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Enhancement in Immune Function and Growth Using E-JUR-94013 Supplementation

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SUMMARY

Animal studies suggest that fish oils are capable of modulating the cell functions of immune system and there is some evidence that the effects of fish oils on immune function are due to fatty acids rather than trace elements or antioxidants. The major objectives of this study were: i) to identify a fish species with high nutritional value able to improve pig feeding conditions; ii) to utilize diets that modulate the immune system early in life in pigs and; iii) to enhance growth rate on a physiological basis. With the aim of maximizing feeding intake after weaning in order to reduce stress and increase growth rate, a study was carried out on 300 pigs supplemented with different fish extracts obtained by advanced biotechnological methods. The results of this work suggest that the lipoproteins obtained from the Trachurus trachurus (E-JUR-94013) species may have a great effect as both an immunomodulating compound (acting mainly on the regulation of IgA synthesis and/or release) and as a hypocholesterolemic compound, reducing the total cholesterol level in the serum of treated pigs. Both effects resulted in better pig growth, demonstrating that E-JUR-94013 can also be used as a natural growth promoter and an immune enhancer.

Key words: Immune system - Lipoproteins - Pig nutrition

INTRODUCTION

In many regions of the world, a large industry has developed around the production of animal feed using not only waste from the human table, but also fish caught especially for the purpose. At first, most of the scientific effort in the industry was directed at measuring the quality of the fish products and analyzing their nutritional value for different classes of livestock (1-3). Once the value was recognized, demand for some of the products began to exceed supply, resulting in the current problems with quality control and efficient use of limited supplies. Over the past 75 years, reviews and general articles on the subjects of fish meal and other fish products (such as animal feed stuffs in general, and pig and chick food in particular) have been published (4-6). In pig feeding, the aim is to ensure satisfactory reproduction, a rapid growth rate, efficient utilization of food and a carcass quality demanded by the market, as dictated by the consumer.

Although many factors in pig production ultimately determine the results, proper feeding is by far the most important. About 70-85% of the total cost of pig production lies in the cost of food, meaning that while ingredients must be governed by nutritional needs, they must also be economically viable (7). Grain only meets part of a pig's needs for a balanced diet (8-11), and information on the content, limitations and feeding levels of other ingredients must be ascertained. In general, supplementation of pig rations with fish products, particularly fish meal, is largely dependent on the availability of alternative high-protein supplements. In Europe, for example, where the production of the latter is rather limited, fish meal has become a standard protein supplement, and therefore much attention has been paid to its quality, which has progressively improved (12). In America, on the other hand, where high-protein vegetable supplements are available in abundance, fish meal has only been given secondary importance, and consequently quality has received much less attention than in, for example, the United Kingdom or Scandinavia.

Of course, each fish product has to be judged on its own merit, as not all of them contain high nutritional value for pigs. The overall guiding principles have been established, and in some countries quality standards for fish products used in animal feed have been developed (13-15). Fish meal is manufactured by boiling, drying and grinding either whole fish or the offal of the fish used for human consumption (16). The chemical composition of the meal will therefore vary. Fish meal manufactured from whole fish, especially herring, is rich in fat if the oil is not extracted, whereas the fish meal of fish waste origin contains less fat, but often has much less mineral material.

Our research center is involved in the development of novel products for human and animal nutrition using biotechnological procedures for the biotransformation of marine natural products into nutritional substances with biomedical and/or veterinary applications (17). In the present paper, E-JUR-94013, a fish extract obtained by advanced biotechnological methods, has proved to be an excellent source of lipoproteins when compared with other commercial fish meals, and also demonstrated less variability with respect to other meals. The results of pig feeding with this novel product allowed us to conclude that E-JUR-94013 can be used successfully as an ingredient of pig rations, and that the use of E-JUR-94013 in pig rations lowers the cost of pork production and improves immune function, potentially leading to a reduction in mortality and morbidity in young pigs.

MATERIAL AND METHODS

Three hundred pigs weaned at 18-21 days of age were transported from an external source to the nursery unit of an experimental farm located in Soria, Spain. The pigs, selected for high lean growth potential, averaged 8.2 kg (range = 7.78-8.89 kg) at the start of the experiment. During an adaptation period of 2 days in their new environment, the pigs were fed with a commercial diet. They were divided into 15 groups of 20 pigs of the same average weight. Pens of pigs were randomly allotted one of five dietary treatments (basal diet, diet 1, diet 2, diet 3 and diet 4), with major ingredients consisting of corn, soybean meal and dried whey (Table 1). Basal diet contained 10 kg/ton limestone and no fish extract; diet 1 contained basal diet, 10 kg/ton limestone and 0.5 kg/ton of fish extract; diet 2 contained basal diet, 5 kg/ton limestone and 0.1 kg/ton of fish extract; diet 3 contained basal diet, 10 kg/ton limestone and 0.5 kg/ton of fish extract; and diet 4 contained basal diet, 5 kg/ton limestone and 0.1 kg/ton of fish extract (Table 1). Diets and water were provided twice daily for a period of 42 days. Food intake was measured daily and body weight was monitored once a week. Sardine, mackerel and Horse mackerel (E-JUR-94013) were used to obtain fish extracts by a lyophilization protocol. Cycles consisted of three phases: freezing (12 h at –20 °C), primary drying (36-48 h at –45 °C), and secondary drying (24-36 h at +30 °C). Conditions in the dryer were varied through the cycle to insure that the resulting product had the desired physical and chemical properties and that the required stability was achieved. A total of 900 blood samples (3 samples/pig, baseline, 21 days and 42 days) were collected *in situ* in a 10-ml tube, sent to laboratory facilities and centrifuged (2,000 x g, 4 °C, 15 min) to isolate and collect serum. Serum samples were transferred to eppendorf tubes and stored at –80 °C until used. Total cholesterol and triglycerides were determined by enzymatic methods using CHOD-PAP and triglyceride without free glycerol, respectively, and enzymatic kits were provided by Boehringer Mannheim. High-density lipoprotein cholesterol was measured enzymatically with the CHOD-PAP kit after precipitation of very low density lipoproteins and low-density lipoproteins with phosphotungstic acid and magnesium ions as described (18), using a high-density lipoprotein precipitant solution supplied by Boehringer Mannheim. Total cholesterol (very low density lipoprotein and low-density lipoprotein) was determined by subtracting high-density lipoprotein cholesterol from total cholesterol. IgA, IgG and IgM were measured by using a turbidimetric method. The determination of immunoglobulins is based on the reaction between immunoglobulin as antigen and the specific antiserum as antibody. This reaction forms an insoluble complex producing a turbidity, which is measured spectrophotometrically at 340 nm. The results are expressed as mean ± standard error of the mean and the significance level is $p = 0.05$. Data were subjected to an analysis of variance using the general linear model procedure according to a 3 x 2 factorial arrangement used to determine the main E-JUR-94013 effects.

RESULTS

As innumerable types and variety of fish are procured by many different methods, and because the raw material can be processed in many different ways, it is obvious that the term "fish products" can only be a general one. Differences in composition of fish products may be attributed to many causes: variation in the composition of the raw material (including the proportion of whole fish and fish offal and contamination with sea animals other than fish), different methods of processing (including

TABLE 1. Composition of dietary treatments used in this study.

Ingredients (kg/ton)	Diet 1 Basal diet	Diet2 Basal diet	Diet 3 Basal diet	Diet Basal diet	Basal diet
Limestone	10	5	10	5	10
Sardine	0.5	0.1	0.5	0.1	0
Limestone	10	5	10	5	10
Mackerel	0.5	0.1	0.5	0.1	0
Limestone	10	5	10	5	10
E-JUR-94013	0.5	0.1	0.5	0.1	0

TABLE 2. Detailed composition (basal diet) of ingredients of the fish meal ration with contribution of cereals and fish meal to the total nutrient content of the ration used in this study.

	Content of ingredients			Content of ration contributed by	
	Barley meal	Fine wheat offal	White fish meal	Cereals 92%	White fish meal 7%
Crude protein (%)	11.6	15.5	63.2	12.2	4.4
Fiber (%)	5.0	5.0	-	4.6	-
Ash (%)	2.7	3.9	21.7	2.9	1.5
Ca (%)	0.09	0.08	7.87	0.08	0.55
P (%)	0.47	0.74	3.61	0.53	0.25
Choline mg/%	115.8	98.4	308.4	100	21.6
Nicotinic acid mg/%	6.45	12.2	5.6	8.1	0.4
Panthothenic acid mg/%	0.7	2	0.9	1.9	0.07
Arginine (%)	0.6	1.0	4.0	0.7	0.28
Lysine (%)	0.6	0.6	5.5	0.55	0.39
Methionine (%)	0.2	0.1	1.8	0.15	0.12
Cystine (%)	0.2	0.3	1.2	0.22	0.09
Tryptophan (%)	0.2	0.2	0.6	0.18	0.05

TABLE 3. Composition of fish extract of different origin obtained by the lyophilization protocol used in the study.

	Herring	Sardine	Tuna	White fish	E-JUR-94013
Crude protein (%)	70.0	65.0	62.0	63.0	76.0
Fiber (%)	1.0	1.0	1.0	1.0	1.0
Ash (%)	12.0	19.0	20.0	22.0	6.0
Ca (%)	3.0	4.5	4.0	6.5	3.2
P (%)	2.0	2.4	2.5	3.5	2.0
Choline mg/%	396	286	308	154	355
Nicotinic acid mg/%	8.8	6.6	6.6	6.6	7.5
Panthotenic acid mg/%	1.1	0.7	0.9	0.7	0.8
Riboflavin mg/%	0.4	0.5	0.9	0.9	1.1
Arginine (%)	4.0	2.7	5.3	4.2	5.0
Lysine (%)	6.3	5.9	5.2	4.3	5.2
Methionine (%)	2.0	2.0	1.7	1.7	1.3
Cystine (%)	1.1	0.8	1.0	0.7	0.8
Tryptophan (%)	0.9	0.5	0.7	0.6	0.7
Glycine (%)	5.0	4.5	4.2	3.7	2.8

efficiency in fat removal, heat treatment, *etc.*), contamination with some waste products or sand, and so on. The results of chemical and mineral composition of basal diet used in this study are summarized in Table 2.

By using an optimized freezing, primary and secondary drying protocol, fishes of different species were tested and chemically analyzed (Table 3) in order to ensure that the required stability was achieved. Table 4 shows the mean food intake and body weight gain of pigs fed the various diets. At the end of the experimental period, food consumption and weight gain were similar for the pigs in the sardine-supplemented group. Significant differences in both feed intake (diet 1: 928 ± 31; $p < 0.05$), (diet 2: 933 ± 35; $p < 0,05$), (diet 3: 958 ± 34; $p < 0.05$) (diet 4: 944 ± 38; $p < 0.05$) and feed:gain ratio (diet 1: 1.88 ± 0.22, $p < 0.01$), (diet 2: 1.86 ± 0.3, $p < 0.01$), (diet 3: 1.8 ± 0.1, $p < 0.01$) (diet 4: 2.02 ± 0.1, $p < 0.01$) (Table 4) were observed in the E-JUR-94013 treated group with respect to controls (1097 ± 28, feed intake; 2.36 ± 0.1, feed:gain ratio). Table 5 shows the effects of the different diets on serum cholesterol of pigs in the fed state. Significant E-JUR-94013 effects were observed on serum cholesterol. Total cholesterol levels were higher in the basal diet-fed pigs than in the E-JUR-94013-fed group. As shown in Table 5, there was a hypocholesterolemic effect in the E-JUR-94013 groups, mainly in the diet 2 and diet 3 groups after 42 days of treatment (83 ± 13, $p < 0.05$; and 86 ± 11, $p < 0.05$, respectively) compared with the control group, and this effect was attributed to a rapid turnover of cholesterol induced by the presence of increased amounts of polyunsaturated fatty acids in the E-JUR-94013-treated group.

When the effect of E-JUR-94013 supplementation was evaluated to determine the rate of immune modulation assessing IgA, IgG and IgM sera levels, a trend towards an increased production of all immunoglobulins was observed (Tables 6-8; Fig. 1). This trend was already statistically significant for the IgA immunoglobulins after 21 days of treatment in all groups supplemented with E-JUR-94013 (diet 1: 17 ± 2 mg/%, $p < 0.05$; diet 2: 15 ± 3 mg/%, $p < 0.05$; diet 3: 16 ± 3 mg/%, $p < 0.05$; diet 4: 17 ± 3 mg/%, $p < 0.05$) (Table 6) with respect to

TABLE 4. Effect of administration of different diets and feed intakes on growth performance.

	Basal diet	Diet 1	Diet 2	Diet 3	Diet 4
Sardine extract treatment					
Start weight (kg)	7.95 ± 0.2	8.05 ± 0.1	8.21 ± 1.2	7.82 ± 0.5	7.90 ± 1
Average daily gain (g)	315 ± 18	335 ± 24	320 ± 36	295 ± 24	305 ± 22
Average daily feed intake (g)	855 ± 25	882 ± 27	825 ± 44	816 ± 37	798 ± 19
Feed:gain	2.7 ± 0.1	2.6 ± 0.4	2.57 ± 0.4	2.76 ± 0.5	2.61 ± 0.3
Mackerel extract treatment					
Start weight (kg)	7.78 ± 0.15	8.49 ± 1.2	8.11 ± 0.2	8.2 1± 0.3	8.29 ± 1.1
Average daily gain (g)	325 ± 22	274 ± 31	365* ± 23	320 ± 21	375* ± 32
Average daily feed intake (g)	803 ± 27	755* ± 44	763 ± 35	763 ± 38	824 ± 44
Feed:gain	2.47 ± 0.12	2.75 ± 0.12	2.09* ± 0.2	2.38 ± 0.4	2.2* ± 0.1
E-JUR-94013 extract treatment					
Start weight (kg)	8.89 ± 0.21	9.12 ± 1.1	8.92 ± 0.4	8.8 ± 1.1	8.38 ± 1.3
Average daily gain (g)	465.8 ± 23	493* ± 28	501* ± 22	524** ± 25	467.2 ± 31
Average daily feed Intake (g)	1097 ± 28	928* ± 31	933* ± 35	958* ± 34	944* ± 38
Feed:gain	2.36 ± 0.1	1.88** ± 0.22	1.86** ± 0.3	1.8** ± 0.1	2.02* ± 0.1

*$p < 0.05$; **$p < 0.01$.

TABLE 5. Effects of diet on total cholesterol levels in the sera of pigs treated with E-JUR-94013 during the experimentation period.

	Basal diet	Diet 1	Diet 2	Diet 3	Diet 4
Cholesterol level (mg/%)					
Baseline	75 ± 12	67 ± 11	69 ± 14	71 ± 10	78 ± 11
21 days	84 ± 19	81 ± 14	72* ± 12	79 ± 13	81 ± 12
42 days	99 ± 15	95 ± 11	83* ± 13	86* ± 11	93 ± 13

*$p < 0.05$.

TABLE 6. Effects of diet on IgA levels (mg/%) in the sera of pigs treated with E-JUR-94013 during the experimentation period.

	Basal diet	Diet 1	Diet 2	Diet 3	Diet 4
Baseline	10 ± 2	11 ± 3	9 ± 2	12 ± 2	11 ± 3
21 days	11 ± 2	17* ± 2	15* ± 3	18* ± 3	17* ± 3
42 days	9 ± 3	22** ± 4	18* ± 1	20* ± 4	18* ± 2

*$p < 0.05$; **$p < 0.01$.

TABLE 7. Effects of diet on IgM levels (mg/%) in the sera of pigs treated with E-JUR-94013 during the experimentation period.

	Basal diet	Diet 1	Diet 2	Diet 3	Diet 4
Baseline	34 ± 5	37 ± 5	39 ± 5	33 ± 4	39 ± 7
21 days	41 ± 6	42* ± 7	44* ± 8	40* ± 8	39* ± 8
42 days	52 ± 8	68* ± 11	72* ± 9	63* ± 8	58* ± 5

*$p < 0.05$.

control (11 ± 2 mg/%). A similar trend was also observed in the IgM production (Table 7) with significant differences after 42 days of treatment in the diet 1 and diet 2 groups (68 ± 11 mg/%, $p < 0.05$; 72 ± 9 mg/%, $p < 0.05$ respectively) in comparison with the control diet (52 ± 8 mg/%). The production of IgG was also affected by E-JUR-94013 supplementation, mainly after 42 days in the diet 1 (443 ± 42 mg/%, $p < 0.01$), diet 2 (384 ± 13 mg/%, $p < 0.05$) and diet 3 (399 ± 11 mg/%, $p < 0.05$) groups with respect to control (334 ± 32 mg/%) (Table 8).

DISCUSSION

The quantity of fish meal to be added to pig rations depends on the following major factors: the intended quality of the ratio (often depending on the class of pig to which the ration is fed and on the level of performance expected); the composition of the fish meal; the composition of the rest of the ration and; the cost of the protein supplements (19). The relatively high cost of fish meal and the frequent scarcity of supplies make it imperative that the amounts used in rations of growing pigs are care-

TABLE 8. Effects of diet on IgG levels (mg/%) in the sera of pigs treated with E-JUR-94013 during the experimentation period.

	Basal diet	Diet 1	Diet 2	Diet 3	Diet 4
Baseline	275 ± 28	284 ± 31	295 ± 34	270 ± 20	278 ± 31
21 days	299 ± 26	322 ± 23	321* ± 31	379* ± 33	281 ± 32
42 days	334 ± 32	443* ± 42	384* ± 13	399* ± 13	348 ± 24

*$p < 0.05$; **$p < 0.01$.

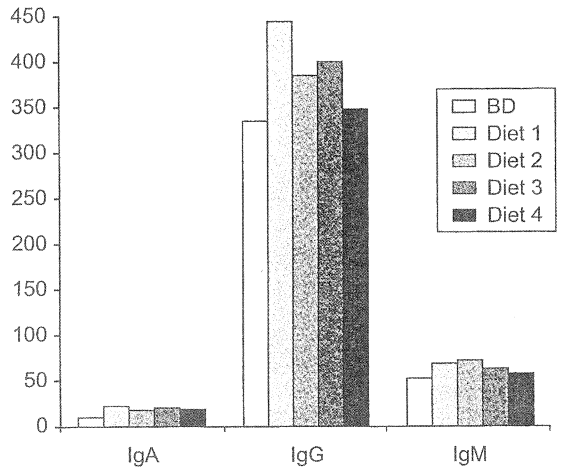

FIG. 1. Effects of different diets on IgA, IgG, and IgM levels (mg/%) in the sera of pigs after 42 days of treatment with E-JUR-94013.

fully measured with the aim of providing just enough to ensure the best possible rate of growth and efficiency of food conversion compatible with a specific quality of carcass (20-22). Above a certain level of supplementation these aims may be antagonistic to each other. An increase, for example, may result in an improvement in growth rate and efficiency of food utilization, but it may also cause deterioration in quality of the carcass and meat. On the other hand, an increase in the amount of fish meal necessary for favorable results may not be viable economically.

Because the method and rate of cooling usually affects the structure and appearance of the final product, the first objective of this study was to establish an extraction procedure and a lyophilization protocol to maintain the stability of the intrinsic properties of E-JUR-94013. During the freezing phase, this goal was reached by freezing the mobile water in the extract during a freezing period of 12 h at –20 °C, avoiding significant supercooling and the formation of ice crystals that are too small. In the primary drying phase, where the chamber pressure is reduced, heat was applied to the product to cause the frozen mobile water to sublime. In order to prevent the degradation and change of the physical characteristics of the dried material, different cycle times and temperature protocols were used. The optimal temperature protocol obtained during this phase was between 36 and 48 h (depending on the amount of product in the drying chamber) at –45 °C. At the end of primary drying, the shelf temperature was increased to desorb bound water such as that of crystallization until the residual water content was at the range required for optimum product stability. During this phase, referred to as secondary drying phase, in order to maintain secondary and tertiary structure of the lipoproteins contained in the E-JUR-94013 extract, the best conditions were 24-36 h (depending on the amount of product in the drying chamber) at 30 °C. By using this freezing, primary and secondary drying protocol, fish of different species were tested and chemically analyzed (Table 3) to insure that the required stability was achieved.

Enzymatically digested proteins (23) and spray-dried blood co-products have been used as important feeding value for early-weaned pigs (24), and some dietary proteins have been shown to influence the concentration of lipids in plasma. Soy protein compared with casein and other animal proteins reduces plasma cholesterol in animal models (25, 26) and in humans (27). Because previous reports demonstrated that both fish oil (28) and fish protein (29) can reduce serum lipid levels, the lyophilization protocol used in the transformation process of E-JUR-94013 allowed us to obtain a combination of both nutrients giving rise to a high lipoprotein content, which might have been responsible for the hypocholesterolemic effect observed in the studied population. The mechanism for the hypocholesterolemic effect may involve a reduction in intestinal cholesterol and bile acids absorption, which leads to an increased excretion of neutral steroids and bile acids in feces, a reduction in hepatic cholesterol concentrations and a rise in the activity of apolipoprotein B/E receptors (30).

Taken together, these results suggest that E-JUR-94013 may act on the immune system mainly affecting the production of immunoglobulins of the IgA class. Since the first level of defense takes place at the intestinal lumen-mucosa interface (31, 32) mainly represented by secretory immunity (sIgA) and, potentially by other components of innate immunity secreted into the intestine, we can speculate that E-JUR-94013 can act on the defensive function of the endogenous microflora by having a modulatory effect on the mucosal immune homeostasis and, therefore, on the mucosal mechanisms of defense.

Future trials must not only determine the effects of E-JUR-94013 on growth, composition and quality, but also

seek to understand the underlying biology that controls such traits. Because the effects of E-JUR-94013 will likely vary across genotypes, learning how this fish extract affects growth will enable us to better determine at what level and for what duration producers should use E-JUR-94013. Understanding of how E-JUR-94013 affects the biology of the pig will allow us to select for components of fat growth and to identify genes that have major effects on pig growth and development. In addition, the nutritional properties of E-JUR-94013 can also help to develop new models for veterinary nutraceuticals.

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