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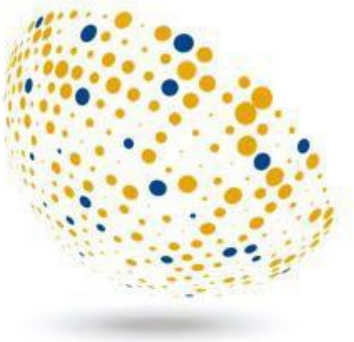

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Fish bone calcium and bone density in the young growing rat.

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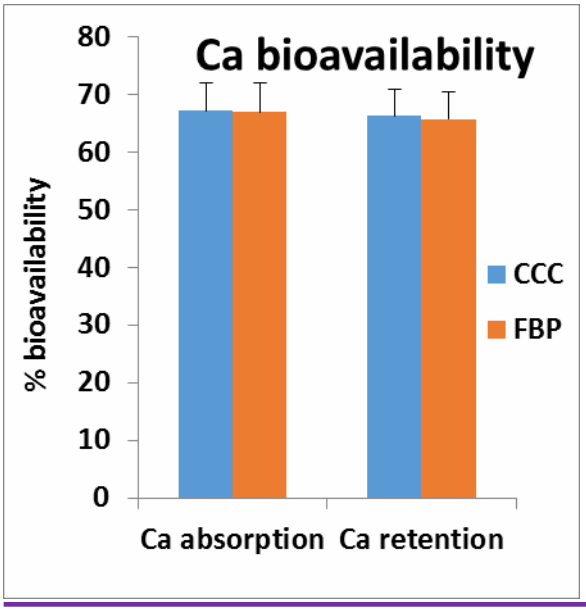
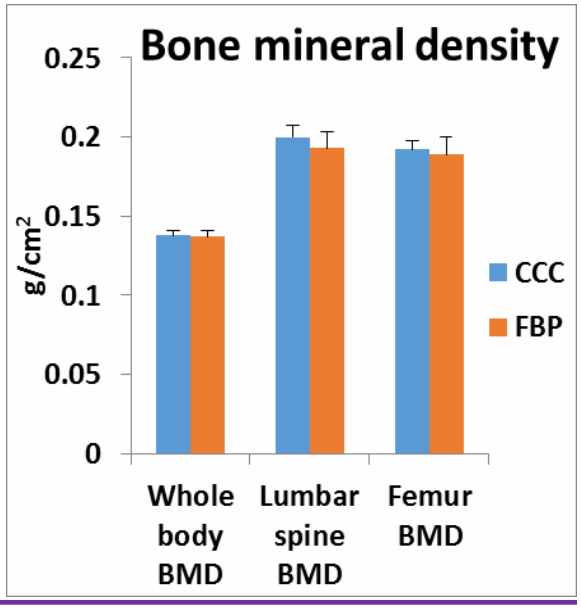
SUMMARY

A proprietary fish bone powder product (FBP) high in calcium was assessed using a growing rat model to provide preliminary data regarding the bioavailability and health benefits of the FBP. Young growing rats fed a nutritionally complete diet in which the dietary calcium was sourced solely from FBP were compared to control rats fed a nutritionally complete diet in which the dietary calcium was sourced solely from calcium carbonate (CCC).

The CCC and FBP rats proved to be nearly identical in most outcomes. Health parameters including energy and water intake, body weight gain, red blood cell counts, and haemoglobin levels did not differ between the two groups. The bioavailability of calcium in FBP was equal to that of the calcium in CCC. The resultant bone mineral density in the young growing rats was also equivalent between the two groups. The results of this study demonstrate that FBP, in a GRAS product format, is an acceptable source of dietary calcium as an alternative to calcium carbonate.

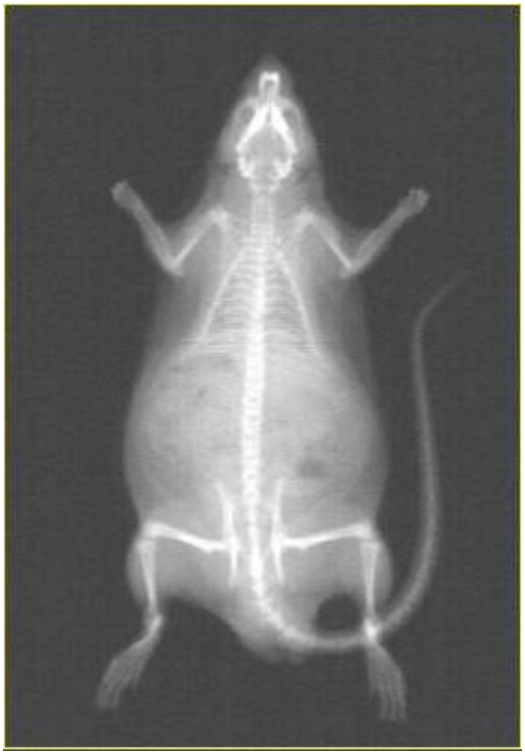




OBJECTIVES

The objectives of this study were to assess the effects in young growing rats of feeding a nutritionally complete diet containing calcium sourced from FBP versus calcium carbonate. The primary parameters to be assessed were:

1. Bone mineral density
2. Calcium absorption
3. Calcium retention



Secondary objectives identified by Massey University as being of scientific interest were added to the study design and carried out at Massey's expense:

1. Energy intake
2. Water intake
3. Body weight gain
4. Feed conversion efficiency
5. Fat:lean body ratio
6. Complete blood count

INTRODUCTION

Bone provides structural support for the body. Calcium is the most abundant mineral in the human body, and 99% of the body's calcium is held in the bone.

Bone tissue is metabolically active and undergoes continuous change throughout life via a series of tightly regulated processes. Bone is destroyed and resorbed by osteoclasts, then replaced through the formation of new bone by osteoblasts [1]. Mineralisation is laid down by osteoblasts under the direction of osteocytes and osteoclast activity.

An imbalance between bone resorption and bone formation results in a decrease in bone mineralisation termed osteopenia. Osteoporosis, which follows on from osteopenia, is a generalised skeletal disorder characterised by decreased bone mass and deteriorated bone architecture. Osteoporosis results in an increased susceptibility to bone fractures and increased post-fracture mortality risk [2].

Osteoporosis is considered a disease of the elderly, but bone mass is acquired far earlier in life. Approximately one-third of adult mineral is deposited in the bone during adolescence. Bone density consolidation continues through young adulthood. Childhood bone parameters at age 8 -11 years are, however, a poor predictor of peak bone mass at age 18 – 19 [3], suggesting that modifiable factors during the earlier adolescent years play key roles in bone development. Evidence is strongest for the positive influences of calcium intake and physical activity [4-6].

Absorption of dietary calcium in the intestine, as well as movement of calcium in and out of the bone, occur under the direction of several hormones including parathyroid hormone, calcitriol (vitamin D), and calcitonin. Other factors, such as dietary fibre and phosphorus, may also affect calcium absorption rates although the reports on these are sometimes conflicting [7, 8]. Many adults fail to meet the recommended daily intake of calcium (800 – 1000 mg per day) due to inadequate diet, impaired absorption, or food intolerances [9, 10].

As calcium is the key component of bone, calcium deficiency is strongly associated with osteoporosis.Ensuring sufficient calcium intake in children and adolescents may be the single most important factor in reducing osteoporosis development later in life. Yet many children, particularly those with a low intake of dairy products, remain deficient in both calcium and vitamin D intake [11-13].

However, calcium supplementation has proven unsuccessful in preventing or halting the progression of osteoporosis. Ingestion of calcium citrate in a tablet form results in a bolus of calcium being absorbed rapidly, resulting in a sharp increase in blood calcium and giving rise to the term "calcium supplement syndrome" [14]. This exaggerated fluctuation in calcium homeostasis results in an increased risk of developing kidney stones and/or cardiovascular disease [15], but has no proven positive effect on bone formation [16, 17]. Because of this, calcium supplements either

for children or adults are no longer recommended by many health organisations worldwide [10, 18, 19].

Instead, increasing dietary calcium and vitamin D (as opposed to supplementation in tablet form) to ensure adequate intake remains a key strategy for reducing osteoporosis. But absorption and utilisation of calcium differs depending on the dietary source. For example, dietary calcium in milk is absorbed differently to ionized calcium, so that even highly fortified calcium-containing milk remains safe for delivering calcium without perturbing calcium homeostasis [20].

Eating fish is positively correlated with calcium intake and improved bone health, both in children and adults [21-24]. However, these effects are likely to be due at least in part to the polyunsaturated fatty acids (PUFA) and vitamin D present in fish, which are known to have positive benefits on bone health [25, 26].

Fish bone, a by-product of the fishing industry, is a rich source of calcium and therefore is a logical source of calcium to be incorporated into other food products. However, there are only a few studies directly assessing fish bone as a calcium source for growing or maintaining bones. In growing pigs, calcium from enzymatically treated salmon bones was absorbed and retained as well as that of calcium carbonate; calcium from enzymatically treated cod bones was slightly less effective [27]. Similarly, in growing rats, calcium from hake bone flour made from a mix of raw and boiled bones was absorbed and retained as well as calcium from *Lithothamnium calcareum* seaweed powder; the two calcium sources also produced equivalent results in bone mineral density and biomechanical properties [28]. Thus, although limited in scope, the available data suggest that fish bone should prove equally as good as standard inorganic calcium sources for inclusion in the diet.

The current study was designed to compare fish bone powder and calcium carbonate in a growing rat model in order to assess both bioavailability (absorption and retention) and incorporation into bone (bone mineral density).

MATERIALS AND METHODS

IANZ accredited assays:

Of the methodologies described below, these were carried out as IANZ-accredited assays: Diet proximate analysis; faecal dry matter; DEXA scanning.

Animals and housing:

Conventional male Sprague-Dawley rats aged ~3 weeks were purchased from the Massey University Small Animal Production Unit (SAPU). Rats were individually housed in plastic cages with wire lids, bedded with sterile wood shavings. All rats had access to food and reverse osmosis distilled (MilliQ) water *ad libitum.* Rats were fed both standard rat chow and control (CCC) powdered diet for the first few days of acclimatisation to their new housing and diet before being switched onto solely the control (CCC) or test (FBP) powdered diet.

During the last week of the four-week study, rats were housed in individual metabolic cages with no bedding material so that uncontaminated faecal and urine outputs could be collected.

Dietary components

Sodium caseinate (CAS) was purchased from Tatua (Morrinsville,NZ). Cysteine, methionine, glutamic acid, glycine, lysine, calcium carbonate, potassium phosphate, potassium sulphate, potassium citrate, and magnesium oxide were purchased from Merck (Darmstadt, Germany). Fish bone powder was provided by United Fisheries with the specifications of shown in Table 1.

Table 1. Proximate analysis of Fish Bone Powder (MCHC).

Component	
Protein	24.75%
Calcium	25.14%
Magnesium	0.45%
Phosphorus	12.86%
Moisture	1.9%
Collagen	14.27%
Mercury	<0.07 ppm
Manganese	16 ppm
Copper	0.19 ppm
Iron	6.2 ppm
Potassium	0.04%
Selenium	0.73 ppm
Zinc	61 ppm
Sulphur	0.25%
Boron	<5 ppm

Tryptophan, ferric citrate, manganous sulphate, zinc oxide, cupric carbonate, chromic potassium sulphate, sodium selenite, cobaltous chloride, potassium iodate, and ammonium molybdate were purchased from Sigma-Aldrich (Auckland, NZ). Cellulose was purchased from Hawkins Watts (Auckland, NZ). Cornstarch, soy oil, and sucrose were purchased from Davis Trading (Palmerston North, New Zealand). Vitamin mix specifically designed to conform to AIN-93G was purchased from AgResearch (Palmerston North, NZ). Trace salt and mineral mixes were prepared at Massey University. The composition of the mineral mix, including the trace salt mix, is shown in Table 2.

Table 2. Components of mineral mix. Amounts are shown in g/kg of mix.

Mineral Mix	**(g)**
Potassium phosphate monobasic	196
Potassium sulphate	46.6
Potassium citrate tri-K monohydrate	70.8
Magnesium oxide	24.0
Trace salt mix	40.0
Cellulose	622.6

Trace Salt Mix	**(g)**
Ferric citrate	756.7
Manganous sulfate	80.0
Zinc oxide	20.0
Cupric carbonate	6.7
Chromic potassium sulphate	6.3
Sodium selenite	0.11
Cobaltous chloride	0.039
Potassium iodate	0.085
Ammonium molybdate	0.93
Cellulose	129.973

Both diets were formulated to meet AIN-93G specifications for protein and fat, and to meet amino acid and micronutrient requirements as specified in the Nutrient Requirements of Laboratory Animals [29]. The diets were matched for macronutrients, micronutrients, and energy, with the sole difference being the source of calcium in the diet. Powdered diets were made up and blended in 10 kg batches as shown in Table 3. Diets were labelled, colour-coded, and stored at -20°C.

Table 3. Components per kilo of test diet. Amounts are shown in grams.

	Control (CCC)	**Test (FBP)**
Sodium caseinate	200	195
Cysteine	2.7	2.7
Glycine	3.3	3.3
Methionine	1.5	1.5
Glutamine	7	7
Cellulose	50	50
Vitamin mix	10	10
Sucrose	40	40
Mineral mix*	50	50
Corn oil	50	50
Cornstarch	573	570.6
$CaCO_3$	12.5	0
Fish Bone Powder	0	19.9

**See Table 2.*

Diet proximate analyses were carried out by an IANZ-accredited testing facility (Nutrition Laboratory, MIFST, Massey University, NZ). Protein was measured using the Leco total combustion method (AOAC 968.06). Fat was measured using Soxtec extraction (AOAC 991.36). Moisture was measured by convection oven drying at 105ºC (AOAC 930.15, 925.10). Ash was measured using a 550ºC furnace (AOAC

942.05). Gross energy was measured by bomb calorimetry. Based on the laboratory proximate analysis of compounds and the information provided by the supplier or available in the literature, the predicted and true levels of individual components in each diet design were found to be comparable (Table 4).

Table 4. Predicted and final levels of key diet components versus minimum recommended amounts ("Req'd") for growth.

	Predicted in dry matter (from formulation)	**Present in CCC diet (proximate analysis)**	**Present in FBP diet (proximate analysis)**
Moisture	0%	6.6%	6.3%
Protein	20%	19.4%	19.8%
Fat	5%	5.1%	4.9%
Carbohydrate	65%	65.5%	64.3%
Energy (kJ per gram)	18 kJ/g	17.5 kJ/g	17.5 kJ/g
Calcium	5 mg/g	4.7 mg/g	4.8 mg/g
Phosphorus		3.5 mg/g	8.2 mg/g

Daily feeding, care, and measurements

The feeding and metabolic studies were carried out as described elsewhere [30]. Briefly, rats were randomised by body weight and allocated to one of the two diets. Powdered feed was replaced with fresh feed once daily. For each rat, the feeder was removed from the cage and its weight recorded before it was emptied and cleaned. The feeder was then filled with fresh diet, its weight recorded, and the feeder placed back into the rat cage. Daily food intake was thus measured as food offered minus food remaining 24 hours later. At this time, each rat's general health was also checked. Water bottles were checked daily to ensure rats had sufficient water. Rats were weighed weekly using a one decimal point balance. Cages, stainless steel feeders, and water bottles were cleaned and replaced once weekly.

Feed conversion ratio was calculated as:

FCR = gain in body weight (g)/100 kilojoules consumed

Food efficiency ratio was calculated [31] as:

FER = gain in body weight (g)/food consumed (g)

Metabolic studies

On day 20 of the study, rats were placed in metabolic cages. Food and water intake continued to be measured. After two days of acclimatisation, rats were kept in the cages for 5 days while urine and faecal materials were collected.

Urine and faecal samples were collected daily and stored at -20°C for 5 consecutive days, with the samples pooled over that period for each individual rat. Each day's urine sample for each rat included the addition of 0.1 mL 1M HCl to acidify the urine and 0.5 mL of water to rinse the collection tube. Just prior to calcium analysis, urine samples were thawed at 4°C overnight and the total volume and weight recorded, and filtration performed to remove contaminants.

Calcium absorption as a percentage was calculated as:

$$CA = [(IC – FC) \times 100]/IC$$

Calcium retention as a percentage was calculated as:

$$CR = [IC – (FC + UC) \times 100]/(IC – FC)$$

where CA = absorption, CR = retention, IC = ingested calcium, FC = faecal calcium, and UC = urine calcium, as described elsewhere [28].

Dual Energy X-ray Absorptiometry (DEXA) measurements

On day 29 – 30 of the study, rats were anesthetised with an intraperitoneal injection of 0.06 mL per 100 grams body weight of the following mixture: 2 parts acepromazine (2 mg/mL), 5 parts ketamine (100 mg/mL), 1 part xylazine (10%), and 2 parts sterile water. Anesthetised rats were scanned for body composition using a Dual-Energy X-ray Absorptiometer (DEXA) as an IANZ-accredited procedure. Femurs and spines collected post-euthanasia were stored frozen at -20°C, and later thawed and scanned using the same DEXA machine for bone mineral density.

Euthanasia and dissection

Following the DEXA scan, rats were received a second dose of anaesthesia prior to blood sampling and euthanasia was carried out by exsanguination and pneumothorax induction. Blood was collected from the superior vena cava through a 19 gauge 1 ½ inch needle into a heparin-flushed 10 mL syringe, then aliquoted into heparin- or EDTA-containing vacutainer tubes. Spine and femurs were removed, and gross pathology of the internal organs assessed.

Blood, urine, and faecal chemistry

Complete blood count (CBC) and haemoglobin analyses on whole blood were carried out using a Beckman Coulter Sysmex XE5000 haematology analyser. Remaining analyses were performed by the Massey University Nutrition Laboratory. Dry matter in faeces was measured by convention oven drying at 105 ºC (AOAC 930.15, 925.10). Faeces were prepared for calcium measurement by AOAC 968.08D method followed by colourimetric analysis. Calcium in urine was measured by cresol phenolphthalein complexone method.

Statistical analyses

Means and standard deviations were calculated for each parameter for the two groups of rats, and two-tailed Student t-tests carried out using Microsoft Excel software.

RESULTS

Rats were randomised by body weight and fed one of two diets (CCC vs FBP) for four weeks. During this time, no signs of ill health or changed behaviour were noted in any of the animals. To determine whether the FBP had any positive or negative effects on basic physiology in the rat, a number of parameters were assessed including body weight gain, food intake, body composition, and blood parameters.

Mean daily food intake by the growing rats increased each week for the first three weeks, levelling out at the fourth. Food intake did not change during the fifth week when the rats were housed in metabolic cages (data not shown). Food intake did not appear to differ between the CCC control and FBP test groups.

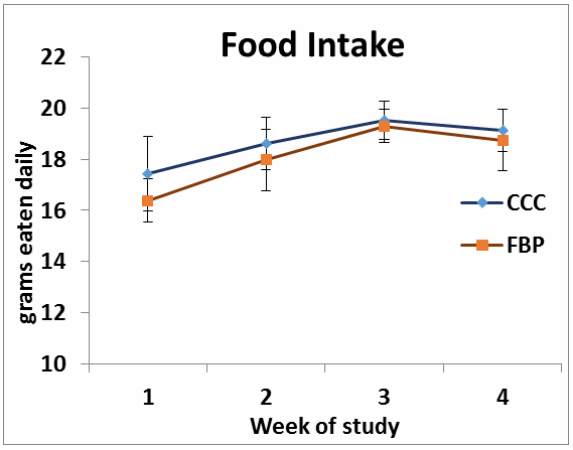


Figure 1: Food intake. Rats were fed the CCC or FBP powdered diet and food intake (in grams) was measured daily. Mean daily intake per rat over each one-week period is shown as mean + SD of N = 10 rats per test group. Rats were placed into metabolic cages on the fifth day of the final week shown.

Body weight of the growing rats was measured weekly. There were no apparent differences between groups in body weight or proportional body weight gain (Figure 2).

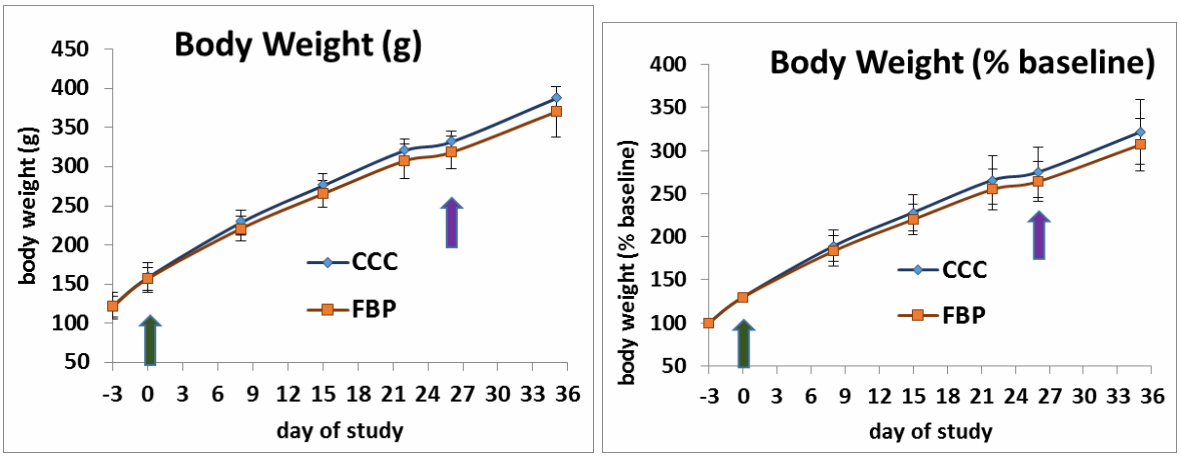


Figure 2: Weekly body weight gain. Body weight (BW) was measured twice weekly. Weight gain in grams (left) or as the percent gain normalised to starting body weight (right) were calculated. Data are shown as mean + SD of N = 12 rats per test group. Green arrows (day 0) on the x-axis indicate the day on which the rats began the CCC or FBP diet; purple arrows (day 26) indicate the day on which rats were placed into metabolic cages.

Rats did not differ significantly by group in their total food intake or energy intake over the course of the study, although the CCC rats ate slightly more and gained slightly more weight (Table 5). The caloric feed conversion ratio and food efficiency ratio, calculated as grams of body weight gained per 100 kJ of energy eaten and grams of body weight gained per gram of food consumed respectively, did not differ between the two groups.

Table 5. Food intake, weight gain, and caloric feed conversion ratio (mean + SD of N=12 rats per group) over a 26 day period in conventional housing. P value shown for two-tailed Student's t-test.

	CCC	**FBP**	*p value*
Food intake (g)	523 ± 22	507 ± 19	0.065
Energy intake (kJ)	9149 ± 381	8865 ± 333	0.065
Weight gain (g)	173 ± 15	161 ± 17	0.078
Feed conversion (%)	1.90 ± 0.19	1.82 ± 0.19	0.33
Food efficiency	0.32 ± 0.03	0.33 ± 0.03	0.33

Food and water intake were more precisely measured during the five days that the rats were housed in metabolic cages. No significant differences between diet groups were observed in food and water intake, faecal and urine excretion, faecal moisture content, or water retention (Table 6).

Table 6. Analysis of nutrient balance study over a 5 day period in metabolic cages, shown as mean + SD of N=12 rats per group.

	CCC	**FBP**	**p value**
Food ingested (g/5 days)	99.0 ± 2.0	98.4 ± 3.4	0.60
Faeces excreted (g/5 days wet wt)	16.4 ± 1.4	16.8 ± 1.3	0.48
Faeces excreted (g/5 days dry wt)	9.8 ± 0.6	9.7 ± 0.6	0.50
Faeces by dry wt (% of diet)	9.9 ± 0.5	9.9 ± 0.5	0.66
Water ingested (mL/5 days)	129.2 ± 30.6	125.6 ± 26.1	0.76
Urine excreted (mL/5 days)	66.5 ± 30.4	66.9 ± 18.9	0.97
Water retention (%)	50.4 ± 11.5	46.5 ± 11.3	0.41

DEXA scanning prior to euthanasia measured total lean and total fat masses of the rats, from which each rat's body composition was calculated. Control rats had slightly less fat and slightly lower lean:fat ratios than FBP rats, but this was not statistically significant (Table 7).

Table 7. DEXA body scan data (mean ± SD).

	CCC	**FBP**	*p value*
Total mass (g)	385 ± 15	375 ± 27	0.27
Fat mass (g)	56 ± 16	65 ± 15	0.19
Lean mass (g)	320 ± 22	301 ± 31	0.10
Fat (% of mass)	15 ± 4	17 ± 4	0.13
Lean (% of mass)	83 ± 4	80 ± 4	0.13
Lean: fat ratio	6.3 : 1	5.0 : 1	0.14

As diet can affect iron uptake, blood samples were assessed for complete blood counts (CBC) at the time of cull. Data were collected for all but two rats, whose blood was too clotted to permit analysis; this is an inherent problem as rat blood clots very rapidly compared to humans [32, 33].

The CBC measurements matched those reported elsewhere for inbred and outbred Sprague Dawley male rats. No significant differences were detected between treatment groups in the number of red blood cells, haemoglobin levels, or other blood erythrocyte or platelet parameters (Table 8). Interestingly, both total neutrophil numbers and the proportion of neutrophils within the white blood cell population were significantly lower in the CCC rats compared to the FBP rats.

Table 8. CBC analysis of whole blood. Data are shown as mean (standard deviation) of N= 11 rats per group.

	Control CCC	**Test FBP**	**p value**	**Ref Range****	**Expected *****
RBC count (x 10^{12}/L)	7.20 + 0.22	7.27 + 0.66	0.73	7.10 – 7.93	6.90 + 0.3
Hg (g/L)	153 + 3	152 + 9	0.78	142 – 172	145 + 8
Hct (L/L)	0.44 + 0.02	0.44 + 0.04	0.92	0.40 – 0.45	48.3 + 2.3
MCV (fL)	61.3 + 1.3	61.0 + 3.9	0.82	53.3 – 58.0	69.9 + 1.7
MCH (pg)	21.3 + 0.4	21.0 + 1.2	0.53	19.3 – 21.7	20.9 + 1.3
MCHC (g/L)	348 + 8	345 + 13	0.60	211 – 384	299 + 1.6
RDW (%)	11.0 + 0.4	11.8 + 2.3	0.26	11.1 – 16.6	n/a
Platelet count (x 10^9/L)	696 + 166	642 + 178	0.47	253 - 964	1358 + 124
MPV (fL)	7.44 + 0.27	7.86 + 0.62	*0.053*	3.20 – 7.25	n/a
WBC count (x 10^9/L)	5.23 + 1.51	6.32 + 2.32	0.21	5.00 – 14.45	9.4 + 3.2
% Neutrophils	1.9 + 4.0	8.8 + 6.8	***0.009****	6.0 – 18.4	n/a
Neutrophils (x 10^9/L)	0.10 + 0.22	0.53 + 0.47	***0.013****	0.35 – 1.70	0.9 + 0.5
% Lymphocytes	97.8 + 4.1	91.4 + 6.5	***0.012****	74.9 – 88.8	n/a
Lymphocytes (x 10^9/L)	5.1 + 1.5	5.8 + 2.2	0.43	4.3 – 12.2	8.3 + 2.9

***** *p ≤ 0.05 by Student's t test*

*** reference ranges reported for inbred adult male Sprague Dawley rats [34]*

**** mean values reported for outbred adult male Sprague Dawley rats [35]*

RBC, red blood cell; Hb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin content; RDW, red cell deviation of width; MPV, mean platelet volume; WBC, white blood cell; n/a, not available.

The main outcomes to be measured in this study were calcium absorption, calcium retention, and calcium deposition in bone. Calcium absorption was assessed by comparing calcium intake versus calcium excretion in the faeces. Calcium retention was assessed by comparing calcium absorption versus calcium excretion in the urine. Calcium retention in bone was assessed by measuring bone mineral density and bone strength.

Faeces and urine were collected while the rats were housed in metabolic cages. Calcium was measured in the diet, faeces, and urine. As shown in Table 9, calcium absorption and retention did not differ between test groups.

Table 9. Analysis of nutrient balance study over a 5 day period in metabolic cages, shown as mean + SD of N=12 rats per group.

	CCC	FBP	p value
Calcium ingested (mg/5 days)	465 ± 9	472 ± 16	0.21
Calcium in faeces (mg/5 days)	153 ± 22	156 ± 24	0.87
Calcium absorption (% of ingested)	67.2 ± 4.9	67.0 ± 5.1	0.95
Calcium in urine (mg/5 days)	4.1 ± 2.9	5.9 ± 2.9	0.11
Calcium retention (% of absorbed)	98.7 ± 0.9	98.2 ± 0.8	0.12
Calcium retention (% of ingested)	66.3 ± 4.7	65.7 ± 4.8	0.89

Rats were scanned by DEXA prior to euthanasia to measure bone mineral density (BMD) and bone mineral content (BMC), and also scanned post-euthanasia to measure BMD and BMC in the dissected femurs and spine. BMC reflects the amount of minerals in the whole bone, while BMD reflects the amount of mineralisation per cm^2 of bone tissue.

Femurs and lumbar spines were analysed as part of the whole-body DEXA. Whole-body bone parameters did not differ between CCC control and FBP test rats (Table 10). FBP rats had significantly less bone mineral in their spines and femurs, and significantly smaller spines and femurs, compared to the CCC rats. However, when bone mineral content was normalised to bone area, the resultant bone mineral density did not differ significantly between the two groups of rats in any of the bones, although the FBP rats were always slightly lower in BMD for each DEXA scan site.

Table 10. Whole body, spine, and femur bone mineralisation.

	CCC	FBP	p value
In vivo DEXA			
Whole-body BMC (g)	9.31 + 0.27	9.09 + 0.63	0.280
Whole-body bone area (cm^2)	66.95 ± 2.83	66.19 ± 3.80	0.582
Whole-body BMD (g/cm^2)	0.138 ± 0.003	0.137 ± 0.004	0.526
Right femur BMC (g)	0.34 + 0.02	0.33 + 0.03	0.294
Right femur bone area (cm^2)	1.49 ± 0.07	1.44 ± 0.10	0.155
Right femur BMD (g/cm^2)	0.228 ± 0.012	0.227 ± 0.012	0.866
Left femur BMC (g)	0.34 + 0.02	0.31 + 0.03 *	***0.017***
Left femur bone area (cm^2)	1.47 ± 0.06	1.39 ± 0.09 *	***0.023***
Left femur BMD (g/cm^2)	0.233 ± 0.009	0.227 ± 0.013	0.173
Mean femur BMC (g)	0.34 + 0.02	0.32 + 0.03	0.072
Mean femur bone area (cm^2)	1.48 ± 0.06	1.41 ± 0.09 *	***0.050***
Mean femur BMD (g/cm^2)	0.230 ± 0.010	0.227 ± 0.011	0.421
Lumbar spine BMC (g)	0.37 + 0.03	0.34 + 0.03 *	***0.036***
Lumbar spine bone area (cm^2)	2.00 ± 0.09	1.89 ± 0.12 *	***0.013***
Lumbar spine BMD (g/cm^2)	0.184 ± 0.010	0.179 ± 0.009	0.222

***** *p ≤ 0.05 by Student's t test compared to CCC.*

Femurs and spines were excised from the carcasses and re-scanned as individual bones. As was observed in the whole body, FBP rats had significantly smaller bones and significantly less bone mineral content, but when normalised to bone mineral density the significance was erased (Table 11). Again, though, there was a trend for bone mineral density in the FBP rats to be lower than the CCC rats.

Table 11. Individual spine and femur bone mineralisation.

	CCC	FBP	p value
ex vivo DEXA			
Right femur BMC (g)	0.39 + 0.02	0.36 + 0.04 *	***0.036***
Right femur bone area (cm^2)	2.01 ± 0.08	1.89 ± 0.13 *	***0.017***
Right femur BMD (g/cm^2)	0.193 ± 0.008	0.191 ± 0.014	0.652
Left femur BMC (g)	0.37 + 0.02	0.35 + 0.03	0.095
Left femur bone area (cm^2)	1.96 ± 0.27	1.90 ± 0.13	0.273
Left femur BMD (g/cm^2)	0.190 ± 0.008	0.187 ± 0.011	0.358
Mean femur BMC (g)	0.38 + 0.02	0.36 + 0.03 *	***0.039***
Mean femur bone area (cm^2)	1.98 ± 0.09	1.90 ± 0.13	0.063
Mean femur BMD (g/cm^2)	0.192 ± 0.006	0.189 ± 0.011	0.449
Lumbar spine BMC (g)	0.41 + 0.03	0.38 + 0.03 *	***0.010***
Lumbar spine bone area (cm^2)	2.05 ± 0.11	1.95 ± 0.11 *	***0.032***
Lumbar spine BMD (g/cm^2)	0.200 ± 0.007	0.193 ± 0.010	0.065

***** *$p \leq 0.05$ by Student's t test compared to CCC.*

DISCUSSION & CONCLUSIONS

The current study examined the effect of replacing calcium carbonate (CCC) in the rat diet with calcium sourced from fish bone powder (FBP). Rats fed the FBP diet did not differ significantly from rats fed the CCC diet in food intake, energy intake, water intake, body weight gain, faecal excretion, urine excretion, faecal moisture content, red blood cell parameters, platelet numbers, or white blood cell numbers, demonstrating that the FBP had no negative health effects and did not impact diet palatability.

The amount of bone present in the body, the bone's mineral content, and the bone's mineral density are three of a number of parameters measured to help diagnose bone health. Bone strength is dependent on both the quantity of minerals present (BMD) and the quality of the bone. Bone quality is a function of the morphology and architecture of the bone, as well as of the material properties of the bone itself. Clinically, the gold standard for measuring bone strength and bone quality is dual energy x-ray absorptiometry (DEXA) [36, 37]. DEXA measures bone area in the body and the amount of mineral in the bone, from which the bone mineral density (BMD) of the individual bone sections can be determined.

DXA scanning of the rat bones, both *in vivo* and *ex vivo*, produced matching results and demonstrated that both diets resulted in similar rates of bone mineral deposition. Control (CCC) rats had slightly, but significantly, larger bones, and this was accompanied by a greater mineral content in those bones; however, when normalised to bone area, the bone mineral density in the spine and femur did not differ between the two test groups. The difference in bone size was statistically significant by Student's t-test analysis due to the precise measurements of the DEXA scan, but represented only a small (~6%) difference between the CCC and FBP rats. It is likely that the slightly larger bones present in the CCC rats was at least part due to their slightly larger (2.6%) body weight. This probably represents natural variability within the population, and would not have any physiological relevance.

Calcium bioavailability as measured by calcium absorption and retention were identical between the two sources of calcium (FBP and CCC) fed to the rats. This finding matches data reported elsewhere in studies comparing fish bone calcium with other sources [27, 28].

The FBP rats had slightly more fat mass, and slightly less lean mass, compared to their CCC counterparts. However, the differences were not statistically significant and likely reflect natural variability within the population. The CBC analysis showed that the CCC rats had significantly fewer neutrophils and significantly more lymphocytes compared to the FBP rats. Similar differences have been reported under conditions of mild stress in rats [38, 39]. However, there was a large amount of inter-individual variability in these parameters. Blood films were made and will be assessed if a similar finding is noted in the adult rat study.

Taken together, the results of this study demonstrate that FBP, if present in a GRAS-format, could be an acceptable source of dietary calcium. While care would need to be taken to ensure that it is not ingested by persons with fish allergies, FBP has the benefit of being highly suitable for persons with dairy allergies.

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