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# Phytase and sodium diformate supplementation in a plant-based diet improves protein and mineral utilization in rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

A basal isonitrogenous and isoenergetic plant-based diet (Control) was supplemented with either 10 g $kg^{-1}$ sodium diformate (NaDF), 4000 FTU $kg^{-1}$ phytase (Phy) or a combination of both additives (NaDF + Phy). Three hundred juvenile rainbow trout with an average weight of 120 g were randomly distributed into 12 fibreglass tanks (300 L). After 65 days of trial, fish fed diets containing phytase, NaDF or the combination of both additives showed a higher growth rate ($P < 0.05$) compared to fish fed Control diet. NaDF increased feed intake ($P = 0.032$), while phytase inclusion resulted in a better feed conversion ratio ($P < 0.0001$) and a higher N retention efficiency ($P = 0.02$) compared with the Control. Apparent digestibility of P, Ca, Mg and Zn was improved by the use of phytase ($P < 0.005$) as well as P, Ca and Mg retention efficiency in fish ($P < 0.0001$). Using 4000 FTU $kg^{-1}$ phytase in plant-based diets resulted in a 13% and 50% reduction in N and P loadings, respectively. The use of NaDF in combination with phytase in a plant-based diet for rainbow trout resulted in a higher weight gain than that when NaDF was used alone.

KEY WORDS: digestibility, nutrient waste output, organic acid salt, phytase, phytate, rainbow trout

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## Introduction

There is a general interest in increasing the level of plant protein ingredients in feeds for farmed fish due to the rising demand, limited supply and high price of fishmeal (FM). However, inclusion is limited due to the presence of a wide variety of antinutritional factors (ANFs) present in such ingredients (Gatlin *et al.* 2007). Antinutritional factors are defined as innate components of an ingredient with an adverse effect on feed intake, digestion and nutrient absorption (Francis *et al.* 2001). Protease inhibitors, phytic acid, lectins, gossypol, glucosinolate and saponin are some of the most common ANFs in plant protein ingredients used in fish feeds (Tacon 1997). Some ANFs are thermolabile and can be disrupted by heating during ingredient and feed processing, while others, such as phytic acid (*myo*-inositol hexaphosphate, IP6), are heat-stable. Phytic acid is the main storage form of phosphorous (P) in seeds (Ravindran 1995), but it is practically unavailable for fish because they lack the intestinal digestive enzyme required to dephosphorylate the IP6 molecule (Jackson *et al.* 1996). As a consequence of low digestibility, most of the IP6-P is finally excreted into the water and may cause eutrophication and consequently changes in the aquatic ecosystem (Baruah *et al.* 2004).

Phytate (IP6) is considered primarily as a factor limiting P availability from plant ingredients; however, evidence shows that the deleterious effects of IP6 in single-stomached animals go beyond just limiting P availability (Ravindran 1995). IP6 is also a strong chelating agent of divalent minerals such as calcium (Ca), magnesium (Mg), zinc (Zn) and iron (Fe), reducing their bioaccessibility in the gastrointestinal tract (Greiner & Konietzny 2006), hence mineral retention in fish (Helland *et al.* 2006). IP6 may also form complexes with cationic groups of proteins

and amino acids present in feedstuffs, reducing their digestibility in fish, poultry and pigs (Kumar *et al.* 2012). In addition, IP6 forms complexes with the digestive proteases within the fish gut, particularly, with gastric pepsin (Morales *et al.* 2011).

Phytases are a special class of phosphatases that catalyse the sequential hydrolysis of IP6 to less phosphorylated *myo*-inositol derivatives and inorganic phosphate (Haros *et al.* 2007). Phytase activity is expressed as phytase units (FTU). One FTU is defined as the amount of enzyme activity that liberates 1 μmol inorganic orthophosphate per minute from 0.0051 mol $L^{-1}$ sodium phytate at pH 5.5 and 37 °C (Engelen *et al.* 1994). IP6 dephosphorylation by phytases can occur during feed processing (Denstadli *et al.* 2006a) or within the fish digestive tract. The net action of phytases on P bioaccessibility within the fish digestive tract may be affected by a number of different factors such as pH and temperature (Morales *et al.* 2011). The main commercial phytases used in animal nutrition are produced from *Escherichia coli* or *Peniophora lycii* strains. Such microbial phytases act efficiently under the acid conditions present in the stomach; however, not all phytases have the same functional pH profile. The bacterial phytase has two pH optima at 2.5 and 4.5, while the fungal phytase has an optimum pH at 5.5 (Elkhalil *et al.* 2007; Morales *et al.* 2011). On the other hand, pH dependence of phytase activity may be related to a certain extent on the solubility of IP6, which decreases as pH increases, forming insoluble salts of phytic acid at neutral pHs (Cheryan 1980). As described by Grynspan & Cheryan (1983), IP6 solubility is largely pH dependent, being more soluble at low pH and tending to precipitate as IP6-Ca at pH above 4.0. This suggests that only a fraction of the native IP6 is soluble and available as phytase substrate. In this sense, a low solubility of IP6 at pH near neutrality could be particularly disadvantageous for IP6 dephosphorylation by dietary phytase in fish species that are not capable to acidify the chyme at pH 4.0, as reported by several authors (Yúfera *et al.* 2004; Sugiura *et al.* 2006). The supplementation of diets with organic acids or their salts has been suggested to improve growth through improved digestion, absorption and retention of a variety of nutrients (Partanen & Mroz 1999). The suggested mode of action of organic acids in the digestive tract includes a reduction of pH in the stomach and in the small intestine, and inhibition of gram-negative bacteria by the undissociated form of the organic acids that passively diffuse through the bacteria cell wall, where it dissociates and inhibits the ability of the bacteria to multiply (Partanen & Mroz 1999).

The reduction of pH in the stomach would increase pepsin activity improving protein digestion (Mroz *et al.* 2000). Likewise, feed supplementation with organic acids has been shown to decrease duodenal pH, improve nitrogen retention and increase nutrient digestibility in pigs (Øverland *et al.* 2000). With respect to enzymatic IP6 dephosphorylation in the digestive tract, the use of organic acids could improve the efficacy of phytase through increased IP6 solubility due to the reduction in stomach pH. Potassium diformate (KDF), a specifically conjugated acid salt, has shown to improve growth performance in piglets and growing–finishing pigs (Øverland *et al.* 2000). The growth-promoting effect of KDF is based mainly on a strong antimicrobial effect against several bacteria, including total anaerobic, lactic acid and coliform bacteria (Øverland *et al.* 2000), and on the potential to improve the digestion, absorption and utilization of ingested dietary nutrients (Roth *et al.* 1998). To date, limited information exists on the effect of KDF on digestibility of nutrients in salmonids. Lückstädt (2008) reported an improved protein digestibility in Atlantic salmon (*Salmo salar*) when 13.5 g $kg^{-1}$ KDF was included to raw fish prior to FM production or prior to extrusion during feed manufacture. Storebakken *et al.* (2010) also found an increase in the digestibility of several amino acids in Atlantic salmon when diets were supplemented with 12 g $kg^{-1}$ KDF. However, there has been no research on the combined use of phytase and diformate in fish.

The aim of this study was to evaluate the effect of adding a microbial phytase and sodium diformate (NaDF), used alone and combined in plant protein-based diets for salmonids, on growth performance, feed conversion efficiency, digestibility and retention efficiency of protein and minerals, as well as the effects of both additives on N and P loading to the water.

## Materials and methods

### *Experimental diets*

The diets were produced at The Centre for Feed Technology, Norwegian University of Life Sciences, Ås, Norway. Four experimental diets were used, one Control diet (Control) and three diets including either 10 g $kg^{-1}$ of NaDF, 4000 FTU $kg^{-1}$ of *E. coli* phytase (Phy) or both (NaDF + Phy). The four diets were produced using gelatine and potato starch as a binder and included yttrium oxide ($Y_2O_3$) as an inert marker for determination of apparent faecal digestibility (Austreng *et al.* 2000). All dry ingredients, except gelatine, were mixed with a Moretti Forni kneading

**Table 1** Diet formulation and analysed chemical composition (based on dry matter)

| Diets | C | NaF | Phy | NaF + Phy |
|---|---|---|---|---|
| Ingredients (g $kg^{-1}$) | | | | |
| Sunflower exp. | 200 | 200 | 200 | 200 |
| Soy protein concentrate | 370 | 370 | 370 | 370 |
| Fishmeal | 70 | 70 | 70 | 70 |
| Fish oil | 180 | 180 | 180 | 180 |
| Gelatine | 70 | 70 | 70 | 70 |
| Potato starch | 92 | 82 | 91.2 | 81.2 |
| Premix[1] | 5 | 5 | 5 | 5 |
| L–Lys[2] | 3 | 3 | 3 | 3 |
| DL–Met[3] | 5 | 5 | 5 | 5 |
| $Y_2O_3$ [4] | 5 | 5 | 5 | 5 |
| NaDF[5] | – | 10 | – | 10 |
| Phytase[6] | – | – | 0.8 | 0.8 |
| Analysed content, $kg^{-1}$ | | | | |
| Dry matter, g | 945.9 | 945.7 | 948.9 | 926.2 |
| In dry matter | | | | |
| Crude protein, g | 427.1 | 432.3 | 430.3 | 423.2 |
| Crude fat, g | 185.8 | 189.7 | 188.3 | 187.3 |
| Starch, g | 101.1 | 97.1 | 103.5 | 98.4 |
| Ash, g | 58.1 | 62 | 58 | 63.2 |
| Phosphorus, g | 7.35 | 7.31 | 7.25 | 7.10 |
| Calcium, g | 5.51 | 5.66 | 5.42 | 5.45 |
| Magnesium, g | 2.72 | 2.7 | 2.69 | 2.62 |
| Zinc, g | 0.17 | 0.16 | 0.16 | 0.16 |
| IP6, g[7] | 13.2 | 13.2 | 9.2 | 8.4 |
| Gross energy, MJ | 22.2 | 22.3 | 22.2 | 21.8 |

[1] Vitamin and mineral premix. Normin Premix Fish. Normin, Norway.
[2] L–Lysine, 996 g $kg^{-1}$, Calbiochem, An Affilate KGaA, Darmstadt, Germany.
[3] DL–Methionine, 990 g $kg^{-1}$, Alfa Aesar, Germany.
[4] Metal Rare Earth Limited, Shenzhen, China.
[5] ADDCON, Bonn, Germany.
[6] Quantum® Phytase 5000 L (5000 FTU $mL^{-1}$), AB Vista, Germany.
[7] *myo*-inositol hexaphosphate (IP6).

machine (Spiry 25, Mondolfo, Italy). The phytase was diluted in 1 L cold water and sprayed on the dry ingredient mixture before the addition of fish oil. The gelatine was mixed in 90 °C water and added to the dry mixture to produce a firm dough that was cold pelleted through an Italgi extruder (P35A; Carasco, Italy) equipped with a 4-mm-diameter die. The formulation and chemical composition of the experimental diets are detailed in Table 1.

## Fish and rearing conditions

The experiment was carried out with rainbow trout (*Oncorhynchus mykiss*) at the Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway. A total of 300 fish with an average weight of 120 g were randomly distributed into 12 fibreglass tanks (300 L) illuminated 24 h per day. Each diet was supplied to three groups of 25 fish over a period of 65 days. Each tank was supplied with recirculating freshwater with an 80% degree of recirculation and a water flow rate of 7.5 L $min^{-1}$. Water temperature ranged between 11 and 15 °C. The fish were fed daily every 30 min for seven hours using automatic band feeders. Uneaten feed was collected daily from the outlet water of each tank and stored at −22 °C prior to analysis, and feed intake was monitored according to the method used by Helland *et al.* (1996). The feeding rate was planned to be 10% in excess and adjusted every day in accordance with uneaten feed.

## Sampling

All sampling was carried out on randomly captured individuals from each tank. Prior to distribution of the fish, 15 fish were taken to determine the proximate composition. Fish were killed by a blow to the head, and the gut was opened to remove the digestive content. Samples of whole fish were kept frozen until analysis. At the end of the experiment (day 65), all fish were anaesthetized with 60 mg $L^{-1}$ tricaine methanesulfonate (MS222; Argent Chemical Laboratories, Redmount, WA, USA) and faeces were stripped as described by Austreng (1978). A sample of five fish per tank was taken to determine the final proximate composition. The faecal and whole fish samples from each tank were pooled and frozen until analysis.

## Chemical analysis

The dry matter of diets, freeze-dried faeces and homogenates of whole fish were determined by drying at 104 °C for 16 h. Ash was determined gravimetrically after combustion at 550 °C for 16 h. Nitrogen was determined by combustion using a Fisons EA1108 elemental analyser (Danvers, MA, USA), following the Dumas process, and crude lipid was determined after hydrolysis with petroleum ether on an Accelerated Solvent Extractor (ASE200) from Dionex (Sunnyvale, CA, USA). Starch was determined as glucose after starch hydrolysis with a heat-tolerant amyloglucosidase in accordance with the procedure of McCleary *et al.* (1994) and gross energy by bomb calorimetry (Parr 1271 Bomb calorimeter; Parr, Moline, IL, USA). For elemental analysis, a mixture of $HNO_3$ and $H_2O_2$ (1 : 2, v/v) was added to the samples and samples were microwaved in a Multiwave 3000 (Anton Paar GmbH, Graz, Austria). The initial effect of 300 W was gradually increased to 1000 W

over 5 min and then maintained for an additional 15 min. Temperature and pressure were kept constant above 230 °C and 73 bars, respectively. Following cooling to a temperature below 60 °C, the samples were analysed for elements using an ICP-AES Thermo Jarrell Ash Polyscan (Thermo Inc., Woburn, MA, USA).

Inositol phosphates were extracted from feed and faeces samples based on the method of Newkirk & Classen (1998). Duplicate samples (0.5 g DM) were extracted with 0.5 M HCl (5 mL) for 3 h at 20 °C under magnetic stirring; 0.5 mL of each sample was placed in an ultracentrifugal filter device (Microcon YM– 30; Millipore, Bedford, MA, USA) and centrifuged at 12 000 ***g*** for 10 min. Approximately 1.0 mL chloroform was added to the filtrate, vortexed and then centrifuged again at 12 000 ***g*** for 10 min. The top layer was transferred to glass vials for IP6 analysis. Inositol phosphates in diets and faeces were separated on an Ultimate 3000 HPLC system (Dionex) equipped with a Dionex CarboPac PA1 guard column and a CarboPac PA1 analytical column. A gradient with 1.5 M methanesulfonic acid and water was used as the mobile phase at 0.8 mL $min^{-1}$ (Blaabjerg *et al.* 2010). The eluents were mixed with 1 g $L^{-1}$ $Fe(NO_3)_3 \times 9H_2O$ in a 20 g $L^{-1}$ solution of $HClO_4$ (0.4 mL $min^{-1}$) in a postcolumn reactor according to the method of Imanari *et al.* (1982). The inositol phosphates were detected at 290 nm and quantified against an external IP6 standard curve using the Chromeleon software (Dionex).

### Calculations and statistical analysis

Thermal growth coefficients were calculated as $1000 \times (BW_{final}{}^{1/3} - BW_{initial}{}^{1/3})/\Sigma d°$, where BW represents the body weight and $\Sigma d°$ represents thermal sum (mean daily temperature in °C × days of the period). Feed conversion ratio (FCR) was calculated as $DM_{feed} \times (BW_{final} - BW_{initial})^{-1}$. Apparent digestibility (ADC) of individual nutrients was calculated as follows: $100 \times [1 - (D_i \times F_i{}^{-1} \times F_n \times D_n{}^{-1})]$, where $D_i$ and $F_i$ represent the concentration of inert marker in diet and faeces, and $D_n$ and $F_n$ represent the concentration of nutrients in diet and faeces, respectively. Nutrient retention was calculated as $100 \times [(BW_{final} \times N_{final}) - (BW_{initial} \times N_{initial})] \times [FCR \times (BW_{final} - BW_{initial}) \times N_{diet}]^{-1}$, where $N_{diet}$ is the content of nutrient in the diet, and $N_{initial}$ and $N_{final}$ represent the initial and final concentration of nutrient in whole minced fish. Solid nitrogen (N) or phosphorus (P) waste loading was calculated as N or P intake × (100 − ADC of N or P) × $100^{-1}$. Dissolved N or P waste loadings were calculated as (N or P intake × ADC × $100^{-1}$) − N or P retained in whole body fish).

The mean values are reported ± the standard deviation of mean (SD) from three replicates by treatment. After verification of the assumptions of normality and homoscedasticity, data were subjected to two-way ANOVA, where Phy and NaDF were the class variables. Differences between means are significant at $P < 0.05$. All the analyses were performed using the STATGRAPHICS software package (STSC Software Group, Rockville, MD, USA).

## Results

### Diets, growth and feed utilization

Diets manufactured were isonitrogenous and isoenergetic, but the addition of NaDF to diets increased the content of ash by 5 g $kg^{-1}$ DM. The content of phytic acid in diets without inclusion of phytase was 13.2 g IP6 $kg^{-1}$ DM, while this value was reduced by 30–36% by the action of phytase during feed manufacturing (Table 1).

With the exception of one fish in the group receiving only phytase, no mortalities occurred during the experiment. After 65 days of treatment, fish fed diets containing NaDF ($P < 0.005$) or phytase ($P < 0.0005$) showed a higher growth rate and final body weight (Table 2) compared with those fed the Control diet. The use of NaDF in combination with phytase in a plant-based diet for rainbow trout resulted in a higher weight gain than that when NaDF was used alone ($P < 0.05$).

Feed intake was significantly increased by the addition of 10 g $kg^{-1}$ NaDF in the diet ($P = 0.032$) compared with the Control group. However, the inclusion of phytase in the diet did not affect feed intake. The data on FCR are presented in Table 2. The addition of phytase to diets improved FCR of fish compared to the diets with no phytase ($P < 0.0001$), whereas the addition of NaDF to diets did not affect FCR.

### Digestibility of nutrients

The effect of Phy and NaDF addition to the plant protein-based diet on nutrient ADC is given in Table 3. The addition of phytase significantly increased ($P < 0.05$) the ADC of dry matter and several minerals, including P, Ca, Mg and Zn. As expected, P was the mineral most affected by phytase, with ADC increasing from 38% in the Control group to 73% and 76% in the 'Phy' and 'NaDF + Phy' groups, respectively. Protein and energy digestibilities were not affected by phytase supplementation. The use of NaDF did not affect the ADCs of any of the parameter evaluated,

**Table 2** Growth, feed intake and feed conversion ratio of rainbow trout fed the experimental diets (mean ± SD, $n$ = 3 tanks diet$^{-1}$)

| | Diets | | | | *P*-value* | | |
|---|---|---|---|---|---|---|---|
| | C | NaDF | Phy | NaDF + Phy | Phy | NaDF | Interaction |
| Initial weight | 120.4 ± 0.1 | 120.7 ± 0.2 | 120.7 ± 0.2 | 120.4 ± 0.2 | 0.404 | 0.404 | 0.351 |
| Final weight | 333.6 ± 2.9 | 346.4 ± 2.3 | 350.7 ± 4.9 | 357.2 ± 5.0 | *0.0003* | *0.0028* | 0.204 |
| TGC × 1000 | 2.25 ± 0.02 | 2.35 ± 0.02 | 2.38 ± 0.03 | 2.43 ± 0.04 | *0.0002* | *0.0023* | 0.197 |
| Feed intake[1] | 179.0 ± 2.7 | 188.6 ± 1.2 | 182.0 ± 4.8 | 186.0 ± 7.1 | 0.948 | *0.032* | 0.314 |
| FCR[2] | 0.84 ± 0.00 | 0.84 ± 0.01 | 0.79 ± 0.00 | 0.79 ± 0.01 | *<0.0001* | 0.4223 | 0.942 |

TGC, thermal growth coefficient.
[1] As dry matter basis.
[2] Feed conversion ratio: feed intake (as dry matter basis)/body weight gain.
* Values in italics indicate significant differences between means.

**Table 3** Apparent digestibility (ADC, %) of dry matter, protein, phosphorus (P), calcium (Ca), magnesium (Mg), zinc (Zn), energy and IP6 in rainbow trout fed the experimental diets (mean ± SD, $n$ = 3 tanks diet$^{-1}$)

| | Diets | | | | *P*-value* | | |
|---|---|---|---|---|---|---|---|
| | C | NaDF | Phy | NaDF + Phy | Phy | NaDF | Interaction |
| Dry matter | 68.5 ± 1.5 | 68.8 ± 2.4 | 72.5 ± 1.3 | 72.3 ± 2.6 | *0.0128* | 0.893 | 0.893 |
| Protein | 90.4 ± 1.2 | 91.7 ± 0.5 | 92.0 ± 0.2 | 91.9 ± 0.6 | 0.134 | 0.347 | 0.134 |
| P | 37.9 ± 0.8 | 38.8 ± 2.5 | 73.2 ± 8.2 | 75.7 ± 4.6 | *<0.0001* | 0.537 | 0.865 |
| Ca | –25.1 ± 9.1 | –20.4 ± 1.4 | 2.8 ± 5.0 | 0.0 ± 7.8 | *0.0002* | 0.796 | 0.355 |
| Mg | 39.2 ± 1.5 | 42.4 ± 1.1 | 54.7 ± 4.6 | 56.8 ± 3.7 | *<0.0001* | 0.187 | 0.861 |
| Zn | 20.5 ± 3.2 | 18.4 ± 6.1 | 32.6 ± 8.0 | 35.1 ± 1.8 | *0.0017* | 1.000 | 0.475 |
| Energy | 79.1 ± 1.4 | 79.3 ± 1.4 | 80.1 ± 0.9 | 79.4 ± 2.7 | 0.626 | 0.626 | 0.423 |
| IP6 | 22.6 ± 16.4 | 17.5 ± 15.1 | 86.5 ± 3.7 | 92.7 ± 3.9 | *<0.0001* | 0.938 | 0.418 |

* Values in italics indicate significant differences between means.

neither in the presence nor in the absence of phytase (Table 3).

### *Nutrient retention and waste loading*

The influence of Phy and NaDF supplementation on fish whole body nutrient content is shown in Table 4. The addition of phytase significantly increased ($P < 0.05$) the body content of P, Ca and Mg, without significantly affecting the content of N and Zn. In addition, the diets containing phytase resulted in a higher Ca:P ratio in whole body fish (≈0.9) compared with that observed in fish fed diet without phytase (≈0.7).

Despite no effect of Phy and Phy + NaDF treatments on protein digestibility and N content in the whole body, the diets containing phytase resulted in a significantly higher ($P = 0.02$) retention efficiency of N in fish compared with the Control diet (Table 5). Results suggest that NaDF did

**Table 4** Content of nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg) and zinc (Zn) in whole body rainbow trout (kg$^{-1}$) at start and after being fed the experimental diets (mean ± SD, $n$ = 3 tanks diet$^{-1}$)

| | | Diets | | | | *P*-value* | | |
|---|---|---|---|---|---|---|---|---|
| | Initial levels | C | NaDF | Phy | NaDF + Phy | Phy | NaDF | Interaction |
| Dry matter, g | 287 | 324 ± 7 | 323 ± 12 | 310 ± 14 | 310 ± 5 | 0.052 | 0.967 | 0.980 |
| N, g | 25.4 | 27.1 ± 0.1 | 27.2 ± 0.7 | 27.0 ± 0.4 | 26.8 ± 0.5 | 0.478 | 0.900 | 0.574 |
| P, g | 4.69 | 3.16 ± 0.08 | 3.12 ± 0.06 | 4.19 ± 0.14 | 4.02 ± 0.20 | *<0.0001* | 0.231 | 0.459 |
| Ca, g | 5.21 | 2.15 ± 0.18 | 2.11 ± 0.03 | 3.93 ± 0.23 | 3.72 ± 0.34 | *<0.0001* | 0.256 | 0.522 |
| Ca:P ratio | 1.11 | 0.68 ± 0.04 | 0.68 ± 0.00 | 0.94 ± 0.02 | 0.92 ± 0.04 | *<0.0001* | 0.583 | 0.713 |
| Mg, mg | 302 | 265 ± 2 | 262 ± 8 | 311 ± 5 | 305 ± 10 | *<0.0001* | 0.300 | 0.703 |
| Zn, mg | 24.6 | 23.7 ± 0.5 | 23.9 ± 1.7 | 22.9 ± 1.0 | 23.9 ± 2.3 | 0.701 | 0.503 | 0.648 |

* Values in italics indicate significant differences between means.

**Table 5** Retention efficiency (% of nutrient intake) of nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), zinc (Zn) and energy in rainbow trout fed the experimental diets (mean ± SD, $n = 3$ tanks diet$^{-1}$)

| | Diets | | | | *P*-value* | | |
|---|---|---|---|---|---|---|---|
| | C | NaDF | Phy | NaDF + Phy | Phy | NaDF | Interaction |
| N | 48.8 ± 0.3 | 48.8 ± 2.4 | 51.2 ± 0.8 | 51.9 ± 2.0 | *0.0194* | 0.703 | 0.703 |
| P | 37.2 ± 2.2 | 37.3 ± 1.9 | 68.5 ± 3.6 | 66.0 ± 5.6 | *<0.0001* | 0.607 | 0.566 |
| Ca | 9.1 ± 6.6 | 9.9 ± 1.3 | 76.4 ± 7.9 | 69.4 ± 12.4 | *<0.0001* | 0.517 | 0.432 |
| Mg | 10.7 ± 0.1 | 10.7 ± 0.6 | 14.8 ± 0.3 | 14.9 ± 0.8 | *<0.0001* | 1.000 | 1.000 |
| Zn | 16.7 ± 0.6 | 17.4 ± 1.7 | 17.0 ± 1.1 | 18.9 ± 3.1 | 0.42 | 0.269 | 0.599 |
| Energy | 52.7 ± 1.5 | 52.9 ± 4.0 | 49.6 ± 3.4 | 51.8 ± 1.2 | 0.255 | 0.487 | 0.549 |

* Values in italics indicate significant differences between means.

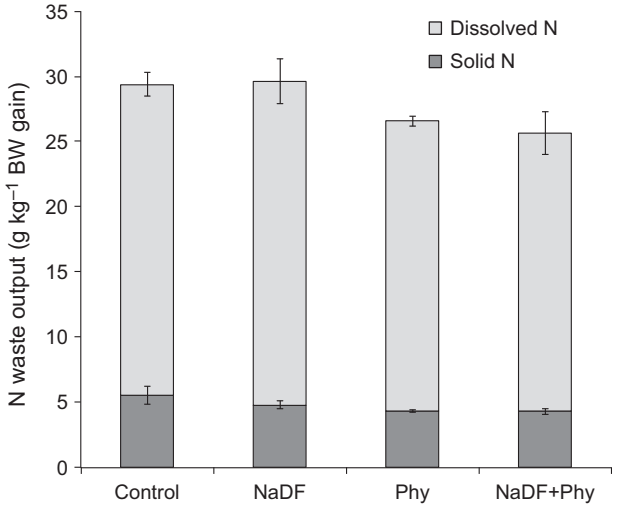


**Figure 1** Solid and dissolved nitrogen waste loading (g kg$^{-1}$ BW fish gain) of rainbow trout fed the experimental diets (mean ± SD, $n = 3$ tanks diet$^{-1}$). ANOVA *P*-values: $P = 0.001$ (Phy); $P = 0.604$ (NaDF); $P = 0.407$ (Interaction).

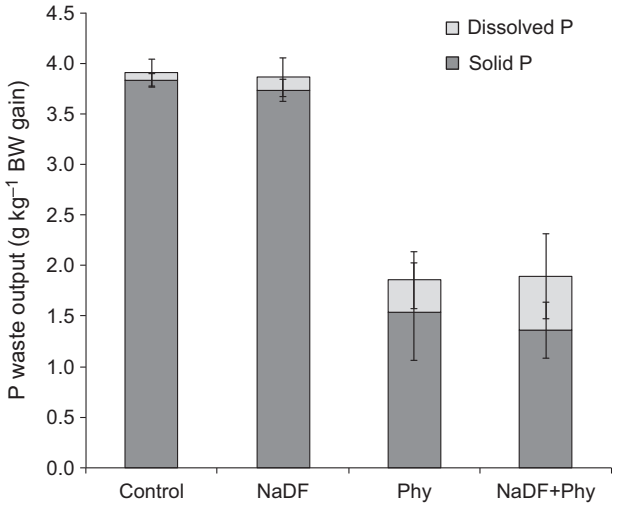


**Figure 2** Solid and dissolved phosphorus waste loading (g kg$^{-1}$ BW fish gain) of rainbow trout fed the experimental diets (mean ± SD, $n = 3$ tanks diet$^{-1}$). ANOVA *P*-values: $P = 0.0001$ (Phy); $P = 0.971$ (NaDF); $P = 0.745$ (Interaction).

not improve nitrogen or mineral retention in fish, while Phy and Phy+NaDF supplementation increased the retention of P, Ca and Mg ($P < 0.0001$). The influence of Phy and NaDF supplementation of a plant protein-based diet on N and P emission is shown in Figs 1 & 2. All fish groups showed a low fraction of solid N discharge (≈5 g kg$^{-1}$ BW gain) compared with the soluble N fraction (≈23 g kg$^{-1}$ BW gain). The use of NaDF as a feed additive did not change N loadings from fish. However, fish fed diets with phytase inclusion showed a clear reduction (13%) in total N waste loading ($P = 0.001$). On the other hand, total P loadings from fish were reduced by 50% by the use of phytase in the plant protein-based diet, mainly affecting the solid P fraction (from ≈3.8 to 1.4 g kg$^{-1}$ BW gain).

## Discussion

In the present experiment, fish triplicated their initial weight and the use of phytase resulted in an increased growth rate and a reduced FCR. It is recognized that phytase inclusion in diets containing native IP6 increases growth rate in fish as a consequence of the hydrolysis of the IP6. Feeding phytase-supplemented diets has previously also shown to improve growth performance in salmonids (Dalsgaard *et al.* 2009; Carter & Sajjadi 2011; Vandenberg *et al.* 2012). Also, the findings observed in the present experiment agree with the general consensus that supplementing IP6-containing diets with phytase neutralizes the negative effects of IP6 and increases growth in fish (Kumar *et al.* 2012), explained mostly by a positive effect of the additive on ADC of minerals and their deposition in the

fish. Cao *et al*. (2007) reviewed that phytase dose at a level of 250–2000 FTU $kg^{-1}$ feed is usually considered optimum for many fish species. However, taking into account the discrepancy in the results reported in the literature, it is difficult to make detailed comparison among different studies. For example, Rodehutscord & Pfeffer (1995) showed that the addition of 1000 U enzyme $kg^{-1}$ diet increased feed intake and weight gain in rainbow trout without improving FCR, while Vielma *et al*. (1998) reported that inclusion of 1500 FTU $kg^{-1}$ feed improved apparent availability of P, bone ash, plasma and body P concentrations. However, Forster *et al*. (1999) supplemented different levels of phytase in a canola protein concentrate for rainbow trout, concluding that 4500 FTU $kg^{-1}$ diet was needed to improve P availability. These differences could be explained considering that the net efficiency of the enzyme depends on a number of factors, such as source of phytase, water temperature, native IP6 content in the diet, as well as gastric pH of fish (Morales *et al*. 2011). Under the experimental conditions of the present study, the inclusion of 4000 FTU $kg^{-1}$ diet resulted in an increase in weight gain from 191% to 197% and improvements in FCR from 0.84 to 0.79, during the 65-day experimental period.

Previous studies indicated that dietary microbial phytase in plant-based diets fed to rainbow trout significantly improved the digestibility of P, with a clear positive dose-dependent response to phytase between 400 and 4500 FTU $kg^{-1}$ diet (Sugiura *et al*. 2001; Vielma *et al*. 2004). Sajjadi & Carter (2004) reported that 2000 FTU $kg^{-1}$ diet using a canola meal-based diet for Atlantic salmon resulted in a higher P digestibility and retention in fish.

Other studies with rainbow trout (Sugiura *et al*. 2001; Cheng & Hardy 2003) reported that phytase addition to soybean meal-based diet increased absorption and retention of not only P, but also Ca, Mg, Mn, Cu, Fe and Zn. A recent study carried out by Vandenberg *et al*. (2012) found that 3000 FTU $kg^{-1}$ diet increased ADC and bioavailability of a range of nutrients from plant protein-based diets for rainbow trout. Similarly, the findings obtained in the present study also indicated that phytase addition improved not only P utilization, but also Ca, Mg and Zn digestibility resulting in an increased retention and bone content of Ca and Mg. This can be explained by the disruption effect of the phytase on inositol phosphate–mineral complexes present naturally in plant ingredients (Morales *et al*. 2013). The observed increase in Zn ADC related to phytase inclusion (from 20.5% to 32.6%) was not reflected in the body content or retention of this mineral. Vielma *et al*. (2004) also reported that Zn digestibility increased in rainbow trout fed soybean meal-based diets supplemented with 4000 FTU $kg^{-1}$ phytase, but found no effect with 2000 FTU $kg^{-1}$ phytase supplementation.

In the present study, the content of phytic acid in diets without inclusion of phytase was 13.2 g IP6 $kg^{-1}$ DM. This value was reduced by 30% to 36% by the action of phytase during feed manufacturing. Dephosphorylation of native IP6 seemed to progress within the digestive tract of the fish fed phytase-supplemented diet and resulted in a significant reduction of its content in faeces (from 32–34 to 4–6 mg $g^{-1}$ faecal DM), and in a significant improvement of the P ADC (from 38% to 76%). This indicates that phytase inclusion in a plant-based diet for rainbow trout can dephosphorylate up to 90% the native inositol phosphate from plant ingredients. These results agree with those reported by Vielma *et al*. (2004) who evaluated a semi-purified diet containing 500 g $kg^{-1}$ soybean meal with phytase levels of 0, 500, 1000, 2000 and 4000 FTU $kg^{-1}$. In that study, phytase decreased IP6 content of faeces from 35 to 5 mg IP6 $g^{-1}$ faecal dry matter and ADC coefficient of P improved from 23% to 83%.

Fish fed diets without phytase inclusion showed IP6 ADC coefficients between 17% and 22% (Table 3). Despite it is recognized that carnivorous species like rainbow trout have low ability to hydrolyse the IP6, some previous studies indicated that this species is capable to dephosphorylate the IP6 to a variable extent. For example, Forster *et al*. (1999) and Denstadli *et al*. (2006b) reported 5% and 15% IP6 digestibility, respectively, in rainbow trout while in some omnivorous species such as tilapia, a 50% digestibility of IP6 has even been observed (Ellestad *et al*. 2002). As suggested by Denstadli *et al*. (2006b), the high ADC of IP6 in the absence of an exogenous phytase could be explained by a triggering effect of IP6 on the phosphatases present in the intestinal epithelium, but more work needs to be conducted in order to elucidate the suggested mechanism.

In the present study, total P discharge from fish was reduced by 50% when fish were fed diets containing phytase. The solid P loading output was the fraction most affected by phytase treatments, diminishing the P discharges to 2.4 g $kg^{-1}$ BW gain. Despite the fact that total P discharges were reduced by phytase, the soluble P fraction was higher when fish were fed diets supplemented with phytase, increasing from 0.1 g to 0.3–0.5 g $kg^{-1}$ BW gain. A possible explanation for this may be that phytase action within the gastrointestinal tract could have increased the bioavailability of P to a level exceeding the P requirement of the fish. Similar results were reported by Dalsgaard

*et al*. (2009) who observed that dissolved fraction of P discharged from rainbow trout fed a diet containing ≈ 1400 FTU $kg^{-1}$ was higher than that observed in fish fed a diet without phytase inclusion.

Taking into account the ADC coefficients of dietary P and energy, the estimated level of ingested P was 0.16 g available P $MJ^{-1}$ digestible energy, which is below the estimated P requirement of 0.25 g available P $MJ^{-1}$ digestible energy recommended for this species by Rodehutscord (1996). Regardless of this, no clinical signs of P deficiency as described by Sugiura *et al*. (2004) were observed. In fact, the higher growth rate observed in fish fed the NaDF diet without phytase inclusion would indicate that P was not a limiting nutrient for growth in Control diet (Table 2). This supports the generally accepted idea that the P requirement for maximal growth rate is lower than that for maximal tissue P levels (Prabhu *et al*. 2013). Thus, the use of 4000 FTU $kg^{-1}$ diet enhanced the P availability of the plant protein-based diet (Table 3), resulting in a higher P retention in fish tissues, but at the expense of increased dissolved P excretion.

There is *in vitro* and *in vivo* evidence suggesting that native IP6 can reduce the bioavailability of the dietary protein by the formation of insoluble IP6–protein complexes (Kumar *et al*. 2012). Even IP6 can non-selectively bind to functional proteins, inhibiting the proteolytic activity of several enzymes such as pepsin and trypsin (Kies *et al*. 2006; Morales *et al*. 2013, 2014).

A number of studies demonstrate that the use of phytase as additive in pretreatment of plant ingredients and feeds leads to an increased apparent protein digestibility in salmonids (Cheng & Hardy 2003; Vielma *et al*. 2004; Vandenberg *et al*. 2012). Nevertheless, other studies did not found such a positive effect (Lanari *et al*. 1998; Storebakken *et al*. 1998; Sajjadi & Carter 2004). Phytase addition in poultry and pig diets also showed the conflicting results observed in fish (Kumar *et al*. 2012). Different responses documented that phytase treatment may be the consequence of a combination of factors related to the phytase doses used in the assays (500–4000 FTU $kg^{-1}$ diet), IP6 content in experimental diets and the stomach pH reached by the different species, which strongly affects IP6 solubility and phytase action.

In the current study, the use of phytase as an additive in a plant-based diet for rainbow trout did not affect the ADC of the protein nor the N content of the final whole body compared to the Control diet without the enzyme. However, fish fed Phy and Phy + NaDF diets showed a higher N retention efficiency in contrast to those fed non-phytase diets. This last finding agrees with several studies performed both in salmonids and in non-salmonid species (Cao *et al*. 2007; Kumar *et al*. 2012). Under the experimental conditions of the present study, a better N retention as a result of phytase addition resulted in a reduction in N excretion from 30 to 26 g $kg^{-1}$ BW gain. These results agree with those obtained by Vielma *et al*. (2004) with the same species, who observed that the addition of a microbial phytase to a diet including 600 g $kg^{-1}$ of soy protein concentrate reduced the N loading from 50.3 to 36.3 g $kg^{-1}$ BW gain. As occurs in terrestrial animals, despite evidence that phytase can produce a positive effect on dietary protein utilization in fish, mainly when high content plant-based diets are used, the direct and indirect effects of the enzyme on protein digestibility and utilization are somewhat contentious. More research is needed to obtain a better understanding of the effects of enzymatic IP6 dephosphorylation on protein utilization in fish.

In the present study, the addition of 10 g $kg^{-1}$ NaDF increased feed intake and weight gain of rainbow trout. Several studies have reported the growth-promoting effect of organic acids and their salts on growth in both terrestrial animals (Øverland *et al*. 2000) and fish (Ringø 1991; Hossain *et al*. 2007; Lückstädt 2008; Zhou *et al*. 2009). In most cases, the positive response to organic acids or their salts on growth can be explained not only by an increase in feed intake, but also related to a higher ADC and nutrient retention efficiency of several nutrients such as protein and minerals that resulting in a better FCR. In pigs, dietary inclusion of KDF increased the ADC of dietary protein, ash and several minerals (Roth *et al*. 1998; Mroz *et al*. 2002). In fish, Lückstädt (2008) reported an improved protein digestibility in Atlantic salmon when 13.5 g $kg^{-1}$ KDF was added to the diet. Similarly, Storebakken *et al*. (2010) reported that dietary inclusion of 12 g $kg^{-1}$ KDF improved protein and individual amino acid digestibility in Atlantic salmon fed a plant-based diet. There is limited information available on the effects of adding NaDF in fish diets. Ringø (1992) found no significant effect of adding 10 g $kg^{-1}$ of sodium formate on the digestibility of protein or lipid in diets for Arctic charr (*Salvelinus alpinus* L.). More recently, Gao *et al*. (2011) reported that supplementing diets with 10 g acid moiety $kg^{-1}$ of sodium formate and butyrate blend (ratio 2 : 1 on acid moiety weight basis) did not improve growth rate or feed utilization of rainbow trout. However, Morken *et al*. (2011) observed that the addition of 10.6 g $kg^{-1}$ NaDF to diets improved the digestibility of lipid, ash, crude protein, total and individual amino acids in rainbow trout.

In contrast with most previous studies that evaluated the effect of organic acids and their salts, the findings obtained in the present work suggest that NaDF did not affect ADC of the dry matter, protein, and energy, P, Ca, Mg or Zn.

Both in terrestrial animals and in fish, the positive effects of dietary organic acids or their salts on nutrient digestibility can be attributed to a decreased gastric pH that favouring the activity of gastric pepsin as well as stimulating pancreatic secretions (Partanen & Mroz 1999). However, in the present study, the salt used had a low acidification capacity; hence, its positive effect on growth could not be totally explained by a reduction in the gastric pH as suggested by Morken *et al*. (2011).

As reported for piglets and growing–finishing pigs (Øverland *et al*. 2000), organic acid salts can improve the general health status of farmed animals by its stronger antimicrobial effect on *E. coli* and *Salmonella* sp., than on *lactobacilli* in fish (Zhou *et al*. 2009). Abu Elala & Ragaa (2014) reported that *Oreochromis niloticus* fed 3 g $kg^{-1}$ KDF exhibited not only enhanced growth performance and apparent protein digestibility, but also a eubiotic effect on the proliferation of indigenous acid lactic bacteria, which plays a prominent role in activation of the immune response against diseases.

Despite different findings reported in previous works regarding to feed intake, growth, feed conversion, nutrients digestibility and retention, which can depend on a number of experimental factors such as fish species, rearing conditions, diet compositions, source of organic acid salts used and their inclusion level, the higher growth rate observed in fish fed NaDF compared with the Control treatment in the present study could be related to antimicrobial effect of the salt. In this way, as suggested by Suzer *et al*. (2008) and Askarian *et al*. (2011), a beneficial effect of the salt on lactic acid bacteria could improve the activity of intestinal digestive enzymes activity in fish and therefore improve nutrient absorption and growth performance. More research on these specific interactions is needed to understand the mode of action of organic acid salts in fish.

## Conclusion

The findings of this study show that microbial phytase can increase fish growth rate, FCR, mineral digestibility and protein retention in fish fed plant-based diets. As a result, the excretions of N and P to the water were reduced, especially for P, which was reduced by 50%. The use of NaDF increased feed intake and growth rate. The combined use of phytase and the organic acid salt in a plant-based diet for rainbow trout resulted in a higher weight gain than that when both additives were used individually.

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