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ORIGINAL RESEARCH

# Metabolic insights in Arctic charr (*Salvelinus alpinus*) fed with zygomycetes and fish meal diets as assessed in liver using nuclear magnetic resonance (NMR) spectroscopy

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**Abstract** Fish meal is the major source of animal protein in feed for cultured salmonid fish, but its availability is limited and it must eventually be replaced by alternative protein-rich feed ingredients. Zygomycetes (*Rhizopus oryzae*) is a fungus with protein-rich biomass that could replace fish meal protein in fish feed. Using an $^{1}$H NMR spectroscopy approach, we studied the metabolic pattern in liver tissues of Arctic charr (*Salvelinus alpinus*) fed a commercial diet with unknown composition (ST), a diet with all protein from fish meal (FM) and a diet with most of the protein from zygomycetes biomass (FZ). No significant difference ($p \geq 0.05$) was found in spectral data between FM and FZ using the OPLS-DA fitted model. However, other models showed that diet ST clearly differed ($p < 0.05$) from diets FM and FZ. Signals for acetate, β-alanine, choline, creatine, formate, glucose, inosine, lysine, SN-glycero-3-phosphocholine and two unknown metabolites were higher in fish fed diets FM and FZ than in fish fed diet ST. These results show that the metabolic profile in liver of Arctic charr will remain unchanged if fish meal protein is replaced with zygomycete protein, suggesting similar physiological responses to both feed ingredients. In contrast, feeding a commercial diet altered the metabolic fingerprint compared with diets FZ and FM, suggesting important differences in ingredient composition and the physiological response to this diet.

**Keywords** Arctic charr · Metabolomics · NMR · Metabolites · Zygomycetes · Fish meal · Diets · Fish

## Introduction

Fish meal constitutes the major source of animal protein in commercial feed for cultured salmonid fish (Sargent and Tacon 1999). However, the availability of fish meal derived from marine fish is limited (Barlow 2000; Tacon 2004). Moreover, the use of fish meal in fish feed is not a sustainable long-term feeding strategy. Therefore, alternative sources of protein that can be used to replace fish meal need to be explored. This is an essential prerequisite in establishing sustainable fish farming (Kristofersson and Anderson 2006).

Zygomycetes (*Rhizopus oryzae*) is a microfungus with biomass that is rich in protein and may have the potential to replace fish meal in fish feed (Edebo 2009). In addition to protein, the fungal biomass is rich in

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carbohydrates (Sues et al. 2005). The carbohydrate fraction is made up of chitin/chitosan heteropolysaccharides, which can vary in content (45–85 %) depending upon fungal strain and cultivation conditions (Zamani 2010). Zygomycetes has been widely used for food production and for production of different organic substances and extra-cellular enzymes (Sues et al. 2005; Dijksterhuis and Samson 2006).

Metabolomics has emerged as a promising novel tool to obtain detailed insights into the impact of nutrition on the metabolic response in animals and humans (Bankefors et al. 2011; Gibney et al. 2005; Brennan 2013). The technique allows rapid identification in biological samples of key metabolites that can be related to nutritional interventions (Moco et al. 2006; Wishart et al. 2007) and provides information that cannot be achieved with more conventional approaches. The proton nuclear magnetic resonance (NMR) technology has been applied extensively in the field of metabolomics in food science research to assess physiological and biological responses to various food items and diets, e.g. chamomile tea (Wang et al. 2005), whole-grain flour (WGF) and refined wheat flour (RF) (Fardet et al. 2007),vegetarian, low-meat and high-meat diets (Stella et al. 2006), and vitamin supplementation (Griffin et al. 2002). The technique has also been successfully applied to determine the biochemical effects of bioactive compounds and the metabolic profile in individuals fed a variety of diets, using organ extracts from different organs (e.g. liver, kidney, stomach, intestine) (Waters et al. 2002; Mun et al. 2004; Ramadan et al. 2005; Serkova et al. 2005).

Liver is the most metabolically active organ. The liver of the fish is involved in metabolism of absorbed nutrients, export of substrates (amino acids, fatty acids, carbohydrates) and synthesis of essential macromolecules (Bystriansky et al. 2007). The aim of the present study was to compare biochemical effects and metabolic profile in the liver of Arctic charr fed a fish meal-based diet (FM), a zygomycete-based diet (FZ), and a commercial diet (ST) using an $^{1}$H NMR spectroscopy approach. Our working hypothesis was that the metabolic fingerprint of feeding diets FM and FZ would be similar due to their comparable nutrient profiles. Thus, we assumed that zygomycete protein could replace fish meal protein with maintained physiological and metabolic effects. Moreover, we hypothesised that the commercial diet would result in a different metabolic fingerprint from diet FM due to inclusion of other (unknown) feed ingredients.

## Materials and methods

### Fish rearing

Arctic char (*Salvelinus alpinus*) fingerlings with an initial body weight of 95 ± 22 g were reared at the Aquaculture Centre North in Kälarne, Sweden. The experimental diets were fed to three replicate groups of fish in flow through fibreglass tanks (1 $m^3$), with 6 fish per tank. During the experiment, water temperature was maintained at 4–6 °C. The water flow was approximately 15 L $min^{-1}$. Dissolved oxygen was maintained in a controlled system with oxygen meters recording the inlet water. The fish were fed twice daily, by using belt feeders, with a total daily allowance of 2 % of body weight, during the 4-week experiment. The fish accepted the experimental diets and no mortality was observed during the experiment. Fish weight was recorded at the beginning and end of the experiment, in order to confirm that the fish had consumed the test diets and had a positive energy balance. The fish gain in weight on all diets, with the highest value for the ST and FM diet and less on the FZ diet, with a mean weight gain of 69.1, 59.2 and 14.1 g, respectively. The experiment was approved by the Ethical Committee for Animal Experiments in the Umeå region.

### Diets

The fish were fed a commercial diet for salmonid fish (diet ST) with unknown composition, a diet with fish meal providing the majority of the protein (diet FM) and a diet with zygomycetes biomass providing the majority of the protein (diet FZ) (Table 1). The ingredients for diets FM and FZ were thoroughly mixed to a stiff dough and pelleted using a meat grinder (MR9-TC22, Nima maskinteknik AB, Örebro, Sweden) with a 2-mm die. The pelleted feed strings were dried and chopped to pellets of approximately 2 mm length.

**Table 1** Composition and proximate analyses of the diets based on fish meal (FM) and zygomycete meal (FZ) and the commercial diet (ST) (g/kg dry weight)

| Ingredients | Diets | | |
|---|---|---|---|
| | FM | FZ | ST |
| Fish meal | 580 | 230 | n |
| Fish oil | 130 | 130 | n |
| Rapeseed oil | 70 | 70 | n |
| Zygomycete biomass | 0 | 480 | n |
| Wheat | 80 | 0 | n |
| Gelatin | 65 | 65 | n |
| Mineral/vitamin mix | 10 | 10 | n |
| Titanium dioxide | 5 | 5 | n |
| Proximate analysis | | | |
| Crude protein | 489 | 471 | 531 |
| Crude fat | 244 | 178 | 221 |
| Ash | 96 | 95 | 89 |

*n* no information available

## Chemical analysis

Samples of feed ingredients and diets were analysed using standard methods (AOAC 1997). Dry matter was determined by drying in an oven at 105 °C for 24 h. Nitrogen (N) was determined by the Kjeldahl method and crude protein (CP) was calculated as $N \times 6.25$. Crude fat (EE) content was analysed using the Soxhlet method after acid hydrolysis of the sample. Ash content was determined by incineration in a muffle furnace at 550 °C for 12 h. Gross energy (MJ $kg^{-1}$) was determined with a bomb calorimeter (Calorimeter Parr 6300, Parr Instrument Company, Moline, IL, USA). Amino acid content in the feed was analysed using the Waters AccQ Tag$^{TM}$ method. In brief, 50 mg feed samples were weighed into a 100 mL Teflon liner for a Paar microwave oven. The samples were hydrolysed in 15 mL 6 M HCl containing 1 % phenol in a microwave oven (Synthos 3000, Anton Paar Nordic AB, Sweden). The temperature programme for the microwave oven was set to increase to 150 °C during 5 min, this temperature was held for 30 min and cooled for 15 min. For analysis of methionine and cysteine, 50 mg feed samples were added to 2 mL cold, freshly prepared formic acid:perhydrol (9:1) and incubated overnight at +4 °C. Thereafter, 2 mL freshly prepared sodium bisulphite (0.17 g sodium bisulphite/mL water) solution were added to each tube and the contents mixed for 15 min. The samples were then hydrolysed as described above.

The hydrolysed samples were neutralised, diluted and derivatised according to the WatersUPLC® amino acid analysis solution protocol. The UPLC system was a Dionex, Ultimate 3000 binary rapid separation LC system with a variable UV-detector (Thermo Fisher, Sweden, Stockholm). Empower 2 (Waters) software was used for system control and data acquisition.

## Extraction and sample preparation for NMR analysis

Four fish from each tank (12 per treatment) were collected and anesthetised with Tricane methane sulphonate (MS-222) solution (50 mg/L). Liver tissues were dissected from the fish and immediately transferred into liquid nitrogen. All the liver tissues were kept frozen at −80 °C for further NMR analyses. Sample preparation was carried out with modifications as previously reported (Atherton et al. 2006; Moazzami et al. 2011). Liver tissue portions (100 mg from the right side of the ventral lobe) were added to 3 mL ice-cold methanol–chloroform (2:1) and the mixture was homogenised for 1 min in a homogeniser (HeidolphDiax, Schwabach, Germany). The mixture was then subjected to sonication for 40 min, followed by vortex mixing every 10 min. A 1 mL portion each of ice-cold water and chloroform were added and the mixture was vortexed again for 1 min and centrifuged at 2,800 g for 30 min. The supernatant (water phase) was collected and transferred to new tubes. The volume was adjusted to a similar level in each tube and the contents were then dried using an

evacuated centrifuge (Savant, SVC 100H, Savant Instruments INC, New York, NJ, USA). The dried pellet was dissolved in 520 μL sodium phosphate buffer (0.25 M, pH 7.0), 50 μL $D_2O$ and 30 μL (internal standard) 1 mM sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (Cambridge Isotope Laboratories, Andover, MA, USA). Finally, 500 μL portions of reconstituted mixture were transferred to NMR tubes for further NMR analysis.

### NMR spectroscopy analysis

The NMR spectra of liver tissue extracts were obtained using a Bruker AV 400 NMR spectrometer (Karlsruhe, Germany) equipped with a 5 mm inverse broadband probe. The NMR spectra were obtained at 25 °C with 264 scans and 32,764 data points, with a spectral width of 8,012.82 Hz. The acquisition time was 2 s and the relaxation delay was 3.0 s. The one-dimensional $^1$H NMR spectra with water pre-saturation were recorded using Noesypr1D (Bruker, BioSpin, Karlsruhe, Germany).

NMR spectral data were processed using Bruker Topspin 1.3 software, Fourier-transformed after multiplication by a line broadening of 0.3 Hz and referenced to TSP [sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate] at 0.0 ppm. The spectral phase and baseline were manually corrected. Each $^1$H NMR spectrum was integrated using Amix 3.7.3 (BrukerBioSpin) into 0.01 ppm integral regions (buckets) between 0.5 and 8.55 ppm. The region spanning 4.41–5.39 ppm containing residual water was removed. Each spectral region was normalised to the intensity of the internal standard (TSP).

The $^1$HNMR signals were identified using the ChenomX software library (ChenomX Inc., Canada), Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu) and the Human Metabolome Database (HMD) (http://www.hmdb.ca).

### Spectral pre-processing and statistical analyses

Nuclear magnetic resonance spectral data were collected and analysed using SIMCA-P+ 12.0.1 software (UME-TRICS, Umeå, Sweden). The data sets were centred and pareto-scaled before statistical analysis with principal component analysis (PCA) and orthogonal partial least squares-discriminate analysis (OPLS-DA). The PCA model was used to look for outliers and to identify any clustering pattern with regard to the different diets. The outliers were determined using PCA-Hotelling $T^2$ Ellipse (95 % CI). The normality of multivariate data was tested using probability plot of PCA model. PCA models including all three diets or two diets at a time were fitted (FM vs. FZ, FM vs. ST, and FZ vs. ST).

The OPLS-DA model was fitted in order to look for the discriminating metabolites in the profile of liver metabolites in response to different diets. Variable influence on projection (VIP) of the NMR spectral regions was used to identify the discriminative metabolites between control and treatment diets. OPLS-DA models including all three diets or two diets at a time were fitted (FM vs. FZ, FM vs. ST, and FZ vs. ST). The NMR spectral regions with VIP $\geq$1 outside the confidence interval or close to zero were considered different between different treatments.

The significance of the OPLS-DA model was tested using cross-validated ANOVA (CV-ANOVA), which is a diagnostic tool for assessing the reliability of OPLS models. The diagnostic is based on an ANOVA assessment of the cross-validatory predictive residuals of an OPLS-DA model. Cross-validated ANOVA tests whether the model has significantly smaller cross-validated predictive residuals than just the variation around the overall average (Eriksson et al. 2008).

## Results

### Principal component analysis

The PCA analysis was performed using the pre-processed $^1$H NMR spectrum data obtained from liver tissue extracts of Arctic charr fed diets ST, FM and FZ. After excluding six outliers, the score scatter plot with first versus second components showed no apparent clustering difference with regard to different treatment diets (Fig. 1). Model parameters were: $R^2X$ component 1 = 0.56; $R^2X$ component 2 = 0.13.

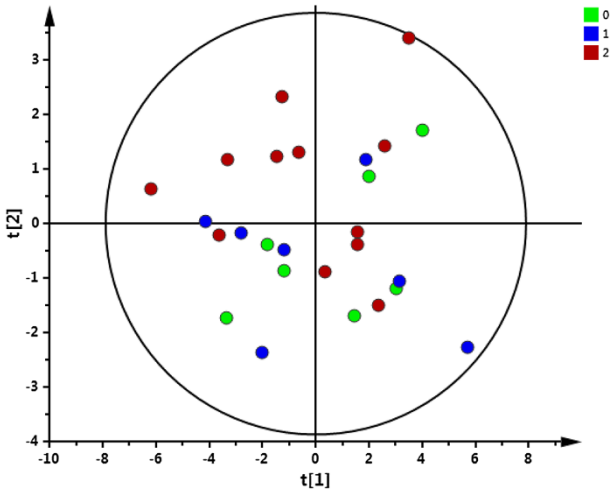


**Fig. 1** Score plot of PCA model fitted using the metabolic profile in liver of Arctic charr fed diet FM (*blue*), diet FZ (*green*) and diet ST (*red*). Model parameters: $R^2X$ component 1 = 0.56; $R^2X$ component 2 = 0.13

## Orthogonal partial least squares discriminant analysis

In order to investigate differences in biochemical variations between the predefined classes of the diets, OPLS-DA analysis was performed including all three diets. The OPLS-DA separated diets FM and FZ from diet ST along the first component. To obtain further insights regarding the biochemical differences between different diets, OPLS-DA models were fitted including just two diets at a time (FM vs. FZ, FM vs. ST, and FZ vs. ST). The OPLS-DA models fitted using spectral data for diets FM and FZ were not significantly different according to CV-ANOVA ($p \geq 0.05$). However, the other models (FM vs. ST and FZ vs. ST) were significantly different ($p < 0.05$). In both OPLS-DA models, diet ST clearly differed from diets FM and FZ (Figs. 2, 3). Model parameters for FM vs. ST were: $R^2Y = 0.98$, $Q^2Y = 0.70$, cross-validated CV-ANOVA $p = 0.042$ and five outliers were excluded based on the corresponding PCA-Hotelling $T^2$ Ellipse (95 % CI). Model parameters for FZ vs. ST were: $R^2Y = 0.98$, $Q^2Y = 0.73$, cross-validated ANOVA $p = 0.014$ and two outliers were excluded based on the corresponding PCA-Hotelling $T^2$ Ellipse (95 % CI). Tables 2 and 3 show the metabolites which were found to be different when diets FM and FZ were compared with diet ST.

## Identification of metabolites

The VIP plot and loading column plot of OPLS-DA were used to identify and compare differences in the metabolites of liver tissue extracts of Arctic charr fed diet FM vs. diet ST and diet FZ vs. diet ST. The spectral regions with VIP values >1 for the corresponding jack-knife-based confidence intervals not near to/or

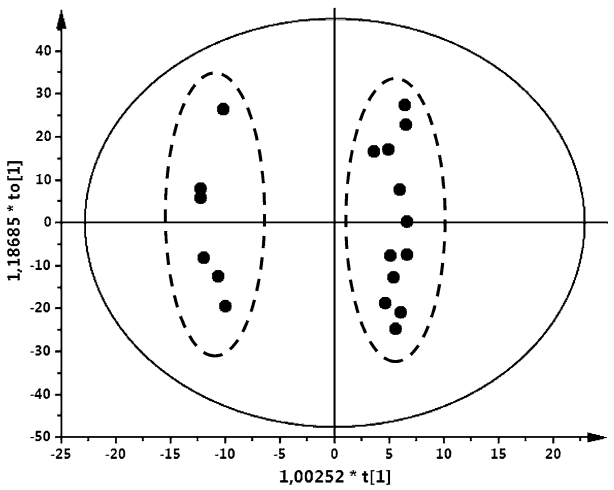


**Fig. 2** Score plot of OPLS-DA model fitted using the metabolic profile in liver of Arctic charr fed diet FM (*left*) and diet ST (*right*). The two diets are clearly separated along the first horizontal component. Model parameters: $R^2Y = 0.98$, $Q^2Y = 0.70$, cross-validated CV-ANOVA $p = 0.042$. Five outliers were excluded based on corresponding PCA-Hotelling $T^2$ Ellipse (95 % CI)

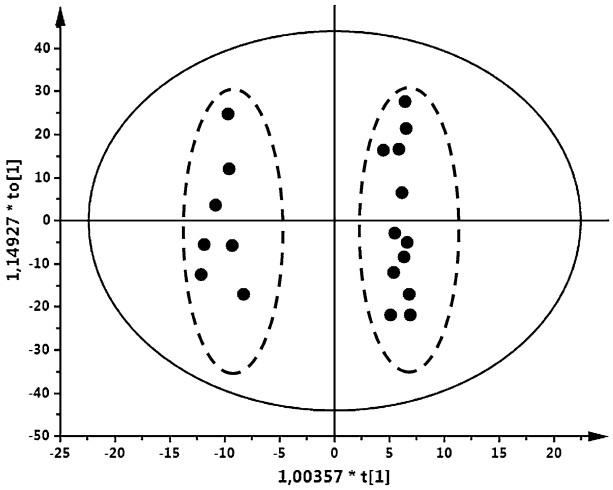


**Fig. 3** Score plot of OPLS-DA model fitted using the metabolic profile in liver of Arctic charr fed diet FZ (*left*) and diet ST (*right*). The two diets are clearly separated along the first horizontal component. Model parameters: $R^2Y = 0.98$, $Q^2Y = 0.73$, cross-validated ANOVA $p = 0.014$. Two outliers were excluded based on corresponding PCA-Hotelling $T^2$ Ellipse (95 % CI)

including zero were considered highly reliable and the corresponding compounds were selected as discriminative metabolites.

Metabolic analysis of diet FM vs. diet ST showed discrimination of various metabolites, i.e. acetate, asparagine, creatine, choline, SN-glycero-3-phosphocholine, formate and an unknown signal (Table 2). The levels of these metabolites were higher in fish fed diet FM than diet ST. However, asparagine was found at lower levels in fish fed diet FM than diet ST.

The effects of diet FZ vs. diet ST were apparent in the metabolites lysine, succinate, β-alanine, asparagine, creatine, SN-glycero-3-phosphocholine, glucose, inosine, formate and an unknown signal (Table 3). The data indicated that the levels of these metabolites were higher in fish fed diet FZ diet than diet ST. However, succinate and asparagine levels were lower in fish fed diet FZ than diet ST.

## Amino acid profile

The levels of amino acids in the different diets are summarised in Table 4. The total amount of essential (EAA) and non-essential amino acids (NEAA) differed slightly between diets FM and ST (370 vs. 386 g/kg diet). For diet FZ, the amino acid content (313 g/kg diet) was 19 % lower than in diet ST. The sum of EAA was highest in diet ST (180.6 g/kg), followed by diet FM (158.2 g/kg) and diet FZ (141.6 g/kg). The main differences were that histidine, leucine, phenylalanine and valine were all higher in diet ST than in diets FM and FZ. The highest content of NEAA was found in diet FM, which had higher contents of alanine, glycine and proline than diets FZ and ST.

**Table 2** Assignment of $^1$HNMR spectrum for liver extract of Arctic charr fed the fish meal-based (FM) and commercial (ST) diets ($n = 24$)

| Metabolites | NMR signal (PPm)[a] | Higher in | VIP (CI)[b] |
|---|---|---|---|
| Acetate | 1.925 | FM | 1.11 (0.79) |
| Asparagine | 2.88, 2.903, 2.945 | ST | 1.29 (0.91), 1.27 (0.89), 1.29 (0.80) |
| Creatine | 3.032 | FM | 2.25 (1.09) |
| Choline | 3.205 | FM | 1.72 (1.38) |
| SN-glycero-3-phosphocholine | 3.232 | FM | 1.59 (1.49) |
| Formate | 8.459 | FM | 1.78 (0.67) |
| Unknown | 2.717 | FM | 2.72 (1.59) |

[a] The $^1$HNMR signal found to be discriminating along the first component of the OPLS-DA model clearly separated diet FZ from diet ST

[b] Spectral regions with VIP values >1 for the corresponding jack-knife-based confidence intervals not near to/or including zero were considered high reliability and the corresponding compounds selected as discriminative metabolites

**Table 3** Assignment of $^{1}$HNMR spectrum for liver extract of Arctic charr fed the zygomycete meal-based (FZ) and commercial (ST) diets ($n = 24$)

| Metabolite | NMR signal (PPm)[a] | Higher in | VIP (CI)[b] |
|---|---|---|---|
| Lysine | 1.712, 1.727, 1.734 | FZ | 1.26 (0.91), 1.26 (0.96), 1.31 (0.90) |
| Succinate | 2.409 | ST | 1.73 (1.48) |
| β-Alanine | 2.563, 2.580 | FZ | 1.29 (0.68), 1.43 (0.46) |
| Asparagine | 2.88, 2.934, 2.945 | ST | 1.71 (0.98), 1.73 (0.95), 1.52 (0.96) |
| Creatine | 3.032 | FZ | 1.54 (0.88) |
| SN-glycero-3-phosphocholine | 3.231 | FZ | 1.94 (1.68) |
| Glucose | 3.481, 3.493, 3.505 | FZ | 1.60 (0.95), 1.55 (0.88), 1.63 (1.01) |
| Inosine | 6.098, 6.112, 8.251 | FZ | 1.91 (0.61), 2.13 (0.75), 1.92 (0.73) |
| Formate | 8.459 | FZ | 2.20 (0.65) |
| Unknown | 2.759 | FZ | 1.99 (0.99) |

[a] The $^{1}$HNMR signal found to be discriminating along the first component of the OPLS-DA model clearly separated diet FZ from diet ST

[b] Spectral regions with VIP values >1 for the corresponding jack-knife-based confidence intervals not near to/or including zero were considered high reliability and the corresponding compounds selected as discriminative metabolites

**Table 4** Amino acid composition of the diets based on fish meal (FM) and zygomycete meal (FZ) and the commercial diet (ST) (g/kg dry matter)

| Amino acid | FM | FZ | ST |
|---|---|---|---|
| *Essential amino acids* | | | |
| Arginine | 24.8 | 21.2 | 25.4 |
| Histidine | 7.7 | 6.6 | 14.6 |
| Isoleucine | 13.3 | 14.8 | 12.4 |
| Leucine | 28.3 | 24.6 | 36.2 |
| Lysine | 28.8 | 25.3 | 27.9 |
| Methionine-S | 14.8 | 14.0 | 11.7 |
| Phenylalanine | 15.1 | 12.9 | 20.8 |
| Threonine | 15.7 | 13.0 | 16.4 |
| Valine | 18.3 | 18.7 | 22.8 |
| *Non-essential amino acids* | | | |
| Alanine | 27.8 | 24.8 | 23.7 |
| Aspartate/aspartic acid | 36.6 | 31.1 | 44.4 |
| Cysteic acid | 5.4 | 5.6 | 7.9 |
| GABA | 0.2 | 0.4 | 0.1 |
| Glutamate/glutamic acid | 61.4 | 46.3 | 65.6 |
| Glycine | 36.3 | 30.2 | 20.9 |
| Ornithine | 1.0 | 1.5 | 0.8 |
| Proline | 23.8 | 17.5 | 19.8 |
| Serine | 19.6 | 14.1 | 22.1 |

Analysis performed according to the Waters amino acid protocol. Results for methionine are expressed as methionine sulphone and cystine as cysteic acid

## Discussion

Using an $^{1}$H NMR metabolomics approach and OPLS-DA, we found that Arctic charr showed a similar metabolic fingerprint when fed a diet with the majority of the protein from fish meal (diet FM) and a diet with the majority of the protein from zygomycete biomass (diet FZ). This suggests that both feed ingredients have

comparable physiological effects when fed to Arctic charr. It has previously been shown that Atlantic salmon and rainbow trout have a similar metabolic profile when fed a zygomycete biomass-based diet and a fish meal-based diet (Mydland et al. 2007; Bankefors et al. 2011). In contrast, the commercial diet (ST, with unknown ingredient composition) included in the present study showed a metabolic fingerprint that differed from that of diets FM and FZ. The different metabolic responses in fish fed the diets in the present study may be related to the utilisation of nutrients, in particular dietary protein, and to energy metabolism (Alasalvar et al. 2002; Grigorakis 2007; Fuentes et al. 2010).

Principal component analysis is unsupervised method that is commonly used to identify how one sample differs from another, which variable contributes significant difference and whether those variables are correlated or independent of one another (Wishart 2008). However, in the present study PCA score scatter plots did not indicate any clear trend in metabolite fingerprint related to the different dietary treatments. On the other hand, OPLS-DA analyses revealed differences in metabolic fingerprints in fish fed diets FM, FZ and ST. OPLS-DA, which is a supervised method, has been shown to be helpful in determining and increasing the separation between groups of observations (Wishart 2008). In the present study, OPLS-DA models were fitted including only two diets at a time. The models used singled out diet ST as being different from diets FM and FZ with respect to the metabolic profile found in liver samples from Arctic charr.

The content of EAA was comparable in diets FM and FZ, while the content of histidine, leucine, phenylalanine and valine was higher in diet ST and the content of methionine was lower. There were also differences in the content of NEAA between diets, with higher contents of most NEAA in diet FM compared with diet FZ, and with higher contents of aspartate/aspartic acid, cysteic acid and serine in diet ST compared with diet FM. Glycine and serine are interconverted in the liver by tetrahydrate-dependent hydroxymethyltransferase. These amino acids are involved in sulphur-containing amino acid metabolism, gluconeogenesis and single carbon metabolism in fish (Fang et al. 2002). In the present study, there was a stronger NMR signal intensity for asparagine, creatine, SN-glycero-3-phosphocholine and formate in fish fed diets FM and FZ than in fish fed diet ST. In addition, there was a stronger NMR signal intensity for acetate and choline in fish fed diet FM than in fish fed diet ST, and a stronger NMR signal intensity for lysine, β-alanine, glucose and inosine in fish fed diet FZ than in fish fed diet ST. A stronger NMR signal intensity for metabolites such as acetate, creatine/creatine phosphate and choline is in line with existing data on Atlantic salmon fed fish meal-based and zygomycete-based diets (Bankefors et al. 2011). The high NMR signal intensity for creatine in diets FM and FZ compared with diet ST in the present study may be related to the energy supply to muscle tissues and may indicate an increase in ATP production.

Choline is involved in various physiological functions such as neurotransmission, betaine synthesis and transportation of lipid molecules, and it acts as a methyl donor in fish tissues (Halver 1989; Mai et al. 2006). Dietary choline has positive effects on weight gain and in the prevention of fatty liver condition in juvenile hybrid striped bass (Griffin et al. 1994). Choline deficiency has been shown to lead to haemorrhagic lesions in the liver and kidney of lake trout, common carp, rainbow trout and chinook salmon (Halver 1957; Ogino et al. 1970; Ketola 1976; Chan 1991).

The high NMR signal intensity for acetate and SN-glycero-3-phosphocholine in Arctic charr fed diet FM compared with diet ST indicates increased activity of the fatty acid metabolic pathway in the former. Increased acetate and SN-glycero-3-phosphocholine in the liver and intestinal tissues has been shown to be related to catabolism of dietary fatty acids in rainbow trout and sea bream (Randall et al. 2013; Robles et al. 2013). In general, fish meal contains high amounts of choline (4.3 g/kg; Barlow et al. 1979), which supports our findings of a higher NMR signal intensity for diet FM compared with diet ST. In the present study, the slightly higher content of crude fat in diet FM than in diet ST cannot explain the difference in NMR signal intensity for acetate between fish fed these two diets, since no difference in NMR signal intensity for acetate was observed between diets FM and FZ, despite the higher content of crude fat in diet FM. However, crude fat analyses do not distinguish between structural lipids, such as membrane lipids, and storage lipids, such as tri-acyl glycerols. A higher content of tri-acyl glycerol would be expected to result in more acetate being formed. Increased catabolism of ketogenic amino acids (Ile, Leu, Tryp, Lys, Phe) can also result in increased acetate concentrations in the liver. However, the highest contents of these amino acids were found in fish fed diet ST and therefore cannot explain the higher NMR signal intensity for acetate in fish fed diets FM and FZ. The NMR signal intensities for inosine and β-alanine were higher in fish fed diet FZ than diet ST, which could reflect the high content of pyrimidines and purines in the zygomycete-based diet (Wasternack 1978). β-alanine

is normally metabolised to acetyl-CoA through the malonate semialdehyde pathway, which can explain the higher acetate concentration in the FZ group. β-alanine is involved in antioxidant and buffer activities in fish (Snyder et al. 2008) and elevated levels of β-alanine are found in tissues due to environmental changes and acute stress (Aragao et al. 2008).

Lysine is an essential amino acid that may be limiting when fish meal is replaced by other protein sources. A limiting dietary lysine content can change protein and energy metabolism in fish (Forster and Ogata 1998). Lysine deficiency can have a negative impact on feed intake, growth and reproductive performance in fish (Li et al. 2009). The higher NMR signal intensity for lysine in fish fed diet FZ than in fish fed diet ST may be related to impaired utilisation of lysine in the latter, due to lower digestibility and a more unbalanced essential amino acid profile in diet ST compared with diet FZ. Asparagine is extensively catabolised in the small intestine of fish, and is mostly derived from synthesis by skeletal muscle (Wu et al. 2008). Due to rapid oxidation, and the non-toxic nature of alanine and asparagine, these amino acids are commonly included to balance the nitrogen content in aquafeeds (Li et al. 2009). The NMR signal intensity for asparagine was higher in fish fed diet ST than in fish fed diets FM and FZ. This can be explained by higher content (approximately 20 and 40 %, respectively) of aspartate and asparagine in diet ST than in diets FM and FZ.

In addition to providing nutrients and energy substrates, zygomycete biomass may have additional value as aquafeed owing to its high chitosan content, since chitosan has the ability to bind fat and act as an emulsifying agent and it has antimicrobial activities (Schulz et al. 1998; Gerasimenko et al. 2004; Arnaz et al. 2009). Chitosan is a non-toxic, biodegradable and biocompatible polysaccharide that is involved in anti-oxidation, promoting permeability across the cell and bio-catalysis processes (Krajewska 1991; Gerasimenko et al. 2004; Arnaz et al. 2009). It is possible that some of the metabolic responses found in the present study could have been related to the physiological effects in the fish. However, further studies are required to investigate whether the inclusion of different levels of zygomycete biomass will affect performance and metabolic responses in Arctic charr.

## Conclusions

Differences in the metabolic fingerprint in liver tissues of Arctic charr fed diets with varying ingredient composition were revealed using an $^{1}$H NMR metabolomics approach and OPLS-DA. The metabolic fingerprint was comparable when fish were fed diets with most of the protein from fish meal or from zygomycete biomass. This suggests that both feed ingredients have comparable physiological effects when fed to Arctic charr. In contrast, a commercial diet with unknown ingredient composition showed a metabolic fingerprint that differed from that of the other diets. These different metabolic responses between the commercial diet and the two experimental diets may be related to the utilisation of nutrients, in particular dietary protein, and to energy metabolism.

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**Conflict of interest** The authors declare that they have no competing interests.

**Author's contributions** TL and AAM conceived and design the study. RA performed the experiments with assistance from AAM. RA, TL and AM analysed the experimental data. RA, AAM, TL and JEL contributed in the writing of manuscript.



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