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ORIGINAL ARTICLE

Fish bones – a highly available calcium source for growing pigs

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Summary

In general, there is a lack of scientific documentation of nutritional value of marine by-products. The bone fraction from fish has been regarded as waste. Due to the high mineral content of fish bones, this material can be well suitable as a natural calcium source. In the present study, apparent calcium absorption of different fish bone sources was tested using growing pigs. The experimental diets consisted of boiled salmon frames, or salmon, saithe or cod bones treated with enzymes. Calcium carbonate ($CaCO_3$) was used as control. The experimental diets were formulated to contain 0.7% total calcium of which the added calcium source to be tested contributed about 71% (study 1) and 86% (study 2). Except for the calcium and phosphorus sources, the animals received similar basal diets. Apparent calcium digestibility coefficient was calculated using yttrium as indicator (both studies) and was based on complete collection of faeces and urine (study 2). The experimental design was parallel and cross-over in study 1 and study 2, respectively. In study 1, piglets getting salmon bone treated with enzymes had significantly higher calcium absorption than piglets getting boiled fish bone or calcium carbonate. Therefore, in the second study only enzymatically treated fish bones were included. The higher calcium absorption from enzymatically treated salmon bone was also found in study 2, but this time not significant. Calcium from boiled salmon bones in study I, and from enzymatically treated saithe and cod bones in study II were absorbed as well as the calcium carbonate control. The results indicate that fish bones may be a useful and well absorbed calcium source. Due to the high mineral content of the bone fraction, salmon bones can be well suitable as a natural calcium and phosphorus source in, for example, food, feed or as supplement.

Introduction

Until now little attention has been paid to the exploitation of marine by-products from the fishing industry. In general, there is a lack of scientific documentation concerning content and function of various components from marine by-products. The Norwegian fisheries produce more than 600 000 tons of by-products annually (RUBIN 2006), which is more than 20% of all the fish caught and farmed in Norway. Today most of the by-products are used as raw materials for feed production; such as fish meal, silage and feed for fur animals. About 166 000 tons are still dumped into the sea (RUBIN 2006). The last year's emphasis has been put in producing high quality products from fish by-products by use of bacterial proteases (Liaset et al., 2003; Sandnes et al., 2003). The bone fraction, which comprise

approximately 10–15% of the total body weight (skin not included) has, however, been regarded as waste. Due to the high mineral content of the bone fraction, this material can be well suitable as a natural calcium source in, for example, food, feed, or as supplement.

Animal and aquatic by-products are valuable sources of protein and minerals for pigs. However, in consequence of the bovine spongiform encephalopathy (BSE), the products originating from terrestrial animals are banned for animal feedstuffs. This is not the case for aquatic by-products such as fish meal or fish bones. Phosphorus of fish meal and bone meal is mainly inorganic and therefore highly digestible for pigs (Rodehutscord et al., 1997). Fish bones could be an alternative source of calcium and phosphorus in pig nutrition. Especially, if there will be shortage of mineral phosphates in the future.

Small fish, which can be eaten whole with bones included, have traditionally been considered a good source of calcium. Already in the 1920s, fish meal was tested as calcium source (Maynard & Miller, 1927a,b). Two recent studies from Denmark have shown that absorption of calcium from small soft-boned fish was comparable to that from skimmed milk both in rats (Larsen et al., 2000) and in humans (Hansen et al., 1998). To our knowledge, there are few reports on the availability of calcium from fish bones, and due to the potential nutritional value of this material, documentation is needed.

The aim of the present studies were to document calcium absorption from fish bones from Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), and saithe (*Pollachius virens*) using growing pigs. Calcium carbonate was used as control.

Materials and methods

Ethics and animal facilities

The experimental protocol for study 1 was approved by a local ethical committee for animal experiments at Norwegian Institute of Fisheries and Aquaculture Research, Bergen, Norway (No 095). The experimental protocol for study 2 was approved by an ethical committee for animal experiments at MTT Agrifood Research Finland, Jokioinen, Finland (No 1805).

In both studies, the pigs were housed individually throughout the study period. In study 1, pens of 0.9 m × 3.0 m, with a combination of plastic walls and bars between the pens, were used. In the feeding area the floor was covered with a cast iron grating (0.9 m × 1.20 m). The opposite end of each pen was covered, and equipped with a glow lamp giving a temperature 3–4°C higher in this area. The experimental unit had temperature varying from 19°C to 23°C and light/dark cycle 12 h/12 h. To obtain a spot sample of urine, the pigs were put into metabolic cages (0.85 m × 0.35 m × 0.50 m) with PVC plastic walls and double galvanized grating floor for a few hours on day 16 in the test period.

In study 2, metabolic pens of 1.43 m × 1.23 m with a slatted plastic floor were used. The experimental unit had temperature varying from 19°C to 26°C, and light/dark cycle 14 h/10 h. Urine samples from the pigs were quantitatively collected from the trays under the pens. Faeces were collected directly into plastic bags attached around the anus of the pigs using adhesive tape and snap-fasteners (van Kleef et al., 1994). The plastic bags were replaced after the pigs had defaecated.

In both studies, fasting blood samples were obtained by removing the food from the pens at 12:30 PM the day preceding the blood sampling. The blood samples were taken the next morning (starting at 9:00 AM) from the pigs' jugular vein. The pigs were given food after the last blood sample was taken.

The animals were not sacrificed at the end of the experiment. Thus, no samples of bones or other organs were harvested.

Calcium source

In study 1, boiled salmon frames and salmon frames rinsed by use of enzymes were used. In study 2, all samples were enzymatically rinsed and frames from saithe and cod were included in addition to salmon.

Bones from fresh salmon (*Salmo salar*) were provided by BioMega AS, Sotra, Norway. After filleting, the carcasses were frozen until processed by use of two different methods in order to remove soft tissue. The frames were either boiled in water at 100°C for 15 min, or treated at BioMega A/S with industrially produced enzymes (proteases) (Sandnes et al., 2003), similar to protamex (Novozymes A/S, Bagsvaerd, Denmark). After the treatment with proteases, the bone is in a moist matrix consisting of both bone and fractions of soft tissue, which were removed manually by using forceps and running cold water. The cleaned bone samples were freeze dried (Hetosicc, Heto, Denmark) for 74 h and thereafter ground (Knifetec mill, Foss Tecator). In the study 1, the fish bones were also heated in an oven at 104°C for 20 h as the fish bone meal still had a moist consistence after freeze drying. The fish bones used in the study 2 were not put through this second drying because

the heating did not reduce the water content significantly. The Saithe bone meal sample came from wild caught fish (*Pollachius virens*), and was bought frozen from Buland Fiskeindustri AS (Buland, Norway) (Liaset and Espe, 2008). The fish bones were enzymatically rinsed at Novozymes' pilot plant (Bagsvaerd, Denmark) according to procedures described in Liaset et al. (2003). The cod bone (*Gadus morhua*) meal was provided by Maritex (Sortland, Norway). These bones were enzymatically rinsed by use of Papain (6000 NFPU/mg, Biochem Europe, Mons, Belgium) and was provided in powder form from Maritex AS (Sortland, Norway). A calcium carbonate supplement [Weifa calcium (limestone), 500 mg tablets, Weifa A/S, Oslo, Norway] was used as calcium source for the control group in the pilot study and was given the same pre-treatment as the fish bones with freeze-drying and heating. Calcium carbonate (limestone, Nordkalk Ltd., Parainen, Finland) served as a control source of calcium in the study 2.

Study design, animal experiment – study 1

The experiment comprised three litters of four 5-week-old male piglets (NOROC, crossbreed between Landrace, Yorkshire and Duroc) with an initial weight of 10.8 ± 1.4 kg. A schematic overview of the design is given in Fig. 1. The experimental design used was a parallel study where the 12 piglets were randomly assigned to one of three experimental diets across litter and according to their initial weight.

Duration of the experimental period was 12 days, containing 4 days of preliminary feeding, 6 days of grab collection of faeces, and fasting prior to blood sampling (Fig. 1). Faeces grab samples were made twice daily (9:30 AM and 3:30 PM) for each piglet. The pigs were weighed four times during the study period (Fig. 1).

The piglets were fed three times per day, at 8:00 AM, at 11:00 AM, and at 3:30 PM and the feed intake was recorded. Weight gain and feed conversion efficiency were calculated based on data collected in the last 6 days of the experiment. Health, appetite, and faeces colour and consistency were monitored. Water was given *ad libitum*.

Wet feces, urine and water samples were stored at −20°C for further analysis. The blood samples were drawn from the jugular vein into two 4 ml vacutainer tubes with gel and without anti-coagulant, and one 4 ml vacutainer tube with EDTA. The total amount of blood withdrawn from each pig was 8–10 ml. The samples were left for 30 min at room temperature before serum separation with centrifuge (2000 *g*, 10 min). The blood samples were stored at −80°C until analysis.

Study design, animal experiment – study 2

A total of eight 6-week-old castrated male piglets [Landrace × Yorkshire (1) and Yorkshire (7)], with an initial weight of 10.8 ± 0.9 kg were used in this trial. A schematic overview of the design is given in Fig. 1. The experimental design used was a 4 × 4 Latin square where the eight piglets were divided into two 4 × 4 Latin squares according to their initial weight. The four experimental diets were circulated between the eight pigs during the four periods

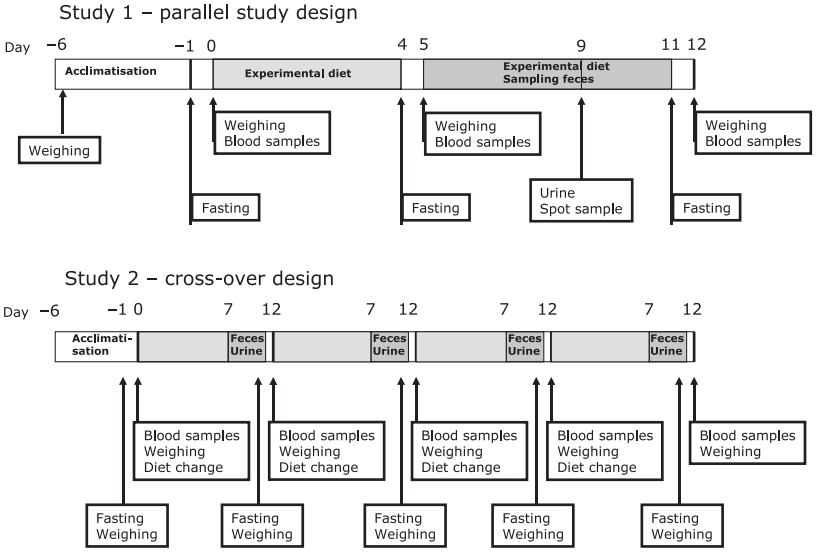

Fig. 1 Study design: schematic overview of study design and sampling of faeces, urine and blood in pigs given different fish bones as calcium source. Study 1 was a parallel study where each pig (n = 12) was exposed to one experimental diet. Study 2 was a cross-over design where each pig (n = 8) was exposed to four experimental diets in a random order. In both studies the pigs fasted prior to blood sampling and weighing.

resulting in eight observations per diet. Duration of the experimental period was 12 days, containing 7 days of preliminary feeding, 4 days of total collection of faeces and urine, and fasting prior to weighing, blood sampling and diet change (Fig. 1). The piglets were fed three times per day with equal amounts of feed, at 8:00 AM, at 12:00 noon, and at 4:00 PM. The feeding level of the piglets was 600, 840, 1080 and 1380 g/day, respectively during the four experimental periods. Feeding level was kept constant during each period. Feeding level for each period was increased according to the weight gain of the piglets. Before feeding, meal-type diets were mixed with water in a ratio of 1:2. Water was also freely available from low-pressure drinking nipples throughout the study.

Faeces were collected directly into plastic bags attached around the anus of the pigs using adhesive tape and snap-fasteners (van Kleef et al., 1994). The plastic bags were replaced after the pigs had defaecated. Collected faeces were frozen at −20°C until analysis. Urine was collected twice daily, one night-time urine (3:00 PM–8:00 AM) and one day-time urine (8:00 AM–3:00 PM), into bottles with 40 ml of 10 mol/l sulphuric acid. A representative sample of approximately 30 ml urine was stored at −20°C pending analysis. Feeds were sampled and pooled from each period. Faeces were sampled and pooled from each day of each period and from each period. Total urine was sampled and pooled from each period. Day-time urine was sampled and pooled from each day of each period.

Fasting (24 h) blood samples were taken from the jugular vein in the beginning and at the end of each experimental period. Two 4 ml vacutainer tubes with gel and without anti-coagulant were taken for serum separation. Samples were left for 30 min at room temperature before serum separation with centrifuge (870 *g*, 15 min, temperature +4°C). One 4 ml vacutainer tube with EDTA was taken for haematocrit measurements. Samples of tap water were taken and pooled for analysis.

Diets

The experimental diets for the study 1 were formulated at Norwegian Institute of Fisheries and Aquaculture Research, Norway, based on vegetable sources low in calcium (Table 1). The diets were formulated to contain approximately 0.7% total calcium. The calcium source to be added contributed approximately 71%. Except for the calcium sources,

Table 1 Feed ingredient composition (kg) in the experimental diets. The fish bone meals were added in different amounts due to various calcium contents, and the weight differences were balanced against barley (study 1) and wheat (study 2). The fish bones used as calcium source were rinsed for soft tissue by boiling (boiled) or by use of industrially produced enzymes (enz)

	Study 1			Study 2			
%	Control	Salmon boiled	Salmon enz	Control	Salmon enz	Saithe enz	Cod enz
Soy extruded	12.00	12.00	12.00	–	–	–	–
Wheat	–	–	–	51.88	51.79	52.14	51.44
Barley	25.90	25.70	25.20	15.00	15.00	15.00	15.00
Maize	–	–	–	15.00	15.00	15.00	15.00
Wholemeal flour	25.00	25.00	25.00	–	–	–	–
Soy protein	12.00	12.00	12.00	11.00	11.00	11.00	11.00
Whey protein	10.00	10.00	10.00	3.50	3.50	3.50	3.50
Ammonium phosphate	0.74	–	–	1.24	–	–	–
Calciumcarbonate	1.59	–	–	1.62	–	–	–
Salmon bone meal	–	3.04	2.48	–	2.95	–	–
Saithe bone meal	–	–	–	–	–	2.60	–
Cod bone meal	–	–	–	–	–	–	3.30
Trace element premix	0.45*	0.45*	0.45*	0.40§	0.40§	0.40§	0.40§
Vitamin mix	0.74†	0.74†	0.74†	–	–	–	–
Lysine	–	–	–	0.35	0.35	0.35	0.35
Amino acid mix	0.60‡	0.60‡	0.60‡	–	–	–	–
Yttrium oxide	0.01	0.01	0.01	0.01	0.01	0.01	0.01

*Providing sodium, copper, manganese, zinc, selenium, iodine, cobalt and iron.

†Providing vitamin mix (fish), vitamin E, vitamin A, and cholin HCl.

‡Providing lysine.HCl, d–l methionine, and threonine.

§Providing sodium, copper, manganese, zinc, selenium, iodine, iron, vitamin A, vitamin D_3, vitamin E, vitamin K, vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, biotin, pantothenic acid, niacin and folic acid.

the animals received similar basal diets. Phosphorus was, however, to be added to the control feed in amounts matching the two other feeds since simultaneous intake of calcium and phosphorus has been shown to affect calcium retention in pigs (Pointillart and Guéguan, 1993). The inert marker, yttrium oxide (VWR International, Oslo, Norway), was added at 0.1 g/kg (0.01% w/w) of diet.

The feeds for study 2 were manufactured at MTT, Agrifood Finland feed mill (Jokioinen, Finland). The diets were formulated to contain approximately 0.7% total calcium, and approximately 86% of the total calcium content originated from the experimental calcium sources added. Dietary ingredients were mainly of vegetable origin with low content of calcium (Table 1). Experimental diets met the Finnish recommendations of ileal digestible amino acids, vitamins and minerals for piglets (Tuori et al., 2002). Yttrium oxide (0.1 g/kg) was used as an indigestible marker for nutrient digestibility determination (Austreng and Storebakken, 2000).

Chemical assessment

Representative samples of the calcium supplements, fish bone, diet, feces and urine were analysed in both studies. The results are given as the mean of duplicate measurements. The moisture content was determined gravimetrically after freeze-drying (Hetosicc, Heto, Denmark). The total fat content of the samples was determined gravimetrically after extraction with ethyl acetate (Losnegård et al., 1979). The ash content was determined gravimetrically by burning all organic substances in a programmable furnace where the temperature was gradually increased from ambient to 550°C and held at this temperature for 20 h until constant weight. Total nitrogen was determined using a nitrogen element analyser (LECO, FP-528 N-analyser, Leco Corporation Svenska AB, Sweden). Protein was calculated as N × 6.25.

Prior to the determination of the elements, subsamples of homogenized feed and faeces were submitted to microwave-assisted wet digestion using 2.0 ml HNO_3 (ultra pure quality) and 0.5 ml H_2O_2, in an Ethos Pro microwave system (Milestone, Holger Teknologi, Oslo, Norway). Flame Atomic Absorption Spectrometry (Perkin-Elmer 3300 AAS, Norwalk, CT, USA) was used for the determination of the elements sodium, potassium, magnesium, calcium and iron (Julshamn et al., 1998; Jorhem and Engman, 2000). Quantification was made by means of external calibration. Hallow cathode lamps (HCL) were used for magnesium, calcium and iron, whereas sodium and potassium were run in emission mode. The effect of the nitrate concentration present in the sample solutions on the calcium signal were tested (Julshamn et al., 1998) and no suppression effect was found. The concentration of phosphorus was determined by electrothermal atomic absorption spectrometry on a Zeeman Atomic Absorption Spectrometer (Perkin Elmer 4110 ZL, Norwalk, CT) equipped with a THGA graphite furnace and an AS 72 autosampler. Palladium and magnesium were used as matrix modifier. An Agilent Quadrupole ICP-MS 7500c instrument (Yokogawa Analytical Systems, Inc., Tokyo, Japan) was used as a specific detector for the essential elements: copper, iodine, manganese, selenium and zinc and the non-essential elements arsenic, cadmium, mercury, lead and yttrium. Six points calibration solutions (5–100 μl/l) were prepared daily by appropriate dilution of 1000 mg/l stock solution of the elements in question (Spectroscan, Teknolab AS, Drøbak, Norway). A diluted solution of a 1000 mg/l (in 10% HCl) certified rhodium stock solution (Spectroscan) was added online and served as an internal standard. Iodine was determined by ICP-MS after the samples were extracted in tetramethyl ammonium hydroxide (TMAH) for 3 h at 90°C (Fecher et al., 1998; Julshamn et al., 2001). The elemental analyses are all accredited by the Norwegian Metrology and Accreditation Service, except the determination of yttrium. The certified reference materials TORT-2, Lobster hepatopancreas (National Research Council of Canada) and NIST Oyster Tissue (National Institute for Standards and Technology, Gaithersburg, MD, USA) were used for quality assurance of the determination of all elements studied, except yttrium. The laboratories at NIFES are frequently participating in proficiency tests. The z-score is an independent assessment of a laboratory's competence, and z-scores within ±2 are considered acceptable. All results obtained for the analytes presented in the present study showed z-scores within ±2.

Measurements and calculations

Apparent percentage digestibility of calcium using yttrium as indicator was calculated in both studies using the following equation: 100 – 100 [(μg/g yttrium in diet × μg/g calcium in faeces)/(μg/g yttrium in faeces × μg/g calcium in diet)] (Kotb and Luckey, 1972). In the study 2, calcium balance was also calculated based on complete collection of faeces and urine. Feed efficiency was calculated as mean body weight gain × 100 × (mean energy intake)$^{-1}$.

The data were analysed using statistical software (STATISTICA 6.1, StatSoft, Tulsa, OK, USA, and SAS version 8.2). In study 1, performance parameters were analysed by one-way (diet) analysis of variance (ANOVA) and Tukey HSD test as post-hoc test. Absorption data were analysed by repeated measures ANOVA, and Tukey HSD post-hoc test.

In the study 2, performance data were subjected to analysis of variance using GLM procedure of SAS (version 9.1). Statistical analyses of the absorption and digestibility data were carried out using the following model:

$$Y_{ijkl} = \mu + S_i + (A_j)S_i + P_k + F_l + S^*P + S^*F + \varepsilon_{ijkl}$$

where μ is the overall mean, S is the effect of square (i = 1–2), A is the effect of animal within the square (j = 1,…,4), P is the effect of period (k = 1,…..,4), F is the effect of feed (l = 1,…..,4) and ε is the normally distributed error with a mean of 0 and the variance of σ^2. The differences between the feeds were further analysed with Tukey HSD test.

Results

Calcium supplements and fish bone meals

The chemical composition of the fish bone samples and the calcium carbonate sample used as calcium sources in the animal studies is given in Table 2. Contrary to the calcium supplements, the fish bone samples also contained fat and proteins. It should be recorded that the salmon frames treated with enzymes in addition to higher calcium content had a lower fat content compared to the heated salmon frames. Also some difference in the concentration of various elements between the two differently treated salmon bones was found (Table 2). The concentration of the non-essential elements in the salmon frames was low and quite similar to the calcium source used in the control diet.

Diets – study 1

The nutritional composition of the diets is given in Table 3. There was some variation in calcium and phosphorus content between the experimental diets. The planning of the diet containing boiled salmon frames was based on analysed data from a different salmon batch than the one used in the experimental diet. This batch contained more calcium (170 g /kg vs. 160 g/kg) than the batch used in the diets, thus explaining most of the difference between the calculated calcium and the analysed calcium concentration in the diet.

The phosphorus content was slightly lower in the control diet than in the experimental diets, but close to the content expected to find in the basal diet.

Diets – study 2

The diet containing cod bone meal as calcium source had higher calcium content than the other diets. The planning of diet with cod bones as calcium source

Table 2 Chemical analysis of proximal, mineral and elemental composition of the different calcium sources; calcium carbonate (control) and bones from salmon, saithe or cod. The fish bones used as calcium source in the experimental diets were rinsed for soft tissue by boiling (boiled) or by use of industrially produced enzymes (enz)

	Study 1			Study 2			
	Control	Salmon boiled	Salmon enz	Control	Salmon enz	Saithe enz	Cod enz
Ash (g/100 g)	78.9	43.0	55.5	98.6	52.2	65.1	67.8
Protein (g/100 g)	0.56	35.7	36.0	0.53	32.1	27.6	26.6
Fat (g/100 g)	0	17.8	3.0	<0.2	5.1	<0.2	<0.2
Calcium (g/kg)	324	157	208	373	195	237	261
Phosphorus (g/kg)	0.8	89	156	<0.04	113	146	151
Magnesium (g/kg)	1.8	2.6	4.1	6.1	3.5	6.5	3.2
Sodium (g/kg)	0.27	3.1	1.9	0.08	1.5	3.7	2.1
Potassium (g/kg)	0.8	89	156	0.3	0.5	0.5	0.03
Iron (mg/kg)	150	20	24	2437	19	27	69
Iodine (mg/kg)	0.06	<0.02	0.28	<0.02	0.39	0.14	0.14
Selenium (mg/kg)	<0.1	0.4	0.3	0.4	0.6	0.5	0.5
Zinc (mg/kg)	1.9	110	170	2.6	199	87	57
Copper (mg/kg)	0.6	4.5	17	0.4	5.0	0.4	0.5
Vitamin D_3 (mg/kg)				<0.02	0.03	0.03	<0.02
Arsenic (mg/kg)	1.1	1.5	0.34	0.28	0.22	0.10	0.11
Cadmium (mg/kg)	0.08	<0.01	<0.01	0.03	<0.01	<0.01	<0.01
Mercury (mg/kg)	<0.01	0.02	0.01	<0.03	<0.03	<0.03	<0.03
Lead (mg/kg)	0.09	0.05	0.11	1.58	<0.04	0.07	<0.04

Table 3 Chemical analysis of composition of the experimental diets (wet weight). Mean values of duplicate measurements. The fish bones used as calcium source were rinsed for soft tissue by boiling (boiled) or by use of industrially produced enzymes (enz)

	Study 1			Study 2			
	Control	Salmon boiled	Salmon enz	Control	Salmon enz	Saithe	Cod enz
Gross energy (KJ/g)	18.0	18.1	18.1	16.5	16.7	16.6	16.5
Digestible energy (KJ/g)				14.5	14.7	14.6	14.5
Fat (g/100 g)	5.0	5.8	5.1	2.0	2.0	1.9	2.0
Protein (g/100 g)	22.6	23.2	23.2	19.6	20.2	20.5	19.8
Ash (g/100 g)	5.3	4.6	5.1	5.2	4.7	4.6	5.5
Lysine (g/kg)				11.4	12.3	11.9	12.1
Methionine (g/kg)				3.3	3.5	3.4	2.3
Cystine (g/kg)				3.6	3.8	3.5	3.6
Threonine (g/kg)				8.0	8.9	8.4	8.7
Calcium (g/kg)	7.5	6.5	7.8	7.2	8.0	7.8	10.8
Phosphorus (g/kg)	5.3	6.9	8.5	9.3	8.5	8.0	9.9
Yttrium (mg/kg)	75	85	81	70	68	70	71

was based on data given by the producer (18.6% calcium). Chemical analysis performed after the trial at NIFES and Norwegian Institute of Fisheries and Aquaculture Research, Fyllingsdalen, Norway, showed that the cod bone meal contained 26.1%, thus explaining the higher calcium content in cod bone diet.

There were no significant differences in protein content of the feed, but amino acid determination showed that the amino acid contents of the control diet were lower than that in the fish bone diets.

Performance – study 1

The body weight (kg) at the end of the experiment did not differ between the groups (Table 4), and there was no significant difference in the average daily weight gains, or the feed-efficiency ratios (data not shown). There was, however, a tendency towards better growth ($p = 0.092$) and feed-efficiency ratio ($p = 0.070$) in the group getting salmon bones treated with enzymes compared to the group getting boiled salmon bones.

The total amount of calcium consumed by the three groups during the 6-day period was 24 ± 2 g (control), 20 ± 2 g (boiled salmon bones), and 25 ± 1 g (salmon bones treated with enzymes). The group given boiled fish bone as calcium source had a significantly lower calcium intake compared to the two other groups (enzymatic treated salmon bones: $p = 0.003$ and control: $p = 0.028$) mainly due to a lower calcium concentration in the feed. There was no significant difference in food intake ($p = 0.92$) or calcium intake ($p = 0.40$) between the groups getting supplements or enzymatic treated fish bone as calcium source.

Performance – study 2

One pig (number 2) died after the second experimental period. Autopsy of the pig revealed abnormal developments in heart which likely were the cause of death, thus, the heart changes were inborn and not related to experimental treatments or conditions. Otherwise the pigs were healthy and ate their diets readily during the study. No differences in haematocrit or plasma calcium values (data not shown) between the treatments were observed.

The daily weight gain (Table 4) and feed efficiency (data not shown) of the pigs fed calcium carbonate

Table 4 Food intake (g/day),daily weight gain (g/day) in piglets given different calcium sources in the diet. The fish bones used as calcium source were rinsed for soft tissue by boiling (boiled) or by use of industrially produced enzymes (enz)

	Study 1			Study 2				
	Control	Salmon boiled	Salmon enz	Control	Salmon enz	Saithe enz	Cod enz	SEM
n	4	4	4	8	8	7	7	
Food intake*	525 ± 46	515 ± 44	538 ± 16	975	975	975	973	1
Daily weight gain*	325 ± 48	248 ± 76	357 ± 62	518[a]	569[b]	557[b]	559[b]	8

*Average values given as mean ± SD (study 1), and least mean square ± SEM (study 2); SEM of the treatments Salmon enz and Cod enz is table value multiplied with 1.173; significant different diet groups are marked with different letters ($p < 0.05$).

	Study 1			Study 2				
	Control	Salmon boiled	Salmon Enz	Control	Salmon enz	Saithe enz	Cod enz	SEM
N	4	4	4	8	8	7*	7*	
Calcium absorption (%), Y	60 ± 2	59 ± 2	70 ± 2	63	66	60	55	3
Calcium absorption (%), tot				74	74	65	64	5
Calcium retention (%), tot				70	71	61	61	5
Phosphorus absorption (%), tot				72	72	69	71	2
Energy absorption (%), tot				88	88	88	88	0.2
Fat absorption, (%), tot				63	69	66	69	3

Table 5 Apparent calcium absorption and retention in growing pigs. The apparent calcium absorption and calcium retention is calculated after complete collection of faeces and urine (tot), and by use of yttrium as inert marker (Y). The fish bones used as calcium source were rinsed for soft tissue by boiling (boiled) or by use of industrially produced enzymes (enz)

*SEM of the treatments C and D is table value multiplied with 1.173.

control treatment was lower than that of the pigs fed diets supplemented with different fish bone meals ($p < 0.05$). No differences were observed between the treatments in food intake (Table 4), faecal or urinary excretion (data not shown).

Apparent nutrient absorption – study 1 and study 2

Apparent absorption is defined as the difference between nutrient intake and faecal excretion and no correction is made for endogenous loss of calcium into faeces. The mean daily apparent calcium absorption is given in Table 5. In study 1, the piglets getting salmon bones treated with enzymes as calcium source had a significantly higher calcium absorption than the piglets getting boiled salmon bones or calcium carbonate (Table 5). There was no significant difference in apparent calcium absorption in the piglets getting boiled salmon bones or calcium carbonate.

In study 2, calcium and phosphorus from the fish bone samples was absorbed as well as from the calcium carbonate source. No statistical differences were observed in calcium and phosphorus absorption between different fish bone meals although numerically calcium absorption from the salmon bone meal was higher than that from the saithe and cod bone meals (Table 5). Calculation of apparent calcium absorption by using yttrium as indicator gave the same order as when the calculation was based on total samples of faeces (Table 5) or when urine samples where included in the calculations (retention). Apparent absorption of energy and fat were the same among the experimental diets, as well as digestible energy content of the diets calculated from gross energy and energy absorption (Table 3).

Urine samples of the pigs ($n = 12$) in study 1 were analysed for yttrium. The mean yttrium concentration was 130 ± 60 μg/l.

Discussion

Calcium from salmon fish bones treated with enzymes had a significantly higher apparent absorption compared to the two other diet groups in study 1. Based on these results, only fish bones treated with enzymes were included in study 2. Also in study 2, calcium from enzymatically treated salmon bones had highest absorption, but the difference was not significant (Table 5). Calcium and phoshorus from enzymatically treated cod and saithe bones, and boiled salmon bones, were absorbed as well as from the calcium carbonate control. Thus, the results indicate that fish bones may be a useful and well absorbed calcium and phoshorus source in growing pigs. Phoshorus absorption of fish bones meals is similar to that of fish meal and animal bone meal in pigs (Rodehutscord et al., 1997). From the Far East, where calcium deficiency is prevalent, there are some reports on fish bone (Deng, 2001) and tuna bone preparations intended as food supplements (Kim et al., 2000a,b). Homogenized fish bone has also been proposed as a weaning food mineral supplement (Martínez et al., 1998). The bioavailability has not been studied in any of these products, and to our knowledge, the present study is the first study testing apparent absorption of calcium from salmon-, cod-, and saithe bone meal. Studies have, however, shown that calcium from small fish that is eaten whole has absorption comparable to skimmed milk, which is regarded a good calcium source (Hansen et al., 1998; Larsen et al., 2000).

In this study the apparent absorption was assessed both using yttrium as an inert marker (both studies) and by complete collection of faeces and urine (study 2). Yttrium oxide is slightly soluble in weak acid, but has a very low solubility under neutral conditions (Budavardi et al., 1996). A fish study

indicated that the solubilized proportion of the marker is not absorbed in the stomach, but is precipitated when the digesta are neutralized in the intestine, and passes unabsorbed through the gastrointestinal tract (Austreng and Storebakken, 2000). The spot samples of urine collected on the fifth day in the experimental period in the study 1 contained traces of yttrium. Pigs have a lower pH in stomach compared to fish, and some yttrium may therefore have been absorbed. In study 2, apparent absorption was calculated both from total collection and by use of yttrium. The calculations based on yttrium were lower than the calculations based on total collection (Table 5), while the order of treatments were the same.

The salmon bone samples contain long chain polyunsaturated (n-3) fatty acids [marine (n-3)]. There is some evidence that marine (n-3) fatty acids have a positive effect on calcium absorption and bone metabolism. Since the salmon bones treated with enzymes had a lower fat content than the boiled salmon bones (Table 3), the potential positive effect of marine (n-3) fatty acids can not be the main reason for higher calcium availability from this diet. During the enzyme processing of marine raw material, the bones are first heated to the optimal temperature for the enzymes used. The temperature of the hydrolysate (also containing solid bones) is thereafter raised to above 60°C to inactivate the enzymes (Gildberg, 1993). Both the heating procedure and the enzymatic treatment (through digestion of bone collagen structure) might soften the bones (Gildberg et al., 2002). Fish bone also contains peptides which are shown to enhance calcium absorption in ovariectomized rats (Jung et al., 2006).

The pigs in study 2 grew significantly better and had a higher feed efficiency in the periods they were given fish bone meal as calcium source. No differences in feed intake or digestible energy content of the diets were found (Tables 3 and 4). The results showed that the lysine content was lower in the control diet compared to the fish bone diets and therefore the lysine intake of the pig on fish bone diets was higher. This likely caused the improvements in performance of the pigs All diets were, however, above the mean lysine requirement of 9.1 g of true ileal digestible lysine per kg of diet (Bertolo et al., 2005).

In study 1 the pigs were fed to satisfaction. Pair-feeding is recommended in absorption studies since feed intake may influence on the results. There is, however, no difference in food or calcium intake between the calcium carbonate group and the group getting salmon bones treated with enzymes (Table 4), thus the feeding regime had no effect on the significant difference in calcium absorption found between these two groups. Both groups getting salmon bones had a high phosphorus intake through the diet (Table 3). Excessive intake of phosphorus in combination with sufficient calcium intake may decrease intestinal calcium absorption due to formation of insoluble complexes in the intestinal lumen (Koshihara et al., 2001).

Calcium may react with fat and form calcium soaps which decrease calcium absorption and result in more calcium and fat excreted in faeces. Fishbone peptides have been shown to inhibit the formation of insoluble calcium salts in neutral pH and decreased loss of calcium in faeces in ovariectomized rats (Jung et al., 2006). The fat and energy content of faeces was therefore tested in order to check for possible calcium soap formation in the control group. The results showed no significant difference between the groups, and can thus not explain the growth differences. Salmon protein hydrolysate has been found to improve growth when fed to nursery pigs at up to 10% of the diet (Gottlob et al., 2006) and studies in fish have shown that inclusion of fish bones in the feed increases feed intake and promote growth (Toppe et al., 2006). The increased growth in the fish bone groups are most likely independent of calcium, since no difference in calcium absorption or retention was found.

Fish bone meal has a potential as human and animal feed ingredient because of the simultaneous presence of highly available calcium and phosphorus, the significant content of other micro- and macro-nutrients (Table 2). It is essentially important to find new sources of feed phosphorus because there will be lack of inorganic phosphorus in the world in the future. One indication of this is rapid rise of feed phosphorus during last years. Another quality of fish bones which make it suitable for ruminants and non-ruminants feed formulation, is the absence of transmissible spongiform encephalopathy disease among marine fish (AAFCO, 2000; cited in Johnsen et al., 2003). Crushed bone has been used for centuries as a phosphorus fertiliser for crops. Currently, fish bone meal is sold in the USA to organic farmers and individual gardeners (Johnson et al., 2003). The calcium concentration is also of importance when evaluating the potential of a source as a food supplement. Calcium carbonate ($CaCO_3$) is the calcium salt most widely used as a nutritional supplement because of its high elemental calcium content (approximately 40%). The fish

bones have a lower calcium content than calcium carbonate (Table 2), but are in the same range as other calcium salts; e.g. calcium citrate, calcium gluconate, calcium lactate, and calcium acetate (10–25% calcium) (Weast, 1979).

Calcium from fish bones from salmon, saithe and cod was absorbed as good as calcium carbonate. There was also a tendency to salmon bones being absorbed better than calcium carbonate. Due to the high mineral content of the bone fraction, salmon bones can be well suitable as a natural calcium source in, for example, food, feed, or as supplement.

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