic acids (LMWOAs), in which a decreased P dissolution rate was observed when moisture decreased from 65% to 40% (Figure 6). The incubation of PBM with a broad action protease in combination with citric acid and EDTA had no effect of the release of soluble P (results not shown).

A scientific paper with detailed information on the multiple lab-bench trials conducted by Prof. C.F. Cai is under preparation. We have completed a first draft and are hoping to complete the preparation of the manuscript in June 2014 and submit it for publication in a scientific journal.

Objective 2.3 Cost-Effectiveness of Production of High Digestible Available P PBM

A visiting MSc student (Anne-Sophie Lemoine) from France was recruited to work on this objective of this multi-year project from January to August 2013. The student carried out a brief technical and economic analysis using the data generated by a series of lab-bench experiments. The goal of this economic analysis is to determine the most cost-effective processing conditions (i.e. optimal combinations of parameters from nutritional and economical perspectives).

The student also carried out a series of bench-scale and pilot-scale trials aimed at producing high bio-available phosphorus poultry by-products meal (Table 10) on the basis of the work done in Objective 2.2 (schematic overview presented in Figure 3).

In the first bench scale study, citric, formic acid were used to optimize the incubation conditions of a high ash poultry by-product meal in order to obtain the highest phosphorous digestibility. The effect moisture content, addition of EDTA (0.1 and 0.2%) and temperature (25, 50 and 75 °C) were assessed. Moisture of the mixture did not affect phosphorus solubility regardless of organic acid (citric acid or formic acid) used for incubation. Incubation temperature affected phosphorus solubility when formic acid was used but had no impact when using citric acid. The optimum temperature was apparently around 25 °C (Fig. 8). EDTA inclusion when incubating with formic acid was about 1.25g per 100 g of PBM. Although no statistical significant influence of an EDTA addition was highlighted for the citric acid incubation, the highest P solubility of the citric acid incubations treatment was obtained when EDTA inclusion level was the highest (3.7g of EDTA per 100g of PBM) (Figure 9). The highest P solubility was obtained at the level of 0.44g formic acid per 100g PBM (Figure 10) and 4.80g citric acid per 100g PBM, respectively (Figure 11). Results from this trial indicated that with optimal combination incubation conditions (Table 11), the digestibility of P in high ash poultry-by product could reach values as high as 83% with formic acid and as high as 65% with citric acid.

An economic analysis carried out to assess the cost effectiveness of available P of poultry bone meal (PBM), meat and bone meal (MBM) and inorganic P supplements was assessed. The available P costs are different between the different ingredients. This cost ranges from less than 1$/kg of available P for sodium dihydrogen phosphate to more than 90$/kg of available P for the non-incubated PBM (Table 12). Based on chemical and conditions required for the incubation process, the cost of incubation process was estimated to be significantly higher than the cost of inorganic P supplement. The results of a preliminary economic analysis suggested that at current price, inorganic P supplements are more cost-effective than would be incubated PBM and MBM. However, PBM and MBM are significant sources of protein, energy, amino acids, fatty acids, etc. Consequently, this needs to be taken in consideration in order to realistically determine the cost-effectiveness of "incubated" PBM and MBM. Therefore, a series of practical (commercial-type) feeds were formulated based on the nutritional requirements of rainbow trout, Nile tilapia and carp. Diets contain or not incubated ingredients and were supplemented to contain NaH_2PO_4 or $Ca(H_2PO_4)_2$ when the formulated diet was predicted to be deficient in digestible P. Although the inclusion of incubated PBM/MBM in fish diet has for consequences an improvement of dietary P digestibility it did not permit the deletion of dietary inorganic P supplementation.

Results from this simulation indicated that depending on the ability of fish to digest the different chemical forms of P, type of animal product (PBM or MBM) and inorganic P supplementation, it is possible to find ingredient combinations for which "incubated PBM or MBM" containing diets could be less expensive than diet formulated with regular PBM/MBM and inorganic P supplements, notably for carp species which lack a true (acid) stomach. However, this price difference did not exceed 10$/MT when the raw material prices ranged between 400 and 1000$/MT. This was only a simulation and *in vivo* experimentations and a real technical and economic analysis must be conducted in order to quantify the impact of incubated PBM/MBM on growth performance of the fish, notably with carp species.

This work was the focus of a document prepared by the MSc student who submitted it for the needs of her MSc thesis at Wageningen University in the Netherlands. A copy of the document is available upon request.

Objective 2.4 In Vivo Assessment of the Digestibility of Incubated PBM and its Effects of Growth Performance

A pilot-scale trial to produce high bio-available phosphorus poultry by-products meal was conducted in the summer of 2013 on the basis of the work done in Objective 2.2.1. Several kg of this novel ingredient were produced. This batch of the novel ingredient was used in a growth-digestibility trial with rainbow trout carried out at the Fish Nutrition Research Laboratory.

The results of the digestibility trial and growth trial are reported in the attached manuscript draft. The results of the trial clearly indicated that in rainbow trout organic acid incubation of PBM offered no advantage compared to no incubation in terms of digestibility of phosphorus and growth performance.

We had hoped to carry out this type of research with an agastric species (e.g. common carp) but obtaining fish for this trial in Canada proved difficult. Similar study may be carried out in Asia in the near future.

Tables and Figures

Table 1. Proximate analysis of the seven animal by-products characterized as part of Objective #1.1 (values reported on an "as is" basis).

Ingredients	Dry Matter	Crude protein	Crude lipid	Ash	NFE	Gross Energy
			%			kJ g^{-1}
Fish meal, herring	95	66	16	15	2	21
Meat and bone meal	96	48	12	29	11	17
Poultry by-products meal, low ash	98	68	18	10	4	24
Poultry by-products meal, high ash	97	68	14	15	4	22
Hydrolyzed feather meal	93	90	2	2	6	22
Spray-dried blood meal	91	93	1	4	2	22
Porcine meat meal	97	60	14	18	8	20

Table 2. Elemental composition of the seven animal by-products characterized in Objective #1.1.

Ingredients	C	N	P	Ca	Mg	K	Na	S	Fe	Zn	Cu	Ni
						% DM						
Fish meal, herring	48.5	11	2.5	4.1	0.1	0.9	0.5	0.7	0.04	0.007	0.002	0.0001
Meat and bone meal	37.9	8	4.0	10.4	0.2	0.6	0.9	0.4	0.02	0.006	0.018	0.0001
Poultry by-products meal, low ash	51.0	11	1.7	2.3	0.2	0.9	0.6	0.7	0.04	0.007	0.002	0.0001
Poultry by-products meal, high ash	48.6	11	2.6	4.2	0.2	0.9	0.6	0.7	0.03	0.007	0.002	0.0003
Hydrolyzed feather meal	50.4	16	0.2	0.4	0.1	0.1	0.1	2.2	0.02	0.010	0.001	0.0006
Spray-dried blood meal	51.0	16	0.4	0.0	0.0	1.2	0.4	0.5	0.31	0.002	0.001	0.0004
Porcine meat meal	43.7	10	3.4	5.9	0.2	0.7	0.6	0.5	0.04	0.012	0.006	0.0002

Table 3. Essential amino acids (EAAs) composition of the seven animal by-products characterized as part of Objective #1.1.

		Essential Amino Acids									
Ingredients	**Total EAA**	**ARG**	**HIS**	**ILE**	**LEU**	**LYS**	**MET**	**PHE**	**THR**	**VAL**	**TRP**
						% DM					
Fish meal, herring	28.8	4.8	1.5	2.6	4.7	3.9	1.4	2.7	2.7	3.3	1.1
Meat and bone meal	18.8	3.4	1.1	1.5	3.2	2.5	0.9	1.8	1.9	2.1	0.4
Poultry by-products meal, low ash	30.1	5.1	1.6	2.4	5.1	4.3	1.6	2.9	3.1	3.2	0.7
Poultry by-products meal, high ash	29.0	5.0	1.5	2.4	4.9	4.2	1.5	2.7	2.9	3.2	0.7
Hydrolyzed feather meal	37.5	6.4	0.7	4.3	7.2	2.7	0.6	4.3	4.2	6.5	0.6
Spray-dried blood meal	46.8	3.6	6.7	0.3	11.5	7.0	0.8	6.1	2.8	6.6	1.3
Porcine meat meal	26.3	5.2	1.3	2.4	4.2	3.8	1.2	2.4	2.3	3.0	0.4

Table 4. Non-essential amino acids (NEAA), taurine (Tau) and hydroxyproline (HyP) of the seven ingredient tested as part of Objective 1.1.

Ingredients		Non-Essential Amino Acids									
	Total NEAA	ALA	ASP	CYS	GLU	GLY	PRO	SER	TYR	Tau	HyP
	% DM					% DM					
Fish meal, herring	39.0	4.4	5.5	0.6	8.9	7.2	4.6	2.9	1.9	0.45	2.7
Meat and bone meal	30.1	3.4	3.9	0.5	6.4	6.0	3.8	2.2	1.2	0.11	2.5
Poultry by-products meal, low ash	41.1	4.6	6.1	0.6	9.7	6.8	4.6	3.5	2.4	0.55	2.3
Poultry by-products meal, high ash	41.2	4.7	5.9	0.6	9.5	7.3	4.7	3.2	2.2	0.50	2.7
Hydrolyzed feather meal	53.0	3.8	5.9	4.0	9.7	6.8	10.0	10.1	2.5	0.03	0.1
Spray dried blood meal	38.0	6.9	10.3	0.5	7.0	4.3	2.8	4.2	1.8	0.01	0.1
Pork meat meal	31.8	4.2	4.9	0.6	8.0	5.8	4.0	2.4	2.0	N/A	N/A

Table 5. Non-protein nitrogen compounds (NPN) measured in the seven ingredient tested as part of Objective 1.1.

Ingredients	Total NH_3	Total DNA	Total RNA[1]	Total N from NPN
	ug/g	ug/g	ug/g	% DM
Fish meal, herring	648	74	128	0.06
Meat and bone meal	286	141	245	0.03
Poultry By-product meal, low ash	545	69	119	0.05
Poultry By-product meal, high ash	607	46	81	0.05
Feather meal, steam hydrolyzed	2000	5	9	0.16
Spray dried blood meal	152	10	18	0.01
Pork meat meal	436	87	152	0.04

Table 6-1. Total nitrogen (TN), TN from essential amino acids (EAA), TN from non-essential amino acids (NEAA) and total NPN in the selected ingredients (% DM) as part of Objective 1.1.

Ingredients	Total N from Protein	EAA-N	NEAA-N	Total NPN[1]		Difference between the N balance	Percentage of missing N
	% DM	% DM	% DM	% DM	% of Total N	% DM	%
Fish meal, herring	11.1	4.7	4.9	0.06	0.51	1.42	12.8
Meat and bone meal	8.0	3.2	3.9	0.03	0.37	0.90	11.3
Poultry by-products meal, low ash	11.2	4.9	5.1	0.05	0.43	1.02	9.1
Poultry by-products meal, high ash	11.2	4.8	5.2	0.05	0.46	1.16	10.4
Hydrolyzed feather meal	15.6	5.8	6.6	0.16	1.06	3.02	19.4
Spray-dried blood meal	16.4	7.5	4.8	0.01	0.08	4.20	25.6
Porcine meat meal	9.9	4.5	5.1	0.04	0.40	0.27	2.7

[1]C from DNA is calculated as 53% C in one DNA mmolecule.

[2]Calculated as 1.74*DNA

Table 6-2. Total nitrogen (TN), TN from essential amino acids (EAA), TN from non-essential amino acids (NEAA) and total NPN in 81 ingredients (% DM) as part of Objective 1.1.

Ingredients	Total N from Pr.	EAA-N	NEAA-N	Total NPN		Difference between the N balance	Percentage of missing N
	% DM	% DM	% DM	% DM	% of total N	% DM	%
Fish meal, high protein	10.6	4.7	4.9	0.06	0.53	0.88	8.3
Danish fish meal	12.1	5.2	4.1	0.05	0.41	2.67	22.1
Peruvian fish meal	12.1	4.9	6.5	0.05	0.41	0.67	5.5
Fish meal, generic	12.0	5.2	4.5	0.05	0.42	2.24	18.6
fishmeal LT-70	11.3	5.0	4.0	0.05	0.44	2.21	19.6
Fish meal, 70% CP, low temperature	11.3	4.3	3.5	0.05	0.44	3.41	30.3
Fish meal, cod, processing waste	11.2	5.8	3.9	0.05	0.45	1.38	12.3
Fish meal, herring, 70% CP	11.2	5.0	4.0	0.05	0.45	2.22	19.8
Fish meal, Alaskan pollock, processing waste	11.3	5.3	3.9	0.05	0.44	2.11	18.7
Fish meal, processing by-products, NOAA	11.0	5.3	3.9	0.05	0.45	1.76	16.0
Fish meal, mackerel	11.1	5.0	4.1	0.05	0.45	1.91	17.2
Fish meal, salmon, mechnically extracted	10.8	5.1	3.9	0.05	0.46	1.78	16.6
Fish meal, anchovy	10.9	7.4	3.0	0.05	0.46	0.41	3.8
Fish meal, sardine	10.7	4.8	3.9	0.05	0.47	2.01	18.7
Fish meal, white, mechanically extracted	10.6	4.6	3.9	0.05	0.47	2.00	19.0
Fish meal, freshwater alewife	10.4	4.5	3.5	0.05	0.48	2.33	22.4
Fish meal, not specified	10.5	4.4	3.8	0.05	0.48	2.35	22.4
Fish meal, menhaden, Special Select	10.2	4.6	3.9	0.05	0.49	1.58	15.5
Fish meal, red fish	10.1	4.5	3.9	0.05	0.49	1.67	16.5
Indian fish meal	9.8	5.0	3.5	0.05	0.51	1.25	12.8
Fishmeal 60	9.9	4.4	4.9	0.05	0.50	0.59	6.0
Fish meal, tuna, mechanically extracted	9.6	4.2	3.4	0.05	0.52	1.90	19.8
Fish meal, 54% CP, not specified	9.3	4.0	3.3	0.05	0.54	1.89	20.3
Fish meal, low protein	8.6	3.8	3.3	0.05	0.58	1.54	17.8
Fish protein concentrate	7.7	3.3	2.9	0.05	0.65	1.58	20.3
Fish protein concentrate, CPSP	13.3	6.9	5.6	0.05	0.38	0.67	5.1
Fish solubles, dried	13.1	5.8	5.0	0.05	0.38	2.32	17.7
Fish solubles, dried	10.9	4.7	4.8	0.05	0.46	1.29	11.9
Fish solubles, dehydrated	10.3	3.5	3.6	0.05	0.49	3.14	30.5
Fish solubles, dehydrated	8.9	3.3	3.2	0.05	0.56	2.40	26.8
Fish solubles, condensed	6.4	1.8	2.4	0.05	0.78	2.22	34.6
Fish silage, salmon, spray-dried	5.1	1.8	2.0	0.05	0.99	1.23	24.3
Fish silage, high oil	9.6	5.1	1.5	0.05	0.52	3.02	31.5
Fish silage, low oil	9.0	5.2	4.2	0.05	0.56	-0.42	-4.7
Fish silage, salmon process waste, wet	7.3	3.4	3.3	0.05	0.68	0.64	8.7
Fish silage, dogfish	4.8	2.2	2.2	0.05	1.04	0.31	6.4
Krill meal	2.4	1.4	1.3	0.05	2.07	-0.31	-12.9
Krill meal	9.2	5.2	3.2	0.05	0.54	0.75	8.1
Shrimp meal, by- products	9.6	4.5	3.4	0.05	0.52	1.70	17.7
Shrimp head meal	6.1	2.5	2.0	0.05	0.81	1.65	26.9
Shrimp meal, whole	6.5	3.1	2.4	0.05	0.77	0.94	14.5
Shrimp meal,dried	10.0	7.6	3.3	0.05	0.50	-0.98	-9.7
Squid meal	7.0	3.0	2.2	0.05	0.72	1.67	23.9
Squid meal	11.5	5.2	4.2	0.05	0.43	2.07	18.0
Spray-dried blood meal	12.6	7.5	4.8	0.05	0.40	0.31	2.4
Blood meal	14.9	7.5	4.8	0.01	0.09	2.71	18.1
Blood cell meal, dried	15.1	8.1	5.3	0.05	0.33	1.67	11.1
Blood cell meal, flash dried	13.2	7.0	4.7	0.05	0.38	1.50	11.3
Blood meal, spray dried	11.5	2.5	4.0	0.05	0.43	4.94	42.9
Porcine blood meal	13.8	6.1	4.9	0.05	0.36	2.75	20.0
Hydrolyzed feather meal	14.4	7.1	4.6	0.05	0.35	2.62	18.2

Feather meal	14.4	5.8	6.6	0.16	1.14	1.85	12.8
Feather meal, hydrolyzed	13.7	5.2	6.1	0.05	0.36	2.30	16.8
Feather meal, steam hydrolyzed	13.1	4.4	5.2	0.05	0.38	3.45	26.3
Feather meal, generic	13.2	4.7	5.6	0.05	0.38	2.78	21.1
Meat and bone meal	13.2	4.8	5.4	0.05	0.38	2.88	21.9
Meat and bone meal, 37% CP	7.7	3.2	3.9	0.03	0.39	0.61	8.0
Meat and bone meal, 45% CP	5.9	4.4	3.3	0.05	0.84	-1.84	-31.0
Meat and bone meal, 50% CP	7.2	2.9	3.3	0.05	0.69	0.93	12.8
Meat and bone meal, 55% CP	8.0	3.2	3.8	0.05	0.62	1.01	12.5
Meat and bone meal, high fat	8.7	3.3	4.0	0.05	0.58	1.35	15.5
Meat and bone meal, 60% CP	8.8	3.4	4.0	0.05	0.57	1.38	15.7
Meat and bone meal, low fat	9.7	4.1	4.4	0.05	0.51	1.16	12.0
Meat meal, generic	9.9	3.9	4.5	0.05	0.50	1.45	14.6
Porcine meat meal	8.9	4.8	4.1	0.05	0.56	-0.06	-0.7
Poultry by-products Meal, low ash	9.6	4.5	5.1	0.04	0.42	-0.05	-0.5
Poultry by-products meal, high ash	10.9	4.9	5.1	0.05	0.44	0.80	7.3
Poultry by-product meal	10.9	4.8	5.2	0.05	0.48	0.85	7.8
Poultry meal, 65% CP	11.7	6.0	4.7	0.05	0.43	1.08	9.2
Poultry by-product meal	10.6	3.2	3.4	0.05	0.47	3.92	37.1
Poultry by-product meal, feed grade low ash	10.4	0.6	3.9	0.05	0.48	5.87	56.2
Poultry by-product meal	9.9	4.2	3.8	0.05	0.50	1.95	19.6
Poultry offal meal	9.7	4.3	4.0	0.05	0.52	1.28	13.3
Milk, skimmed, powder	9.6	3.8	4.0	0.05	0.52	1.84	19.1
Milk, whole, dried	5.3	2.5	1.8	0.05	0.95	0.92	17.4
Whey, dried	3.9	1.8	1.4	0.05	1.27	0.67	17.0
Whey, dried, low-lactose	2.2	1.3	0.7	0.05	2.28	0.14	6.4
Whey, permeate, dried	2.2	1.0	0.7	0.05	2.30	0.47	21.8

Table 7. Total carbon (TC), TC from essential amino acids (EAA), non-essential amino acids (NEAA), carbohydrate (CHO), fat, DNA and RNA in the selected ingredients (% DM) as part of Objective 1.1.

Ingredients	Total C % DM	CHO % DM	Fat % DM	EAA-C % DM	NEAA-C % DM	CHO-C[1] %DM	Fat-C[2] %DM	DNA and RNA-C[3] % DM	Difference between the C balance % DM	Percentage of missing C %
Fish meal, herring	48.5	2.3	16.4	14.5	15.7	1.0	12.6	0.01	4.68	9.6
Meat and bone meal	37.9	11.2	12.3	9.4	12.1	4.9	9.5	0.02	1.94	5.1
Poultry by-products meal, low ash	51.0	3.7	17.7	15.0	16.6	1.6	13.6	0.01	4.14	8.1
Poultry by-products meal, high ash	48.6	3.7	13.5	14.5	16.6	1.6	10.4	0.01	5.51	11.3
Hydrolyzed feather meal	50.4	5.9	2.3	19.1	21.2	2.6	1.8	0.00	5.74	11.4
Spray-dried blood meal	51.0	1.7	1.1	24.3	14.9	0.7	0.8	0.00	10.20	20.0
Porcine meat meal	43.7	8.4	13.7	13.1	n/a	3.7	10.5	0.01	n/a	n/a

[1]C from CHO is calculated as 44% C.

[2] Values were calculated from Table 7.

[3]C from fat is calculated as 77% C.

Table 8. Results of preliminary study on the effect of grinding on the bioavailability P content (estimated with the *in vitro* PBA assay) of animal protein ingredients before or after grinding (Objective 2.1)

Ingredients	Original Particle Size	After Grinding
	%	
Poultry bone meal (Batch 1)	4.32 ± 0.02^{B}	6.53 ± 0.07^{A}
Poultry bone meal (Batch 2)	4.39 ± 0.02^{B}	5.59 ± 0.27^{A}
Low Ash Poultry By-Products Meal	1.37 ± 0.03^{A}	1.32 ± 0.02^{A}
Regular Poultry By-Products Meal	1.7 ± 0.00^{A}	1.69 ± 0.00^{A}

Values in the same row sharing the same subscript are not significantly different

Table 9. Bio-availability of P (estimated with the *in vitro* PBA assay) of different animal by-products prior and after grinding (Objective 2.1)

Ingredient	Size	Total P %	Bioavailable P %	P Bioavailability %	Mean particle size mm^2
Fish meal, herring W	Original	2.75±0.12	1.93±0.12	70^{A}	0.06
	Ground	2.78±0.06	2.02±0.03	73^{A}	0.06
Fish meal, herring Y	Original	2.83±0.15	1.70±0.06	60^{B}	0.22
	Ground	2.84±0.49	1.96±0.05	69^{A}	0.18
Fish meal, herring Z	Original	2.76±0.18	1.70±0.04	65^{B}	0.26
	Ground	2.72±0.01	1.91±0.02	70^{A}	0.09
Meat bone meal Q	Original	3.75±0.48	2.27±0.23	61^{B}	0.23
	Ground	3.68±0.06	2.70±0.06	73^{A}	0.19
Poultry bone meal L	Original	7.04±0.30	5.12±0.13	73^{B}	0.14
	Ground	7.00±0.11	5.42±0.09	77^{A}	0.10
Poultry bone meal M	Original	6.56±0.37	4.49±0.29	68^{B}	0.20
	Ground	6.85±0.16	5.72±0.12	83^{A}	0.08
Poultry bone meal N	Original	6.91±0.36	4.66±0.19	67^{B}	0.18
	Ground	7.11±0.02	5.95±0.10	84^{A}	0.07
Poultry by-products meal A	Original	2.71±0.02	1.72±0.03	63^{B}	0.14
	Ground	2.64±0.03	1.89±0.10	71^{A}	0.12
Poultry by-products meal B	Original	2.56±0.49	1.58±0.07	62^{B}	0.19
	Ground	2.53±0.01	1.92±0.06	76^{A}	0.13
Poultry by-products meal C	Original	2.66±0.27	1.74±0.11	65^{B}	0.17
	Ground	2.67±0.08	2.00±0.05	75^{A}	0.16
Poultry by-products meal D	Original	2.88±0.10	1.87±0.04	65^{B}	0.14
	Ground	2.70±0.07	1.96±0.02	73^{A}	0.10
Poultry by-products meal E	Original	2.97±0.13	1.58±0.10	53^{B}	0.12
	Ground	2.65±0.11	2.01±0.12	76^{A}	0.07

Table 10. Proximate composition of the high-ash poultry by-product meal (Objective 2.3)

Nutrients	g/kg
Moisture	29
Crude Protein	693
Lipid	113
Ash	143
Total P	24

Table 11. Optimum incubation conditions depending on the type of incubation (Objective 2.3)

Type of incubation	Acid (g/100 g PBM)	EDTA (g/100 g PBM)	Temperature (°C)	Moisture Content (%)
Formic Acid	0.45	1.85	25	31
Citric acid	4.63	3.70	50	31

Table 12 : Available P costs ($/kg of available P) depending on different P sources and fish species (Objective 2.3)

Ingredients	Rainbow Trout	Common Carp	Nile Tilapia
Poultry by-product meal			
Non-incubated PBM	69.02	103.10	48.62
Citric acid incubated PBM	22	40.66	27.11
Formic acid incubated PBM	24.89	49.45	29.17
Meat and bone meal			
Non-incubated MBM	83.29	82.99	72.22
Citric acid incubated MBM	27.71	33.52	20.65
Formic acid incubated MBM	28.71	40.18	24.29
Inorganic P supplement			
Ca monobasic phosphate	3.72	3.85	3.56
Ca dibasic phosphate	3.34	3.34	3.34
Monopotassium phosphate	2.97	3.07	2.84
Sodium dihydrogen phosphate	0.98	1.01	0.93

Figure 1. In vitro bio-availability of P of poultry by-products meals (Total P = 2.5 to 3.0%) with different average particle sizes (Objective 2.1)

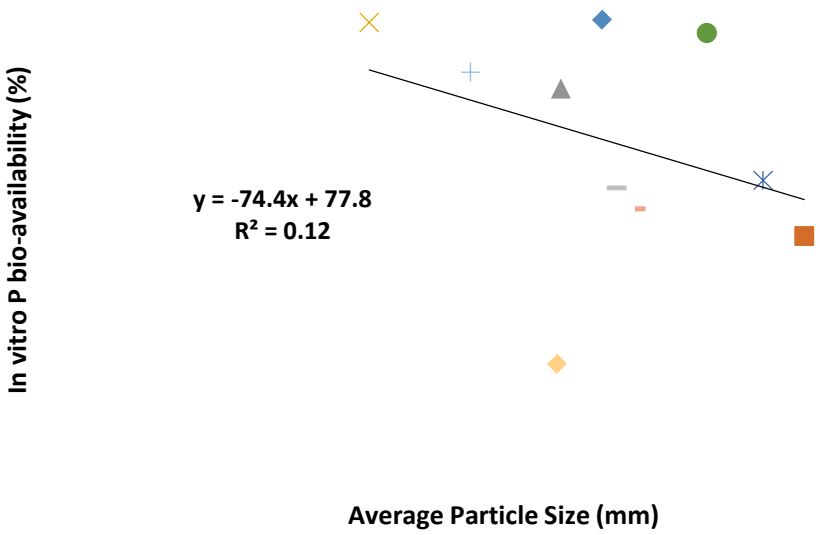


Figure 2. In vitro bio-availability of P of poultry bone meals (Total P = 6.6 to 7.1%) with different average particle sizes (Objective 2.1)

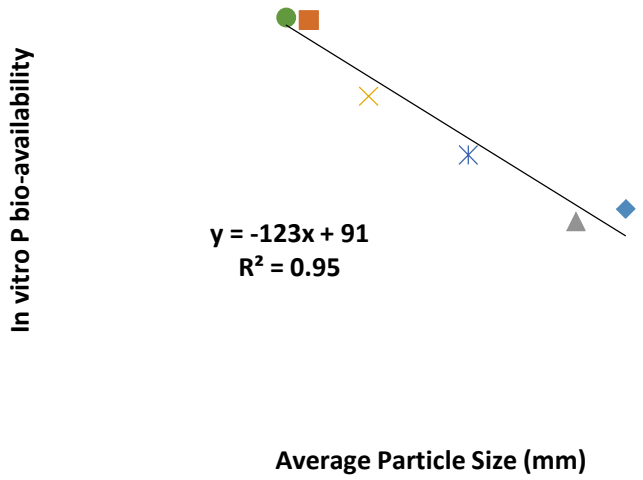


Figure 3. General approach used in the incubation trials (Objective 2.2).

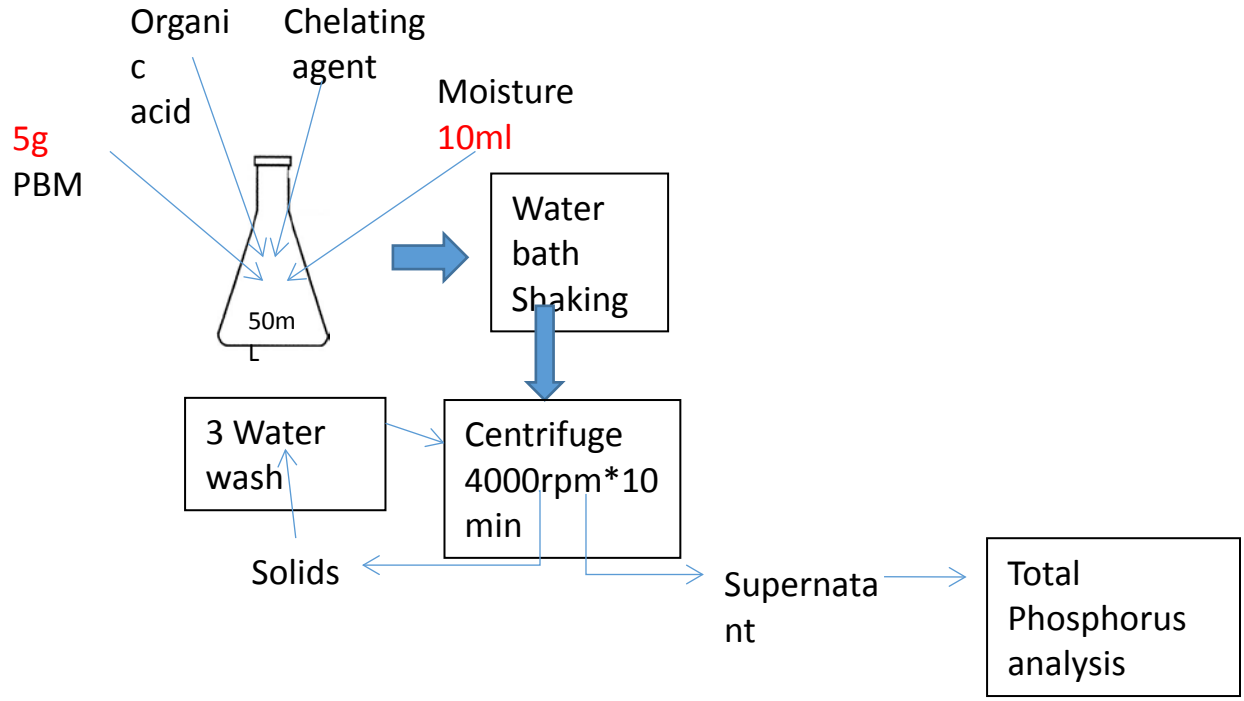


Figure 4. Effects of citric acid concentration (% w/w) on the amount of phosphorus solubilized from poultry by-products meal in the presence or absence of EFDA (Objective 2.2).

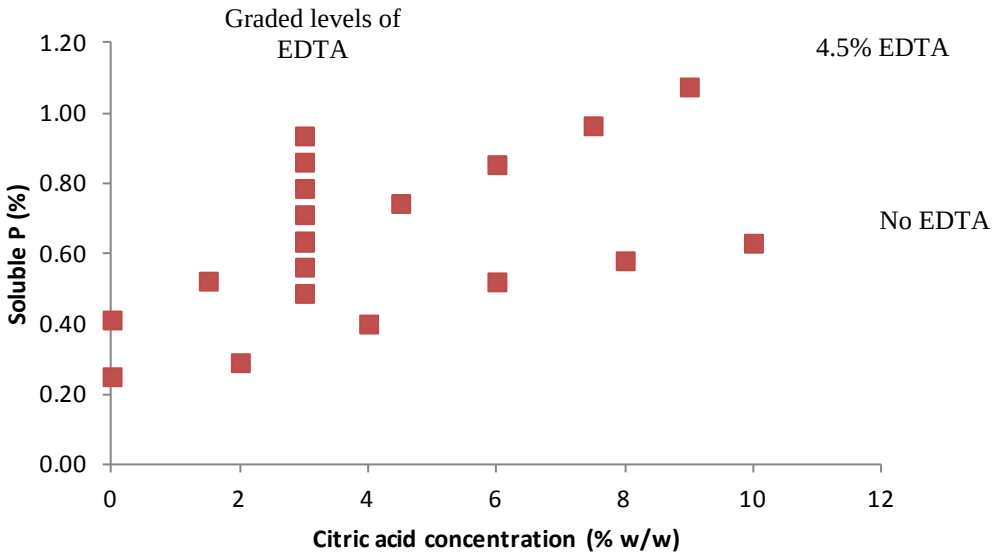


Figure 5. Soluble P obtained after incubation with 10 g 100 g-1 of acetic acid (Ace), benzonic acid (Ben), butyric acid (But), citric acid (Cit), formic acid (For), fumaric acid (Fum), lactic acid (Lac), malic acid (Mal), oxalic acid (Oxa), propionic acid (Pro), sorbic acid (Sob), and tartaric acid (Tar) respectively in condition of 3.8 g 100g-1 of EDTA and 65g 100g-1 of system moisture

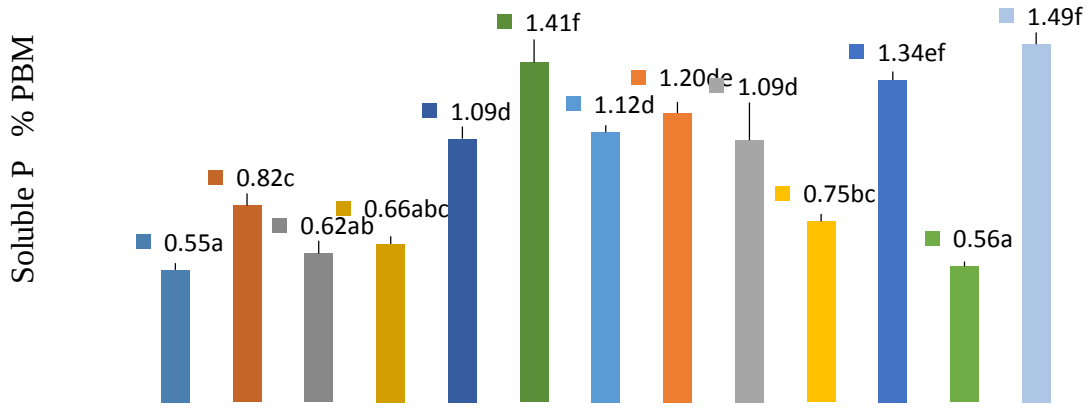


Figure 6. Effect of low molecular weight organic acids and moisture level on kinetic of phosphorus solubility from PBM.

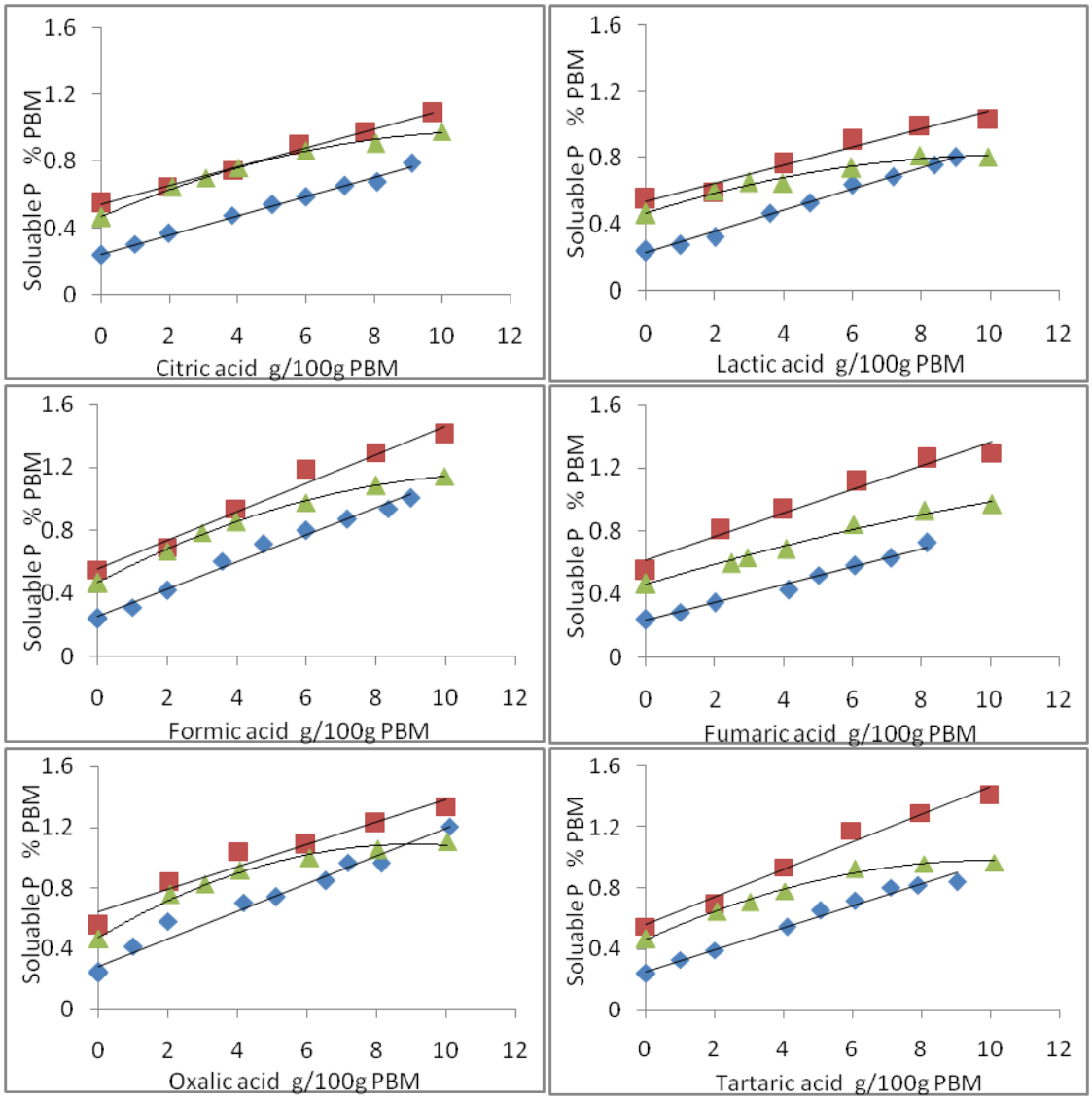


Figure 7. Effect of time, temperature, moisture and EDTA on bone P release from PBM

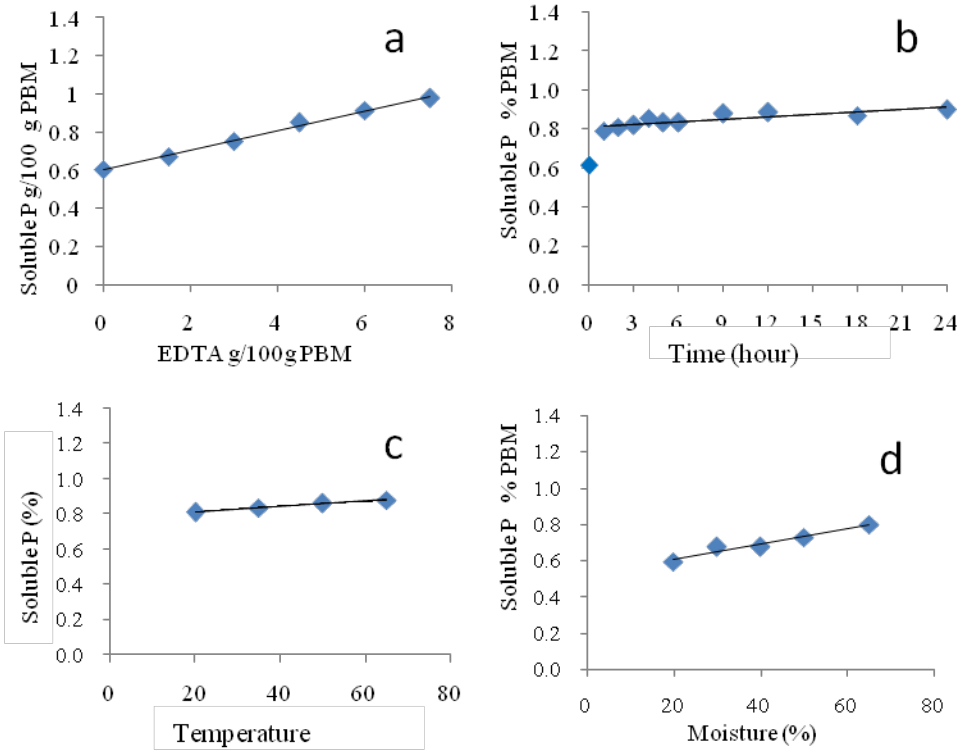


Figure 8: Incubation temperature (°C) and its consequences on P solubility

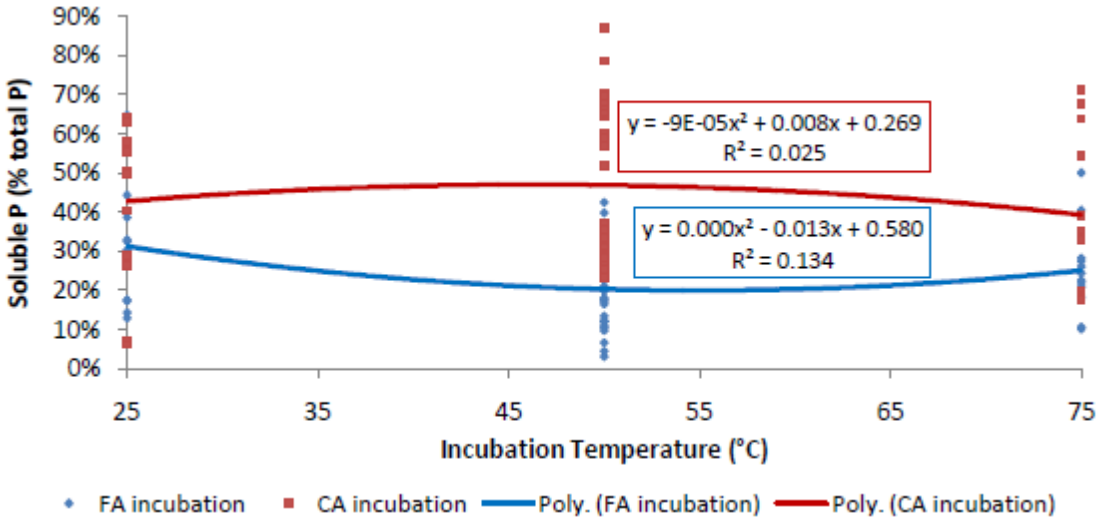


Figure 9: EDTA inclusion level (g/100 g PBM) and its consequences on P solubility

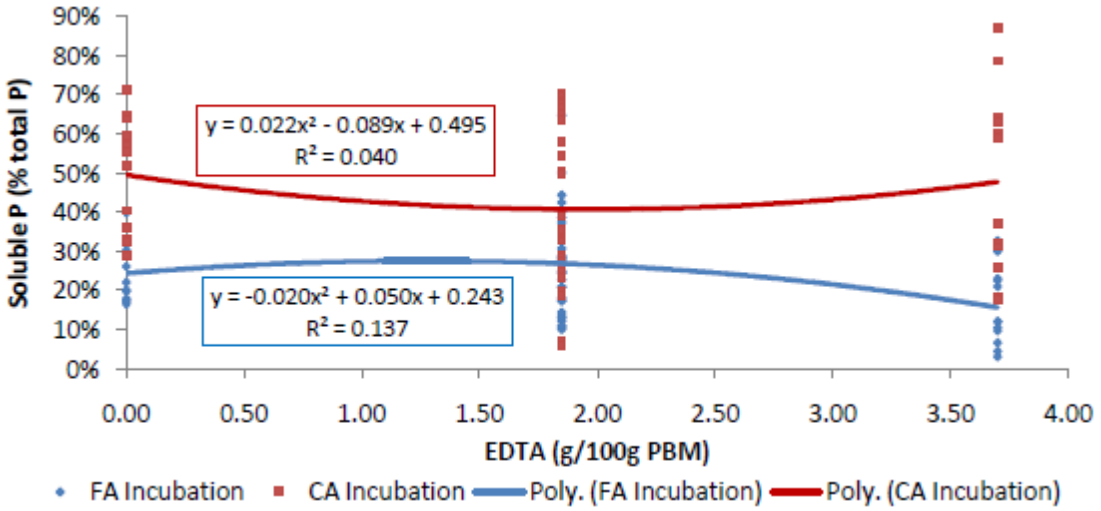


Figure 10: Soluble P (% total P) depending on the formic acid inclusion level (g/100g PBM)

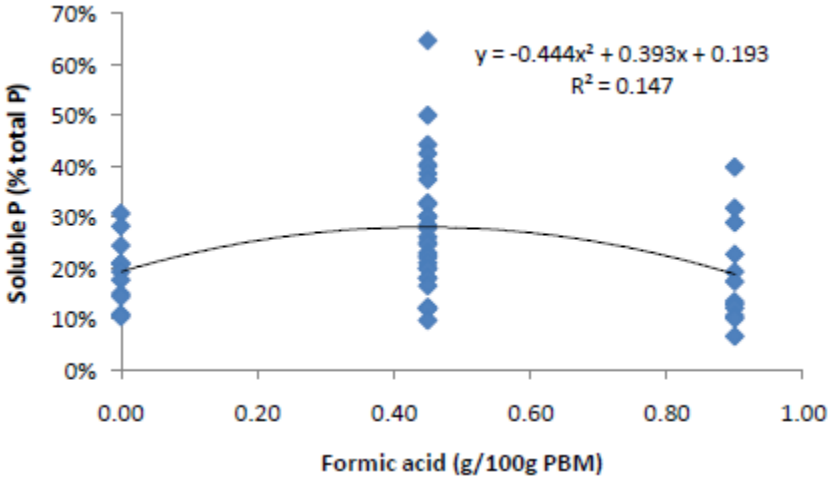
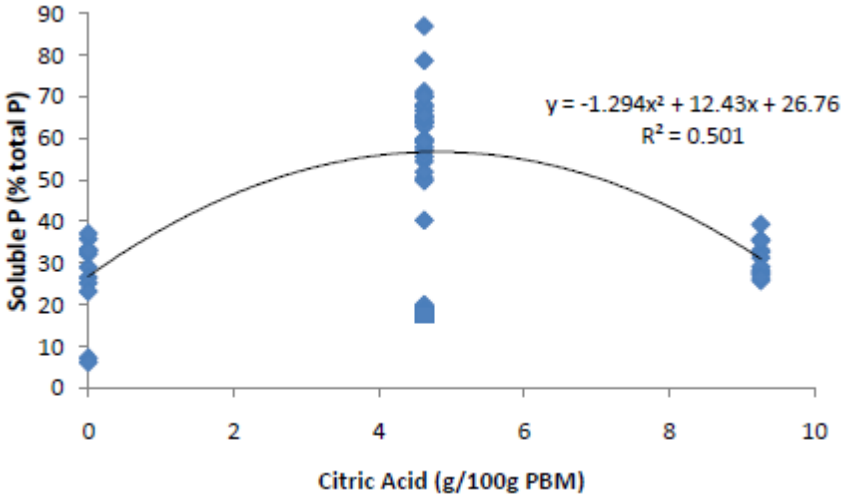


Figure 11: P solubility (% total P) depending on the inclusion level of citric acid



Publications

Published Peer-Reviewed Publications

Sarker, P.K., D.P. Bureau, K. Hua, M. D. Drew, I Forster, K. Were, B. Hicks and G.W. Vandenberg. 2013. Sustainability issues related to feeding salmonids: a Canadian perspective. Reviews in Aquaculture 5: 1–21.

Hua, K. and D.P. Bureau. 2012. Exploring the possibility of quantifying the effects of plant protein ingredients in fish feeds using meta-analysis and nutritional model simulation-based approaches. Aquaculture 356-357: 284–301.

Committee on Nutrient Requirements of Fish and Shrimp (R.W.Hardy, D.M. Gatlin, D.P. Bureau, L. Dabramo, D.A. Davis, J.E. Halver, A. Korgdahl, F. Medale, S.Y Shiau, D. Tocher) National Research Council. 2011. Nutrient Requirements of Fish and Shrimp. Animal Nutrition Series. National Academies Press, Washington, DC. (10 authors, one author faculty at the University of Guelph).

Hua, K. and D.P. Bureau. 2010. Quantification of differences in digestibility of phosphorus among cyprinids, cichlids, and salmonids through a mathematical modelling approach. Aquaculture 308: 152-158.

Poppi, D.A., M.V. Quinton, K. Hua and D.P. Bureau. 2011. Development of a test diet for assessing the bioavailability of arginine in feather meal fed to rainbow trout (*Oncorhynchus mykiss*). Aquaculture 314, 100-109.

Presentations

Bureau, D.P. 2014. Talking about nutrition. SalmoFood's Client Liaison Meetings, Puerto Varas, Chile, 10-11 April 2014 (presentation made four times to different client groups).

Bureau, D.P. 2014. Review of protein ingredients: What's limiting, what's new, where is research taking us? Workshop on Developing an Alternative Feeds Research Program Livestock Research Innovation Corporation (LRIC), Guelph, Ontario, Canada, 27 February 2014.

Bureau, D.P. 2013 Current issues with raw material availability and nutritive value in aquafeeds. Biomin's Asia Nutrition Forum, HCM City, Vietnam, 19 October 2013.

Bureau, D.P. 2013. Models as tools to improve efficiency of aquaculture operations. VI Symposium Nicovita, 17-18 April 2013, Santa Rosa,Ecuador.

Bureau, D.P. 2013. Nutrition as an important factor for environmentally sustainable aquaculture production. The Ninth Symposium of the World's Chinese Scientists on Nutrition and Feeding of Finfish and Shellfish (SWCSNFFS), Xiamen, P.R. China, 12-16 November 2013.

Bureau, D.P. 2013 Fish nutrition research updates. Martin Mills Cage Growers Meeting, 23

March 2013, Midland, Ontario.

Bureau, D.P. 2013. The nutritive value of processed animal proteins to different aquaculture species. EFPRA Congress, 13-15 June 2013, Prague, Czech Republic.

Bureau, D.P. 2013. Usage of animal products in salmonid fish species. SONAC International Aqua Event, 29 May 2013, Burgum, The Netherlands.

Bureau, D.P. 2012. Current challenges of shrimp nutrition: Overview of state-of-the-art and feedback from an outsider. Lallemand Aquaculture Seminar 2012: Intestinal Health in Aquaculture, a sustainable way toward performance, 21-22 June 2012, Bangkok, Thailand.

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Final Report Attachment

Summary of Experiments R05-75 and R12-88
(FPRF Project Objective 2.4)

Effects of Citric Acid and Formic Acid Pre-Treatment of Poultry By-Product Meal (PBM) on Growth and Mineral Retention of Rainbow Trout

1. Introduction

Feed supplementation with organic acids has been shown to improve mineral and nitrogen retention and increase nutrient digestibility (Overland et al., 2008). Notably, supplementation of diets with citric acid increased weight gain and specific growth rate (SGR) and reduced feed conversion ratio (FCR) in Beluga (Khajepour and Hosseini, 2011), red sea bream (Sarker et al., 2005) and rainbow trout (Vielma et al., 1999; Pandey and Satoh, 2008). Our previous *in vitro* study showed a greater increase in soluble phosphorous (P) when poultry by-product meal (PBM) was incubated with formic acid compared to citric acid. However, the effect of formic acid on growth and nutrient retention of fish has not been adequately evaluated (Vielma and Lall, 1997). To our knowledge, no information regarding the use of organic acid pre-treated PBM in diets for rainbow trout currently exists. This experiment was designed to assess the effect of *in vitro* incubation of PBM with citric acid and formic acid on growth and nutrient utilization efficiency of rainbow trout.

2. Materials and methods

2.1 Fish and experimental conditions

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Alma Aquaculture Research Station (Elora, Ontario). Fish were maintained in a flow-through system consisting of 60 L fiberglass tanks, individually aerated and supplied with well water at a rate of approximately 3 L/min and equipped with fecal settling columns (Guelph System) as described by Cho et al. (1982). Water temperature was maintained at 11.8 ± 0.5 ^{o}C and 12.6±0.4 ^{o}C for the digestibility and growth trials, respectively. Photoperiod was maintained at 12 h light: 12h dark in a windowless laboratory. The animals were kept in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1984).

2.2 Experimental diets and feeding protocol

A reference diet (Table 1) was prepared and combined with each test ingredient (PBM pre-treated with either water, citric acid or formic acid) at a 70:30 ratio (as is basis) to produce three test diets. Yttrium oxide at an inclusion level of 100 ppm was added to the reference diet to serve as a digestibility indicator. The diets were mixed using a Hobart mixer (Hobart, Don

Mills, Ontario) and pelleted using a laboratory steam pellet mill (California Pellet Mill, San Francisco, CA). The feed pellets were subsequently dried using forced air at room temperature for 24 h. The diets were kept at 4 °C until used.

The PBM was obtained from Rothsay (Moorefield, Ontario). The proximate composition of the PBM and the reference and test diets are shown in Table 2. Citric acid and formic acid (analytical grade) were obtained from Sigma-Aldrich (Oakville, Ontario). Pre-treatment of PBM was achieved by diluting 10 g citric acid or formic acid with 670 mL distilled water and adding this mixture to 1 kg PBM. After mixing thoroughly, the treated PBM was incubated at 50 °C for 3 h and then air dried. PBM was also incubated with distilled water in order to formulate the control diet. The test diets (formulated using PBM pre-treated with water, citric acid or formic acid) were used to conduct the growth trial. Fish in both trials were hand-fed to satiety three times daily on weekdays and once daily on weekends.

2.3 Digestibility trial

Groups of 15 fish with an initial average weight of 21 g/fish were randomly distributed into 24 tanks. The four experimental diets (reference, control, citric acid, formic acid) were randomly allocated to two collection units each (each unit collects feces from 3 tanks). The fish were acclimated to the experimental system and dietary regime for four days prior to collection. A total of four fecal samples per diet were collected. Two fecal samples per diet were collected during the first collection period (10 days). The experimental diets were then randomly re-allocated to new collection units for the second period and two additional fecal samples per diet were collected in the following 10-day period. One hour after the last daily meal, the drainpipe and the settling column were brushed out to remove feed residues and feces from the system. One-third of the water in the tanks was drained to ensure that the cleaning procedure was complete. At 08:30 h the following day, the settled feces and surrounding water were gently withdrawn from the base of the settling column into a large centrifuge bottle. These feces were free of uneaten feed particles and considered to be a representative sample of the feces produced throughout the 24 h period. The feces were centrifuged at 4000×*g* for 10 min and the supernatant discarded. The feces were then freeze-dried, ground and stored at −20° C until analysis.

2.4 Growth trial

Groups of 15 fish with an initial average weight of 35.9 g/fish were randomly distributed into nine tanks, with 3 replicate tanks per diet (control, citric acid and formic acid). Tank was considered the experimental unit. Fish were acclimated to the experimental conditions for one week prior to the start of the experiment. During this period, they were fed a maintenance ration of a commercial trout feed (Martin Mills Inc., Elmira, Ontario) once daily. Throughout the duration of the experiment (58 days), feed intake was recorded weekly and fish were weighed every 28 days. At the beginning of the experiment, a pooled sample of 12 fish was taken for determination of initial carcass composition. At the end of the experiment, five fish per tank were randomly sampled for carcass composition analysis

and an additional 10 fish per tank were individually weighed and dissected in order to obtain the hepatosomatic index (HSI) and viscerosomatic index (VSI). Fish were killed by a lethal dose of tricaine methane sulfonate (200 mg/L water). Fish to be analyzed for carcass composition were cooked in an autoclave, ground into a homogeneous slurry using a food processor, freeze-dried, reground and stored at -20 ^{o}C until analysis. Fish sampled for analysis of carcass composition were immediately dissected and the intestine was divided into three equal parts, not including the rectum, using pieces of thin thread. An additional tie was made between the stomach and the intestine. pH of the digesta in the stomach and the two intestinal regions was measured using a Reagecon (Shannon, Ireland) Series GC Glass pH Micro Combination Electrode (GCMF 11-100, 4 mm tip) on a Jenway (Essex, U.K.) pH meter at 21° C. The pH probe was inserted into the digesta in the middle of the section being studied and the stable reading was recorded.

2.5 Chemical analyses

Diets, ingredients, feces and carcass samples were analyzed for dry matter (DM) and ash according to AOAC (1995), crude protein (CP, N × 6.25) by LECO (LECO Corp., St. Joseph, MI, USA), lipid with an Ankom XT20 fat analyzer (Ankom Technology, Macedon, NY, USA) using petroleum ether and gross energy (GE) using a Parr 1271 automated bomb calorimeter (Parr Instruments, Moline, IL, USA). Mineral composition of carcass samples and yttrium oxide content of diets and feces were analyzed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) by Laboratory Services Division of the University of Guelph (Guelph, Ontario).

2.6 Calculations

Growth rate, expressed as thermal-unit growth coefficient (TGC), was calculated for each tank as: $TGC = 100 \times [(FBW^{1/3} – IBW^{1/3}) \times (\text{sum } T \times D)^{-1}]$, where: FBW=final body weight (g/fish); IBW=initial body weight (g/fish); sum T × D = sum degrees Celsius × days.

Feed efficiency (FE, gain:feed) was calculated for each tank as: FE = live body weight gain/dry feed intake, where: feed intake = total dry feed/number of fish; live body weight gain = (FBW/final number of fish) – (IBW/initial number of fish); FBW = final body weight (g); IBW = initial body weight (g).

Retained nitrogen (RN, g/fish) and recovered energy (RE, kJ/fish) were calculated for each tank as: $RN = (FBW \times N\ content_{final}) – (IBW \times N\ content_{initial})$ and $RE = (FBW \times GE\ content_{final}) – (IBW \times GE\ content_{initial})$, respectively, where: FBW = final body weight (g/fish); IBW = initial body weight (g/fish); $N\ content_{final}$ = nitrogen content (%) of the final carcass sample; $N\ content_{initial}$ = nitrogen content (%) of the initial carcass sample; GE_{final} = gross energy (kJ/g) content of the final carcass sample; $GE_{initial}$ = gross energy (kJ/g) content of the initial carcass sample.

Nitrogen retention efficiency (NRE), energy retention efficiency (ERE) and retention phosphorus efficiency (PRE) were calculated for each tank as a percentage of ingested nitrogen (IN): NRE (% IN) = [[(FBW × N $content_{final}$) – (IBW × N $content_{initial}$)]/IN] × 100; ERE (% IE) = [[(FBW × GE $content_{final}$) – (IBW × GE $content_{initial}$)]/IE] × 100, and PRE (% IP) = [[(FBW × P $content_{final}$) – (IBW × P $content_{initial}$)]/IN] × 100 where: FBW = final body weight (g/fish); IBW = initial body weight (g/fish); N $content_{final}$ = nitrogen content (%) of the final carcass sample; N $content_{initial}$ = nitrogen content (%) of the initial carcass sample; GE_{final} = gross energy (kJ/g) content of the final carcass sample; $GE_{initial}$ = gross energy (kJ/g) content of the initial carcass sample; IN = ingested nitrogen (g/fish); IE = ingested energy (kJ/fish); P $content_{final}$ = phosphorus content (%) of the final carcass sample; P $content_{initial}$ = phosphorus content (%) of the initial carcass sample.

The apparent digestibility coefficients (ADC) for the nutrients and GE of the experimental diets were calculated according to Cho et al. (1982): ADC = 1 – (F/D × Di/Fi), where: D = % nutrient (or kJ/g gross energy) of diet; F = % nutrient (or kJ/g gross energy) of feces; Di = % digestion indicator (yttrium) of diet; Fi = % digestion indicator (yttrium) of feces.

ADCs of the test ingredients (ADC_{ingr}) were calculated based on the digestibility of the reference diet and the test diets using the equation proposed by Forster (1999) and mathematically simplified as per the recommendation of Bureau and Hua (2006): $ADC_{ingr}=ADC_{test\ diet} + [(ADC_{test\ diet} – ADC_{ref\ diet}) × (0.7 × D_{ref}/0.3 × D_{ingr})]$, where: D_{ref} = % nutrient (or kJ/g GE) of reference diet mash (as is); D_{ingr} = % nutrient (or kJ/g GE of test ingredient (as is).

The HSI and VSI were calculated as: HSI (%) = (liver weight/body weight) × 100 and VSI (%) = (viscera weight/body weight) × 100.

2.7 Statistical analysis

All results were assessed for normality by the Shapiro-Wilk test, homoscedasticity by SNHT and expressed as mean values. When the data did not show normality, transformation using Box-Cox was performed prior to analysis. The dependent variables were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's HSD test using the XLSTAT® software Version 2014.5.01. For all analyses, the level of significance adopted was $P \leq 0.05$.

3. Results

The ADCs of DM, CP, GE and P were significantly higher for rainbow trout fed the reference diet ($P<0.05$) compared to fish fed the three test diets, but no significant difference in lipid digestibility was observed between diets. The ADC of ash was significantly lower ($P<0.05$) for fish fed the control diet compared to the reference diet, but not significantly different from the diets containing PBM pre-treated with either citric or formic acid.

Furthermore, the ADC of ash for rainbow trout fed the diets containing PBM pre-treated with citric or formic acid was not significantly different from that of fish fed the reference diet (Table 3).

There were no significant differences ($P>0.05$) in the ADCs of DM, CP, lipid, ash, GE or P among the PBM test ingredients. However, it appears that pre-treatment with formic acid may have resulted in a slight, but non-significant, improvement in the digestibility of PBM in rainbow trout (Table 4).

Inclusion of PBM pre-treated with organic acids in diets for rainbow trout did not significantly affect growth, feed intake, feed efficiency or HSI and VSI. Excellent growth rates (TGC) were achieved with all experimental diets (Table 5). Similarly, there were no significant effects of pre-treatment of PBM with citric or formic acid on proximate and mineral carcass composition or nutrient utilization efficiency of rainbow trout (Tables 6 and 7).

Feeding diets formulated with PBM pre-treated with citric or formic acid did not significantly affect the pH or dry matter content of digesta from the stomach, mid- or distal intestine of rainbow trout (Table 8).

4. Conclusion

Pre-treatment of PBM with citric acid or formic acid did not significantly improve the digestibility of this ingredient in rainbow trout. Furthermore, inclusion of PBM pre-treated with these organic acids in diets for rainbow trout did not affect growth performance, proximate or mineral carcass composition, nitrogen, energy or phosphorous utilization efficiency or pH of digesta in the stomach and intestine. In summary, the pre-treatment methods employed in this study did not appear to improve the nutritive value of PBM for rainbow trout.

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Table 1. Ingredient composition of the reference diet.

Ingredients	%
Fish meal, herring, 70% CP	18.5
Soybean meal, dehulled	6.0
Blood meal, porcine, spray-dried	6.0
Corn gluten meal, 60% CP	12.0
Feather meal	5.0
Wheat middlings	12.9
Soy protein concentrate (HP300)	12.0
Vitamin premix[a] (Martin Mills)	1.0
Mineral premix[b] (Martin Mills)	0.5
Wheat gluten	8.0
Fish oil	14.0
Vegetable oil	4.0
Yttrium oxide	100ppm
Total	100

[a]Provides per kg of diet: retinyl acetate (vitamin A), 3750 IU; cholecalciferol (vitamin D), 3000 IU; dl-a-tocopheryl acetate (vitamin E), 75 IU; menadione sodium bisulphite (vitamin K), 1.5mg; ascorbic acid polyphosphate (Stay C^{TM} 25% ascorbic acid), 75 mg; cyanocobalamine (vitamin B12), 0.03 mg; biotin, 0.21 mg; inositol, 450 mg; folic acid, 1.5 mg; niacin, 15 mg; Pantothenic acid, 30 mg; pyridoxine·HCl, 7.5 mg; riboflavin, 9.0 mg; thiamin·HCl, 1.5 mg.
[b]Provides per kg of diet: sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; ferrous sulphate ($FeSO_4 \cdot 7H_2O$, 20% Fe), 65 mg; potassium iodide (KI, 24% K), 11 mg; manganese sulphate ($MnSO_4$, 36% Mn), 89 mg; zinc sulphate ($ZnSO_4 \cdot 7H_2O$, 40% Zn), 150 mg; copper sulphate ($CuSO_4 \cdot 5H_2O$, 25% Cu), 28 mg; di-sodium selenite (Na_2SeO_3), 10 mg.

Table 2. Proximate composition of the PBM, reference diet and test diets.

PBM or Diet	DM (%)	Composition (dry matter basis)					
		CP[a] (%)	Lipid (%)	Ash (%)	GE (kJ/g)	P (%)	Y_20_3[b] (μg/g)
PBM	97.4	72.0	11.3	13.9	21.8	2.46	-
Reference	94.9	53.0	20.7	5.9	24.5	0.87	88
Control	95.0	55.8	18.1	8.7	23.7	1.37	59
Citric acid	95.0	58.5	17.7	8.6	23.6	1.26	58
Formic acid	95.0	58.3	17.8	8.6	23.7	1.37	60

[a]CP crude protein (N X 6.25)
[b]Yttrium oxide

Table 3. Apparent digestibility coefficients of the reference diet and three test diets fed to rainbow trout.

Diet	Apparent digestibility coefficients[1]					
	DM (%)	CP (%)	Lipid (%)	Ash (%)	GE (%)	P (%)
Reference	79[a]	92[a]	86[a]	55[a]	83[a]	62[a]
Control	76[b]	87[b]	84[a]	45[b]	79[b]	51[b]
Citric acid	77[b]	85[b]	85[a]	48[ab]	80[b]	49[b]
Formic acid	77[b]	88[b]	85[a]	48[ab]	80[b]	53[b]
Significance[2]	0.0012	<0.0001	NS[4]	0.0018	0.0003	0.0017
S.E.M.[3]	0.0041	0.0058	0.0040	0.0110	0.0035	0.0152

[1]Mean (n= 4 replicates). Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.
[2]Significance of the one-way ANOVA.
[3]S.E.M.=standard error mean.
[4]Not statistically significant (P≥0.05).

Table 4. Apparent digestibility coefficients of PBM pre-treated with water (control), citric acid or formic acid in rainbow trout.

Ingredient	Apparent digestibility coefficients[1]					
	DM (%)	CP (%)	Lipid (%)	Ash (%)	GE (%)	P (%)
Control	68[a]	78[a]	76[a]	38[a]	72[a]	43[a]
Citric acid	69[a]	79[a]	78[a]	40[a]	73[a]	39[a]
Formic acid	71[a]	80[a]	83[a]	40[a]	75[a]	45[a]
Significance[2]	NS[4]	NS	NS	NS	NS	NS
S.E.M.[3]	0.0101	0.0091	0.0178	0.0088	0.0210	0.0210

[1]Mean (n=4 replicates). Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.
[2]Significance of the one-way ANOVA.
[3]S.E.M.=standard error mean.
[4]Not statistically significant (P≥0.05).

Table 5. Weight gain, growth rate, feed intake, feed efficiency (FE) and viscerosomatic (VSI) and hepatosomatic (HSI) indices of rainbow trout (initial average weight=35.9 g/fish) fed the experimental diets for 58 days.

Diet	Gain (g/fish)	TGC[1]	Feed intake (g/fish)	FE[2] (gain/feed)	VSI (%)	HSI (%)
Control	143.0[a]	0.311[a]	122.6[a]	1.16[a]	20.4[a]	1.36[a]
Citric acid	140.0[a]	0.313[a]	125.7[a]	1.12[a]	19.8[a]	1.46[a]
Formic acid	138.3[a]	0.304[a]	120.7[a]	1.13[a]	18.8[a]	1.39[a]
Significance[3]	NS[5]	NS	NS	NS	NS	NS
S.E.M. [4]	2.711	0.005	2.085	0.01	0.38	0.02

[1]TGC=thermal-unit growth coefficient.

[2]FE=feed efficiency.

[3]Significance=significance of the one-way ANOVA. Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.

[4]S.E.M.=standard error mean.

[5]NS=not statistically significant ($P \geq 0.05$).

Table 6. Chemical body composition of the whole carcass of rainbow trout (initial average weight=35.9 g/fish) fed the experimental diets for 58 days.

Diet	H_2O (%)	CP[1] (%)	Lipid (%)	Ash (%)	GE[2] (kJ/g)	P (%)	Mg (%)	Ca (%)	K (%)	Na (%)	S (%)	Fe (%)
Control	69.8[a]	15.8[a]	11.8[a]	1.9[a]	8.6[a]	0.36[a]	0.025[a]	0.34[a]	0.30[a]	0.08[a]	0.15[a]	0.001[a]
Citric acid	69.6[a]	16.3[a]	12.2[a]	2.1[a]	8.7[a]	0.40[a]	0.027[a]	0.37[a]	0.30[a]	0.08[a]	0.15[a]	0.001[a]
Formic acid	69.8[a]	16.4[a]	12.0[a]	2.1[a]	8.6[a]	0.49[a]	0.027[a]	0.40[a]	0.31[a]	0.08[a]	0.16[a]	0.001[a]
Significance[3]	NS[5]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
S.E.M. [4]	0.21	0.17	0.36	0.07	0.07	0.04	0.001	0.02	0.002	0.001	0.002	0.000

[1]CP=crude protein.

[2]GE=gross energy.

[3]Significance=significance of the one-way ANOVA. Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.

[4]S.E.M.=standard error mean.

[5]NS=not statistically significant ($P \geq 0.05$).

Table 7. Retained nitrogen, recovered energy, retained phosphorus, nitrogen retention efficiency, energy retention efficiency and phosphorus retention efficiency of rainbow trout (initial average weight = 35.9 g/fish) fed the experimental diets for 58 days.

Diet	RN[1] (g/fish)	RE[2] (kJ/fish)	RP[3] (g/fish)	NRE[4] (% IN)	ERE[5] (% IE)	PRE[6] (% IP)
Control	3.3[a]	1170[a]	0.4[a]	33[a]	43[a]	17[a]
Citric acid	3.4[a]	1160[a]	0.5[a]	32[a]	43[a]	19[a]
Formic acid	3.4[a]	1134[a]	0.6[a]	33[a]	44[a]	24[a]
Significance[7]	NS[9]	NS	NS	NS	NS	NS
S.E.M.[8]	0.1	28.19	0.1	0.44	0.63	0.2

[1]RN=retained nitrogen.
[2]RE=recovered energy.
[3]RP=retained phosphorus.
[4]NRE (% IN)=nitrogen retention efficiency (% ingested nitrogen).
[5]ERE (% IE)=energy retention efficiency (% ingested energy).
[6]PRE (% IP)=phosphorus retention efficiency (% ingested phosphorus).
[7]Significance=significance of the one-way ANOVA. Means in the same column sharing a common superscript are not significantly different according to Tukey's HSD test.
[8]S.E.M.=standard error mean.
[9]N.S.=not statistically significant ($P \geq 0.05$).

Table 8. pH and dry matter content of digesta of rainbow trout (initial average weight = 35.9 g/fish) fed the experimental diets for 58 days.

Diet	pH[1]				Dry matter[1]		
	Diet	ST[2]	MI[3]	DI[4]	ST	MI	DI
Control	5.67	3.89[b]	7.67[a]	7.90[a]	29.39[a]	17.82[b]	18.19[b]
Citric acid	5.45	3.90[b]	7.79[a]	8.12[a]	28.34[a]	16.70[b]	16.85[b]
Formic acid	5.29	3.72[b]	7.92[a]	8.25[a]	28.38[a]	17.88[b]	18.02[b]

[1]Means in the same column or row sharing a common superscript are not significantly different according to Tukey's HSD test.
[2]ST=stomach.
[3]MI=mid-intestine.
[4]DI=distal intestine.