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Norwegian University of Life Sciences
NO–1432 Ås, Norway
Phone +47 64 96 50 00
www.umb.no, e-mail: postmottak@umb.no

Youling Gao

Norwegian University of Life Sciences • Universitetet for miljø- og biovitenskap
Department of Animal and Aquacultural Sciences
Philosophiae Doctor (PhD) Thesis 2011:23

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# Improved nutritional value of fish feed with plant protein ingredients by means of organic acid salts and solid state fermentation

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Ås, April 2011

Youling Gao

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# ABSTRACT

**Gao, Y., 2011.** Improved nutritional value of fish feed with plant protein ingredients by means of organic acid salts and solid state fermentation. Norwegian University of life Sciences, Philosophiae Doctor Thesis 2011:23, ISSN: 1503-1667, ISBN: 978-82-575-0987-3.

The objective of this work was to improve the nutritional value of plant protein sources to be used in fish feed by means of organic acid salts and solid state fermentation (SSF). Four studies were conducted to determine the effects of a blend of organic acid salts (OAB) in diets for rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*), SSF rapeseed meal in different types of diets for Nile tilapia, and thermal-hydro (steam) treatment on nutritional value of fermented rapeseed meal in diets for Nile tilapia.

The first study was designed to evaluate the effects of adding an OAB (a mixture of sodium formate and butyrate) to a fish meal-based diet and a diet where 36% of the fish meal protein had been replaced by plant proteins (a mixture of soybean meal and pea protein concentrate). The diets were fed to rainbow trout reared in freshwater. Adding OAB to the plant protein-based diet before extrusion significantly reduced the digestibility of dry matter, organic matter, crude fat, and most amino acids. Supplementation of OAB after extrusion reduced the digestibility of crude fat both in the fishmeal and plant protein-based diets. Inclusion of OAB in both fishmeal and plant protein-based diets before extrusion significantly increased feed conversion ratio (FCR), and middle intestine to body weight ratio. Partly replacing fishmeal with plant proteins reduced the digestibility of dry matter, organic matter, crude fat, phosphorous and several amino acids, and increased FCR.

The second study was carried out to determine the potential of laboratory-scale aerobic SSF with *Aspergillus niger* to increase the nutritional quality of rapeseed meal, and to evaluate the potential for use of untreated (RSM) and fermented rapeseed meal (FRSM) in diets for Nile tilapia. In Exp. 1, three moist diets were produced: a diet based on fish meal and soybean meal (SBM), and two diets in which SBM was fully replaced by RSM or FRSM. In Exp. 2, three extruded diets were produced with the same formulation as used in Exp. 1. Phytic acid and glucosinolates in RSM were reduced, the content of crude protein increased and total amino acids was decreased by SSF. In Exp.1, the growth rate of fish fed the SBM diet was significantly lower than that of tilapias fed the RSM and FRSM diets. FCR of fish fed RSM diet was significantly poorer than that of fish fed the SBM and FRSM diets. Fish fed the RSM diet had significantly higher whole body protein than fish fed the SBM diet. In Exp. 2, the growth rate showed no significant difference among the dietary treatments, while FCR

differed among all 3 treatments (SBM<RSM< FRSM). The nitrogen retention of fish fed SBM diet was significantly higher than that of fish fed the RSM diet, which was higher than that for FRSM. The digestibility of nitrogen in fish fed SBM diet was higher than for tilapia fed the RSM and FRSM diets. The digestibility of Mg was significantly higher in fish fed the SBM and FRSM diets than that in tilapias fed RSM. The differences in nutritional value in the two experiments are rationalized by differences in the SSF process, and by the two methods of feed production used.

The third study was conducted to evaluate the effects of supplementing the OAB to the diets, in combination with medium-scale SSF of RSM on growth performance and apparent nutrient digestibilities in Nile tilapia. The results showed that supplementing diets with the OAB did not have other effects than increasing the stomach weight in percentage of body weight. SSF of RSM reduced the feed intake, growth rate, liver weight percentage, increased stomach weight percentage and FCR, and reduced whole-body dry matter, crude fat and increased ash contents.

The fourth study was conducted to evaluate the effect of short-term steam treatment for pasteurisation of FRSM on growth performance, whole body composition and apparent nutrient digestibilities in Nile tilapia. Two diets were prepared with and without steam treatment after SSF of RSM. Steam treatment increased FCR, while growth rate, nutrient digestibilities and protein retention were not affected.

In conclusion, supplementing the diets with an OAB did not improve the growth performance of rainbow trout and Nile tilapia. Laboratory-scale SSF of RSM changed the nutritional value and improved the FCR when used in a moist diet, while less efficient FCR was found when used in an extruded diet. Medium-scale SSF of RSM reduced growth rate, but improved FCR. Short-term steam treatment did not improve the nutritional quality of FRSM as a dietary ingredient for Nile tilapia.

# SAMMENDRAG

**Gao, Y., 2011.** Forbedret næringsverdi i fiskefôr med planteproteinfôrmidler ved hjelp av organiske syresalter og faststoff fermentering. Universitetet for miljø og biovitskap, Doktoravhandling 2011:23, ISSN: 1503-1667, ISBN: 978-82-575-0987-3.

Målet med dette arbeidet var å forbedre ernæringsmessig kvalitet av proteinrike fôrmidler fra planter for bruk i fiskefôr ved hjelp av organiske syresalter og faststoff fermentering (SSF). Fire studier ble gjennomført for å undersøke effektene av organiske salter i fôr til regnbueørret (*Oncorhynchus mykiss*) og niltilapia (*Oreochromis niloticus*), SSF-behandlet rapsmel i ulike fôr til niltilapia, og fuktig varmebehandling (damp) på den ernæringsmessige verdien av fermentert rapsmel (FRSM) i fôr til niltilapia.

Det første studiet ble utført for å evaluere effektene av å sette organiske syresalter (OAB, en blanding av natriumformat og butyrat) til et fiskemelbasert fôr og et fôr hvor 36 % av protein fra fiskemel var erstattet av planteprotein (en blanding av soyamel og erteproteinkonsentrat). Fôrene ble gitt til regnbueørret i ferskvann. Tilsetning av OAB før ekstrudering av fôrene med planteprotein førte til en signifikant reduksjon i fordøyelighet av tørrstoff og organisk materiale, råfett, og de fleste aminosyrene. Tilsetning av OAB etter ekstrudering reduserte fordøyeligheten av råfett både i fôrene med fiskemel og planteprotein. Tilsetning av OAB førte til signifikant høyere fôrforbruk per kg tilvekst, og økt vekt av midttarmen, i prosent av kroppsvekt, både i fôr med fiskemel og planteprotein. Delvis utbytting av fiskemel med planteprotein reduserte fordøyelighet av tørrstoff, organisk materiale, råfett, fosfor og flere aminosyrer, og økte fôrforbruk per kg tilvekst.

Det andre studiet ble utført for å bestemme potensialet av laboratorium-skala aerob SSF ved hjelp av *Aspergillus niger* for å forbedre ernæringsverdien av rapsmel (RSM), og for å evaluere potensialet for bruk av RSM og fermentert rapsmel (FRSM) i fôr til niltilapia. Tre mykfôr ble produsert i forsøk 1: et fôr basert på fiskemel og soyamel (SBM), og to fôr hvor SBM ble helt erstattet med RSM og FRSM. I forsøk 2 ble tre ekstruderte fôr produsert med lik sammensetning som i forsøk 1. Fytinsyre og glukosinolater ble redusert, innholdet av råprotein økte, og det totale innholdet av aminosyrer ble redusert ved SSF. I forsøk 1 var veksthastigheten signifikant lavere hos fisk som fikk fôr med SBM enn hos tilapia som fikk RSM og FRSM i fôret. Fisk som fikk fôr med RSM trengte mer fôr per kg tilvekst enn tilapia som fikk fôr med SBM og FRSM. Fisk som fikk fôr med RSM hadde signifikant høyere innhold av protein i kroppen enn de som fikk fôr med SBM. Det var ingen signifikante forskjeller i veksthastighet i forsøk 2, mens fôropptaket per kg tilvekst var forskjellig for alle

tre fôr (SBM<RSM<FRSM). Nitrogenretensjonen hos fisk som fikk SBM i fôret var signifikant høyere enn den hos fisk som fikk RSM, som igjen var høyere enn retensjonen hos tilapia som fikk fôr med FRSM. Fordøyelighet av nitrogen var høyere hos fisk som fikk SBM i fôret enn hos tilapia som fikk fôr med RSM og FRSM. Fordøyelighet av magnesium var signifikant høyere hos fisk som fikk fôr med SBM og FRSM enn hos tilapia fôret med RSM. Forskjellene i ernæringsmessig kvalitet mellom de to forsøkene er forklart ved ulikheter i SSF, og ved at to ulike metoder for fôrproduksjon ble benyttet.

Det tredje studiet ble utført for å evaluere hvilke effekter tilsetning av OAB til fôret, kombinert med SSF utført i halvindustriell skala, hadde på vekst og fordøyelighet av næringsstoffer hos niltilapia. Resultatene viste at tilsetning av OAB til fôret ikke hadde andre effekter enn å øke magens vekt i forhold til vekten av hele fisken. SSF av RSM førte til redusert fôropptak, veksthastighet, levervekt i forhold til kroppsvekt, økt magevekt, mindre effektiv fôrutnyttelse, og redusert innhold av tørrstoff, råfett, og økt innhold av aske i hel fiskekropp.

Det fjerde studiet ble utført for å undersøke hvorvidt korttids varmebehandling for pasteurisering av FRSM med damp påvirket vekst og kjemisk sammensetning hos niltilapia, og fordøyelse av næringsstoffer. To fôr ble produsert, med FRSM som enten var ubehandlet eller oppvarmet med damp. Oppvarming med damp førte til høyere fôrforbruk per kg tilvekst, mens veksthastighet, fordøyelighet av næringsstoffer og proteinretensjon ikke ble påvirket.

Hovedkonklusjonene er at tilsetning av OAB til fôrene ikke førte til bedret vekst hos regnbueørret eller niltilapia. Behandling med SSF i laboratorie-skala endret ernæringsmessig sammensetning av RSM og førte til bedre utnyttelse av fôret når FRSM ble benyttet i mykfôr, mens det motsatte ble observert når FRSM ble benyttet i ekstrudert fôr. SSF av RSM i halvindustriell skala førte til redusert tilvekst, men forbedret fôrutnyttelse. Korttids varmebehandling med damp økte ikke næringsverdien av FRSM som råvare i fôr til niltilapia.

# CHINESE ABSTRACT

**摘要：**本研究通过添加有机酸盐和固体发酵（SSF）的方法，来改善植物蛋白源在鱼饲料中的应用状况。研究内容包括：测定甲酸钠和丁酸钠混合剂（OAB）在饲料中对虹鳟鱼（*Oncorhynchus mykiss*） 和尼罗罗非鱼（*Oreochromis niloticus*）的生长性能等影响；比较发酵菜籽饼在不同类型饲料中对尼罗罗非鱼的影响；评价湿热（蒸汽）处理过的发酵菜籽饼对尼罗罗非鱼的影响。

第一个研究评价 OAB（甲酸钠和丁酸钠混合剂）在鱼粉料和植物蛋白料中（豆粕和豌豆浓缩蛋白混合物替代 36%的鱼粉）对饲养于淡水的虹鳟鱼的作用效果。结果表明：膨化处理前添加 OAB 到植物蛋白料中显著降低了干物质、有机物、粗脂肪和大部分氨基酸的消化率；膨化处理后添加 OAB 到鱼粉料和植物蛋白料中均降低了粗脂肪的消化率。在膨化处理前添加 OAB 到鱼粉料和植物蛋白料显著提高了饲料系数（FCR），中肠和体重比值。此外，用植物蛋白部分替代鱼粉显著降低了干物质、有机物、粗脂肪、磷和一些氨基酸的消脂率，但提高了 FCR。

第二个研究测定实验室级别的固体需氧发酵对菜籽饼的营养品质改善效果，并评估菜籽饼（RSM）和发酵菜籽饼（FRSM）在尼罗罗非鱼饲料中的应用前景。试验一制备了三种湿性颗粒饲料：基于鱼粉和豆粕的 SBM 料，以及两种分别用 RSM 和 FRSM 完全替代豆粕的饲料（RSM 料和 FRSM 料）。试验二采用相同的配方制备三种膨化饲料。试验结果表明：SSF 降低了菜籽饼中植酸、硫代葡萄糖甙和总氨基酸的含量；在试验一中，SBM 组的生长率要显著低于 RSM 组和 FRSM 组，RSM 组的 FCR 显著高于 SBM 组和 RSM 组，RSM 组的全鱼粗蛋白含量要显著高于 SBM 组；在试验二中，三组鱼的生长率无显著差异，但 FCR 有显著差异（SBM 组<RSM 组<FRSM 组），SBM 组的氮贮留率要显著高于 RSM 组，同时 RSM 组要高于 FRSM 组，SBM 组氮的消化率要高于 RSM 组和 FRSM 组，SBM 组和 FRSM 组的 Mg 消化率要显著高于 RSM 组。两个试验结果有差异可能由于不同发酵过程和不同饲料制备方法所致。

第三个研究评价饲料中添加 OAB 结合菜籽饼的固体发酵（中等级别）对尼罗罗非鱼的生长性能和表观消化率的影响。结果表明：饲料中添加 OAB 除了增加胃与体重的比值外，无其它效果；经中等级别固体发酵处理的菜籽饼显著降低了摄食量，生长率，肝体比值，提高了胃与体重比值和 FCR， 降低了全鱼的干物质和粗脂肪，并提高了灰分含量。

第四个研究测定了短暂的蒸汽消毒处理发酵菜籽饼对尼罗罗非鱼生长，体成分和表观消化率的影响。试验采用经蒸汽消毒处理过的发酵菜籽饼和未经蒸汽消毒处理过的发酵菜籽饼制备了两种饲料。结果表明短暂的蒸汽消毒处理提高了 FCR，但对生长和营养物质消化率，以及氨贮留率没有影响。

本研究因此得出如下结论：在饲料中添加甲酸钠和丁酸钠混合剂没有改善虹鳟鱼和尼罗罗非鱼的生长性能及饲料利用；实验室级别的固体发酵改变了菜籽饼的营养品质，用于湿性颗粒料中能有较好的 FCR，但若应用在膨化饲料中 FCR 效率较低。菜籽饼中等级别的固体发酵处理降低了生长率、改善了 FCR；短暂的蒸汽消毒处理并没有改善发酵菜籽饼的营养品质。

# ABBREVIATIONS

| | |
|---|---|
| ANFs | Anti-nutritional factors |
| ATP | Adenosine-triphosphate |
| DM | Dry matter |
| EAA | Essential amino acids |
| FCR | Feed conversion ratio |
| FRSM | Fermented rapeseed meal |
| IP | Inositol phosphate |
| IP5 | Myo-Inositol pentaphosphates |
| IP6 | Phytic acid (myo-Inositol hexaphosphate) |
| KDF | Potassium diformate |
| NSPs | Non-starch polysaccharides |
| OAB | Organic acid salt blend |
| RSM | Rapeseed meal |
| SBM | Soybean meal |
| S.E.M | Standard error of the mean |
| SGR | Specific growth rate |
| SME | Specific mechanical energy |
| SSF | Solid state fermentation |

# LIST OF PAPERS

I. Gao, Y., Storebakken, T., Shearer, K.D., Penn, M., Øverland, M., 2011. Supplementation of fishmeal and plant-protein meal based diets for rainbow trout with a mixture of sodium formate and butyrate. Aquaculture 311, 233-240.

II. Gao, Y., Nabulime, M.M., Hanssen, J.F., Mydland, L.T., Denstadli, V., Gjøen, H.M., Storebakken, T. Solid state fermentation of *Aspergillus niger* improves the nutritional value of rapeseed meal as a feed ingredient for Nile tilapia (*Oreochromis niloticus*). Manuscript.

III. Gao, Y., Chowdhury, D., Hanssen J.F., Gjøen, H.M., Mydland, L.T., Øverland, M., Storebakken, T. Fermentation of rapeseed meal, and supplementation with sodium butyrate and formate in diets for Nile tilapia (*Oreochromis niloticus*). Manuscript.

IV. Gao, Y., Øverland, M., Storebakken, T. Steam treatment does not improve nutritional value of solid state fermented rapeseed meal in diets for Nile tilapia (*Oreochromis niloticus*). Manuscript.

# 1. INTRODUCTION

Plant protein sources like soybean meal and rapeseed meal are becoming more widely used in fish feeds. However, they can have negative effects on fish growth, feed intake and health when they replace fishmeal at high levels. The presence of anti-nutritional factors and the relative unbalanced amino acid profile compared to fishmeal are regarded as the main reasons for the negative effects (Francis et al., 2001).

Organic acids are widely distributed in nature as normal constituents of plant, animals and are common metabolites of microbial fermentation in the digestive tract. Organic acids and their salts (Na, K or Ca) appear to have the potential to improve growth performance in several farm animal species. Efforts have been made to understand the effects of organic acids and their salts on nutrient digestibility, growth performance and health of fish, including Arctic charr (Ringø, 1991), Atlantic salmon (Lückstädt, 2008a; Ringø et al., 1994), rainbow trout (deWet, 2005; Pandey and Satoh, 2008; Rungruangsak and Utne, 1981), tilapias (Ramli et al. 2005; Ng et al., 2009; Zhou et al., 2009), catfish (Owen et al., 2006), red sea bream (Hossain et al., 2007; Sarker et al. 2007, 2005) and Indian major carp, Rohu (Baruah et al. 2007a, b). The results of these studies indicate that nutrient digestibility, growth performance and gut health can be improved by the addition of some organic acids to the diet, particularly formic acid (Reviewed by Lückstädt, 2008b). In addition, butyric acid is superior in providing energy for intestinal epithelial growth (Topping and Clifton, 2001), and in pH-reducing capacity due to its high pKa value (4.82) compared with formic acid (Partanen and Mroz, 1999). Thus, using formic acid or butyric acid and their salts may have different beneficial effects when fed to fish, and may overcome some negative effects of plant protein sources. Thus, they are already increasingly used in fish feeds.

Tilapia has relatively high production worldwide. There is high demand both from the high end fillet market, and for the poor in developing countries as a major animal protein sources. World tilapia aquaculture reached 2.4 million metric tons in 2006 (Fitzsimmons, 2008), and more than 22 tilapia species are being cultured. Nile tilapia (*Oreochromis niloticus* L.) is the most cultured species, with a total production volume of 2 million metric tons in 2006 (FAO, 2006). The increasing trend in tilapia consumption requires improvement in tilapia production, and improvement in tilapia feed has a large potential. In traditional extensive farming of tilapia, natural pond organisms are the major sources of nutrients for the fish. As the industry expansion continues, extensive farming is being replaced by semi-intensive or intensive farming. Locally available, low-cost feedstuffs have been introduced to

these farming systems. However, it seems that modern, genetically improved tilapia farming needs higher quality feed, but they should still be based on low-cost feedstuffs.

Rapeseed meal is a candidate owning to an abundant supply and high nutritional value. Rapeseed meal is the world's second-leading oil extraction meal after soybean meal, and the world production was 34.1 million metric tons in 2010/11 (USDA, 2011). Rapeseed meal contains approximately 43% crude protein, 12% crude fat on a dry matter basis (Bell and Jeffers, 1976), and the essential amino acid profile of rapeseed meal is favourable compared with other plant protein sources (Friedman, 1996). In addition, protein from rapeseed meal is less expensive than fishmeal and soybean meal. However, the challenge of using rapeseed meal is that they contain anti-nutritional factors (ANFs). Glucosinolates and phytate are the main ANFs in rapeseed meal (reviewed by Francis et al., 2001). The high content of fibre in rapeseed meal also makes it less suitable for monogastric animals. It has been shown that Canola meal, from rapeseed selected for low contents of erucic acid in the fat and low glucosinolate content in the meal, contains approximately 14.5% cellulose, 5.0% hemicellulose and 8.3% lignin (Mwachireya et al., 1999). Hence, rapeseed meal should be processing before it is used in fish feed.

Solid state fermentation (SSF) is a traditional technology that can change the nutritional properties of rapeseed meal. It has been shown that SSF reduces the content of glucosinolates (Vig and Walia, 2001), phytate (Egounlety and Aworh, 2003; El-Batal and Karem, 2001; Nair and Duvnjak, 1991; Vig and Walia, 2001), and crude fibre (Vig and Walia, 2001). Moreover, synthesis of amino acids (Reviewed by Kumagai, 2006) and fatty acids (Singh, 1991) during fermentation have been observed. Thus, with SSF treatment, the nutritional quality of rapeseed meal can be improved so that it can be used in tilapia feed as a novel protein source. So far, no effort has been made to evaluate fermented rapeseed meal in diets for tilapia.

# 2. OBJECTIVES OF THE STUDY

The overall objective of the current work was to improve the use of plant protein sources in fish feed by adding organic acid salts and by SSF. The sub-objectives of the current research were:

- To investigate the effect of a plant protein based-diet with soybean meal and pea protein concentrate on nutrient digestibility, growth performance and gut health of rainbow trout (Paper I).
- To investigate the effects of an organic acid salt blend (OAB) of sodium formate and sodium butyrate in diets on digestibility of nutrients, growth performance, and intestinal morphology of rainbow trout (Paper I), and on growth performance, body composition, digestibility of nitrogen and minerals, and nitrogen retention of Nile tilapia (Paper III).
- To compare two different methods of adding OAB to the extruded diets, before and after extrusion (Paper I).
- To determine if laboratory-scale (Paper II), and medium-scale (Paper III) SSF with *Aspergillus niger* is a useful tool to increase the nutritional value of rapeseed meal.
- To evaluate the potential for use of untreated and fermented rapeseed meal in diets for Nile tilapia when replacing soybean meal (Paper II and III).
- To determine if short-term pasteurization by steam treatment improves the nutritional value of solid state fermented rapeseed meal in diets for Nile tilapia (Paper IV).

# 3. BACKGROUND

## 3.1 Organic acids and their salts

### *3.1.1 General information*

Organic acids, widely distributed in nature as normal constituents of plants and animals and are common metabolites of microbial fermentation in the digestive tract. They are natural components that could stimulate growth performance and health in all farm animal productions. Organic acids that are commonly used as dietary acidifiers in aquaculture include formic, acetic, propionic, butyric, lactic, sorbic, malic and citric acids (reviewed by Lückstädt, 2008b). Liquid forms of pure organic acids are corrosive and difficult to handle during feed manufacture. The salts of organic acids, which are solid and less corrosive and easier to handle, are increasingly used as an additive in feed.

Organic acids and their salts (sodium, potassium or calcium) appear to have the potential to improve growth performance of some animals. The positive effects of organic acid salts on growth performance and animal health, especially when added in sufficient amounts in diets for pigs, have been well documented (Canibe et al., 2001; Jongbloed et al., 2000; Øverland et al., 2000; 2008; Partanen and Mroz, 1999). The positive effects of organic acids and acid salts on animal growth performance and health were exhibited via three different mechanisms: feed, digestive tract and metabolism (Freitag, 2008).

Table 1. Mechanisms of organic acids and their salts (modified from Freitag, 2008)

| Site of action | Effects |
|---|---|
| Feed | pH reduction<br>Antimicrobial effects<br>Reduced buffering capacity |
| Digestive tract | pH reduction in stomach<br>Increase in efficiency of pepsin (pH optimum 2.5 and 3.5)<br>Antimicrobial effect<br>Complexing agent ($Ca^{2+}$, $Mg^{2+}$, $Fe^{2+}$, $Cu^{2+}$, $Zn^{2+}$)<br>Antimicrobial effect |
| Metabolism | Energy source |

During feed storage, there may be a certain level of contamination with fungi, bacteria and yeasts. The lower pH inhibits microbe growth and metabolism, thus reducing the risk of contamination from pathogenic organisms and their metabolites. Al-Natour and Alshawabkeh

(2005) found that the addition of formic acid at 1.5% of the diet to newly hatched broiler chicks significantly decreased the contamination of the diet with *Salmonella gallinarum*. Moreover, the ingested diets containing organic acid reduced the pH in the stomach of the animals. The pepsin efficiency was increased due to a rapid reduction in pH. Further, supplementing diets with organic acids increased gastric pepsin activity (Eidelsburger et al., 1992), since $H^+$ ions activate pepsinogen.

Antimicrobial effects of organic acids have been detected in various studies. The study by Ojo et al. (2005) concluded that the growth of *Bacillus subtilis*, *Streptococcus faecalis, Streptococcus pneumoniae, Corynebacterium diphteriae, Micrococcus luteus, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Shigella dysenteriae* and *Serratia marcescens* was inhibited by acetic, ascorbic, citric and formic acids. Eswaranandam et al. (2004) evaluated the inhibition of citric, lactic, malic, and tartaric acids on growth of *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella gaminara*. The authors observed that malic acid showed the best inhibition. In the field of animal nutrition, there is acceptance of an antimicrobial action of organic acid after they are ingested (Fig.1). In the stomach, undissociated and dissociated forms of organic acids coexist because the pKa values of most organic acids range from 3 to 5. Undissociated forms of organic acids diffuse across cell membranes of pathogens, inactivating bacterial decarboxylases and catalases, thus destroying the cytoplasm or inhibiting growth. Dissociated forms of organic acids ($H^+$ ions and anions) serve as a pH barrier against pathogen colonization on the brush border of the intestine.

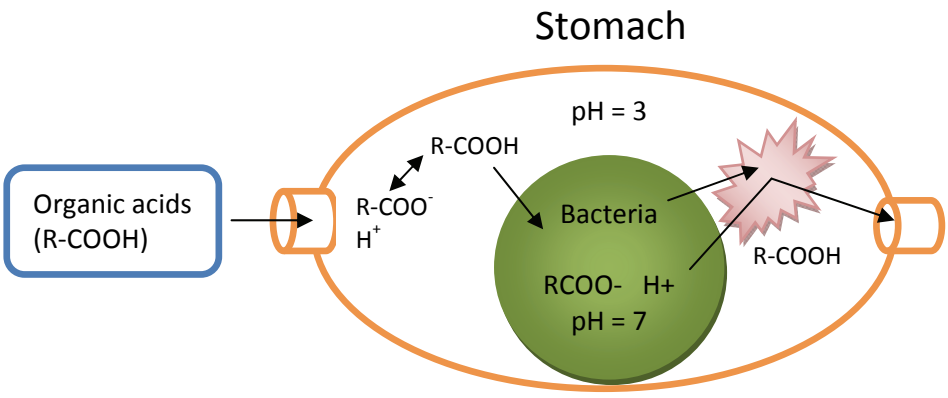


Figure 1. Model antimicrobial action of organic acid in the stomach (redrawn from Mroz, 2005)

Organic acids contribute to a considerable amount of energy. Bergman (1990) showed that after they were absorbed through the intestinal epithelia by passive diffusion, organic acids supply the energy for the epithelial cells, and contribute to 20-30% of the total energy requirements for the omnivorous or herbivorous animals. Further, short chain acids can also be used for ATP generation in the citric cycle.

### *3.1.2 Formic acid and its salts*

Formic acid (HCOOH) is a colourless, transparent liquid with a pungent odour (Partanen and Mroz, 1999). In addition to the free acid form, the salts of formic acid are a solid and less volatile form and are becoming commonly used in feed due to ease of handling (Partanen and Mroz, 1999)..The application of pure acid and salts of formic acid in fish feed has been well documented. The results have varied and beneficial effects on fish growth and health have been observed in some studies. However, some studies have failed to find positive benefits from formic acid or salts. Recently, Zhou et al. (2009) observed that the addition of dietary potassium diformate (KDF; 0.3 to 1.2%) had no significant effect on hybrid tilapia growth, feed conversion ratio or survival. Whereas, 0.3% and 0.6% KDF in diets improved the relative abundance of some intestinal allochthonous bacteria such as *Mycobacterium* sp. partial MHSD12-like, *Mycobacterium peregrinum*-like, *Pseudomonas* sp. HMPB4-like. The authors thus speculated that dietary KDF could stimulate a beneficial bacterial colonization of the intestine. Another study, conducted by Ng et al (2009), showed that there was no significant difference in the growth, feed conversion ratio and nutrient digestibility among treatment groups despite a trend towards improved results with tilapia fed organic acid supplemented diets (0.2%). Total bacteria in faeces and adherent gut bacteria were significantly reduced in the fish fed the organic acid diets compared with fish fed the control diet. The authors concluded that dietary organic acids exerted a strong anti-microbial effect and have the potential to exert beneficial effects on growth, nutrient utilization and disease resistance in tilapia. Ramli et al. (2005) tested KDF as a growth promoter in tilapia. In this study, fish were fed the diets containing different concentrations of KDF (0%, 0.2%, 0.3% and 0.5%). The results showed that KDF significantly increased feed intake and weight gain and improved feed conversion ratios. Protein efficiency ratio was also significantly improved and the improvement was greatest with the addition of 0.2% and 0.5% KDF. Survival rates of fish after a challenge with *Vibro anguillarum* on day 10 were also significantly higher compared with the negative control and the effect was dose dependent. The authors thus concluded that 0.2% KDF is an efficient tool to control bacterial infections in tropical tilapia culture.

Based on the above information, it can be concluded that the most commonly used salt of formic acid in aquaculture is KDF and the application of this dissociating salt is attracting increasing interest. KDF has been shown to improve disease resistance in tilapia, but the effect on growth performance and feed utilization appears to be limited. The information

regarding the use of other salts of formic acid in aquaculture is limited. Thus, further studies are needed to better understand the effects of formic acid and its salts in diets for farmed fish.

### *3.1.3 Butyric acid and its salts*

Butyric acid ($CH_3CH_2$ $CH_2COOH$) is an oily liquid with a disagreeable rancid odour (Partanen and Mroz, 1999). Butyrate, together with propionate and acetate, are produced within the intestinal lumen by bacterial fermentation of carbohydrates, but also in a minor part by dietary and endogenous proteins, such as mucous, and sloughed epithelial cells (Topping and Clifton, 2001). The butyrate not only supplies energy for the epithelial cells, but also influences a wide array of cellular functions affecting gut health (Hamer et al., 2008). Butyrate can be absorbed by diffusion, and together with other short-chain fatty acids, contribute 20-30% of the total energy requirement of omnivorous or herbivorous animals, such as pigs (Bergman, 1990). The absorption of butyrate increases as pH decreases or concentrations increase in the intestinal lumen (Hollander et al., 1986). It has been shown that part of butyrate is converted to ketone bodies and free amino acid in the intestine. In addition to those compounds, butyrate is readily oxidized to $CO_2$, supplying energy for epithelial cells. Butyrate is also transported to the liver where it is metabolized to be butyryl-CoA, and then acetyl-CoA, longer chain fatty acids or ketone bodies (Bergman, 1990).

The application of butyric acid or its salts in the diets has been tested on some monogastric animals including pigs (Bokori et al., 1989; Gomes et al., 2007), poultry (Fernandez-Rubio et al., 2009; Mallo et al., 2008; Van Immerseel et al., 2005), and rabbits (Hullar et al., 1996). There is no data on the use of butyric acid or its salts in feed for fish. The beneficial effects of feeding butyric acid to other animals suggest that it may have potential use in fish feed as an additive. Thus, research is necessary to find out if butyric acid and its salts have a potential for use in diets for fish.

## 3.2 Rapeseed meal

### *3.2.1 General information*

*Brassica* oilseeds have been grown by humans for thousands of years. Nowadays, there are several *Brassica* oilseed species grown in the major rapeseed producing areas of the world (Booth and Gunstone, 2004). *B. rapa* (turnip rape) is a cold-hardy species that is grown in western Canada. Ecotypes of this species are also grown in the Indian subcontinent. *B. juncea*

is widely grown in northern India, China and parts of Australia due to its adaptability to dry growing conditions. *B. carinata* is largely restricted to Ethiopia and the surrounding countries in East Africa. *B. napus* (Swede rape) is the most commonly grown rapeseed in Europe, Canada and China. It originated through spontaneous interspecific hybridization between *B. rapa* and *B. oleracea* L (Friedt and Snowdon, 2009). Therefore an amphidiploid genome comprising the full chromosome complements of its two progenitors is produced after hybridization. The common name of *Brassica* oilseeds is rapeseed. The Canadian cultivar Canola, that contains less than 2% erucic acid in its oil and less than 30 μmol $g^{-1}$ aliphatic glucosinolates in its defatted meal (Shahidi, 1990), is most investigated in animal nutrition studies. Rapeseed currently is the world's second – leading oil crop after soybean, third-leading sources of vegetable oil after soybean and oil palm and second-leading oil extraction meal after soybean. World production of rapeseed, rapeseed oil and rapeseed meal were 58.4, 22.7 and 34.1 million metric tons annually in 2010/11 (USDA, 2011). The leading producers are China, Canada, India, European Union, Ukraine and Australia (IndexMundi, 2010).

Rapeseed meal is a by-product of rapeseed oil extraction. Traditionally, the rapeseed is crushed and then solvent extracted in order to separate the oil from the meal. This process called pre-press solvent extraction, usually involves: seed cleaning, optional tempering and dehulling, flaking, conditioning, mechanical extraction by pre-pressing and extrusion, expansion, most likely followed by solvent extraction, desolventizing and toasting of the meal (Booth and Gunstone, 2004). The details of the oil extraction steps are illuminated in Fig. 2.

### *3.2.2 Nutrition properties of rapeseed meal*

Typically, conventional rapeseed meal contains 402-428 g $kg^{-1}$ crude protein and 31-41 g $kg^{-1}$ crude fat on a dry matter basis (Bell and Jeffer; 1976; Sklan et al., 2004), and Canola meal contains 389-486 g $kg^{-1}$ crude protein, 34-145 g $kg^{-1}$ crude fat (Glencross et al., 2004b; Newkirk, 2009) (Table 2). The amino acid composition of conventional rapeseed meal and Canola meal are similar, and they contain more histidine, methionine, cystine, valine and threonine than soybean meal (Table 3). The composition of rapeseed meal, or Canola meal, is influenced by the type of cultivar, producing region and oil extraction method. A study by Glenocross et al. (2004a) showed that expeller-extracted Australian Canola meal contained 406 g $kg^{-1}$ crude protein, 145 g $kg^{-1}$ crude fat and 22 MJ $kg^{-1}$ gross energy of dry matter, while solvent-extracted Canola meal contained 432 g $kg^{-1}$ crude protein, 33.7 g $kg^{-1}$ crude fat and 19.7 MJ $kg^{-1}$ gross energy of dry matter. Sun et al. (2008) reported that the crude protein

content of differently processed Canola meals ranged from 327 to 435 g $kg^{-1}$ of dry matter and the crude fat was ranged from 11.8 to 283 g $kg^{-1}$ of dry matter.

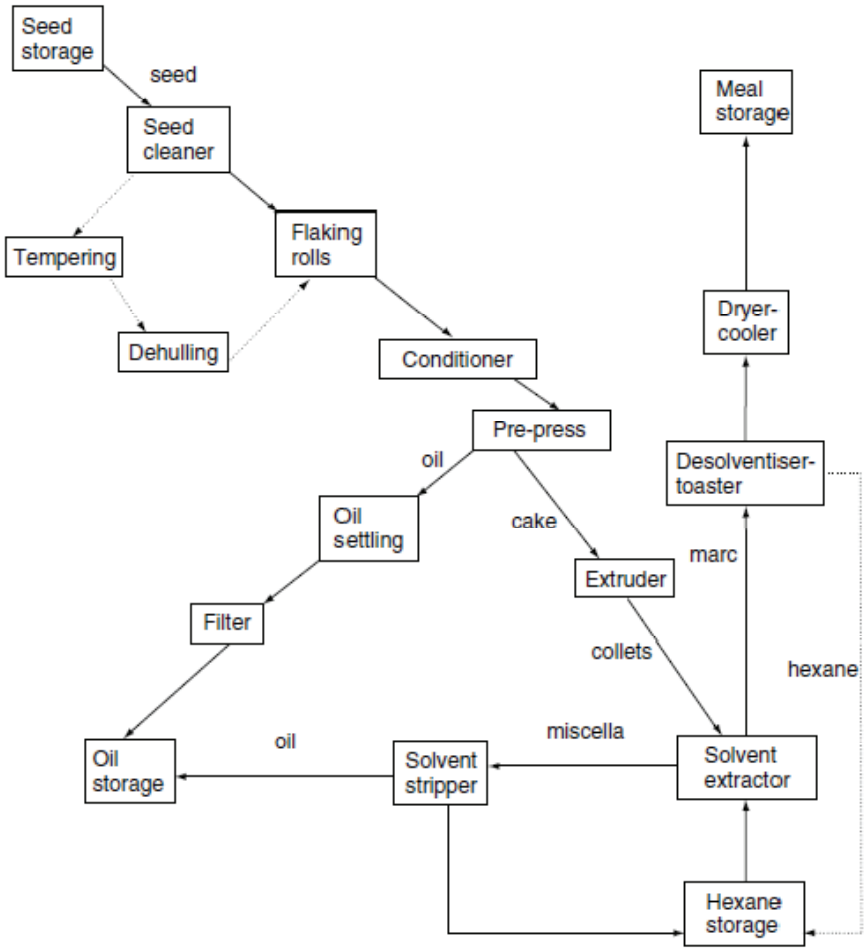


Figure 2. Rapeseed oil extraction and rapeseed meal production (Booth and Gunstone, 2004).

Table 2. Typical chemical composition of conventional rapeseed and Canola meals (dry matter basis)

| Component | Rapeseed meal[a] | Canola meal[b] |
|---|---|---|
| Crude protein, g $kg^{-1}$ | 402-428 | 389-486 |
| Oil, g $kg^{-1}$ | 31-41 | 34-145 |
| Linoleic acid, g $kg^{-1}$ | | 7 |
| Ash, g $kg^{-1}$ | 70-100 | 63-83 |
| Crude fibre, g $kg^{-1}$ | 121-163 | 136 |
| Tannins, g $kg^{-1}$ | | 17 |
| Sinapine, g $kg^{-1}$ | 24 | 11 |
| Phytate, g $kg^{-1}$ | 20 | 26-44 |
| Glucosinolates, mmol $kg^{-1}$ | 60-2,100[c] | 1.1-82 |
| Gross energy, kJ $kg^{-1}$ | 18,060-21,418 | 19,700-22,000 |

[a]Bell and Jeffers (1976); Sklan et al. (2004).
[b]Newkrik (2009); Glencross et al. (2004a; 2004b).
[c]Reviewed by Tripathi and Mishra (2007).

Table 3. Essential amino acid composition of conventional rapeseed meal, Canola meal and soybean meal (proportion as % of crude protein)

| | Rapeseed meal[a] | Canola meal[b] | Soybean meal[a] |
|---|---|---|---|
| Arginine | 6.11 | 5.78 | 6.44 |
| Histidine | 2.81 | 3.11 | 2.40 |
| Isoleucine | 3.98 | 4.33 | 4.69 |
| Leucine | 6.97 | 7.06 | 7.49 |
| Lysine | 5.98 | 5.56 | 6.22 |
| Methionine | 1.78 | 2.06 | 1.40 |
| Cystine | 1.23 | 2.38 | 0.65 |
| Tryptophan | 1.16 | 1.33 | 1.2 |
| Phenylalanine | 4.01 | 3.83 | 4.8 |
| Valine | 5.11 | 5.47 | 5.00 |
| Threonine | 4.50 | 4.39 | 3.8 |

[a]Bell and Jeffers (1976).
[b]Newkrik (2009).

### *3.2.3 Anti-nutritional factors of rapeseed meal*

The challenge of using rapeseed meal is the content of anti-nutritional factors (ANFs), which includes protease inhibitors, glucosinolates, phytate and tannins (Francis et al., 2001). Glucosinolates and phytate are the main ANFs in rapeseed meal. Therefore the following discussion will focus on these two.

The details of glucosinolates and their role in animal nutrition have been reviewed by Tripathi and Mishra (2007). The glucosinolates are sulphur-containing secondary plant metabolites, and 120 different glucosinolates have been identified (Chen and Andreasson, 2001). The major glucosinolates found in *B. napus* and *B. campestris* rapeseed meals are progoltrin, gluconapin, glucobrassicanapin, napoleiferin, glucobrassicin and neoglucobrassicin (Bell, 1984). All glucosinolates share a common structure of β-D-thioglucose groups, a sulphonated oxime moiety and a variable side-chain derived from methionine, tryptophan or phenylananine (Fig 3).

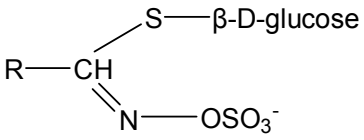


Figure 3. General structure of glucosinolates (Tripathi and Mishra, 2007).

Glucosinolates themselves are biologically inactive molecules. However, their breakdown products are biologically active and may exhibit unpleasant effects on animal growth and health. The breakdown of glucosinolates starts with hydrolysis, which is catalyzed by the myrosinase enzyme. The details of hydrolysis are shown in the Fig 4. Myrosinase is present in plants and can be produced by the intestinal microflora in animals (Nugon-Baudon et al., 1990). Once glucosinolates meet myrosinase, the hydrolysis starts. The final products of hydrolysis are isothiocyanate, nitrile, epithionitrile, thiocyanate and oxazolidine-2-thione (Fig 4).

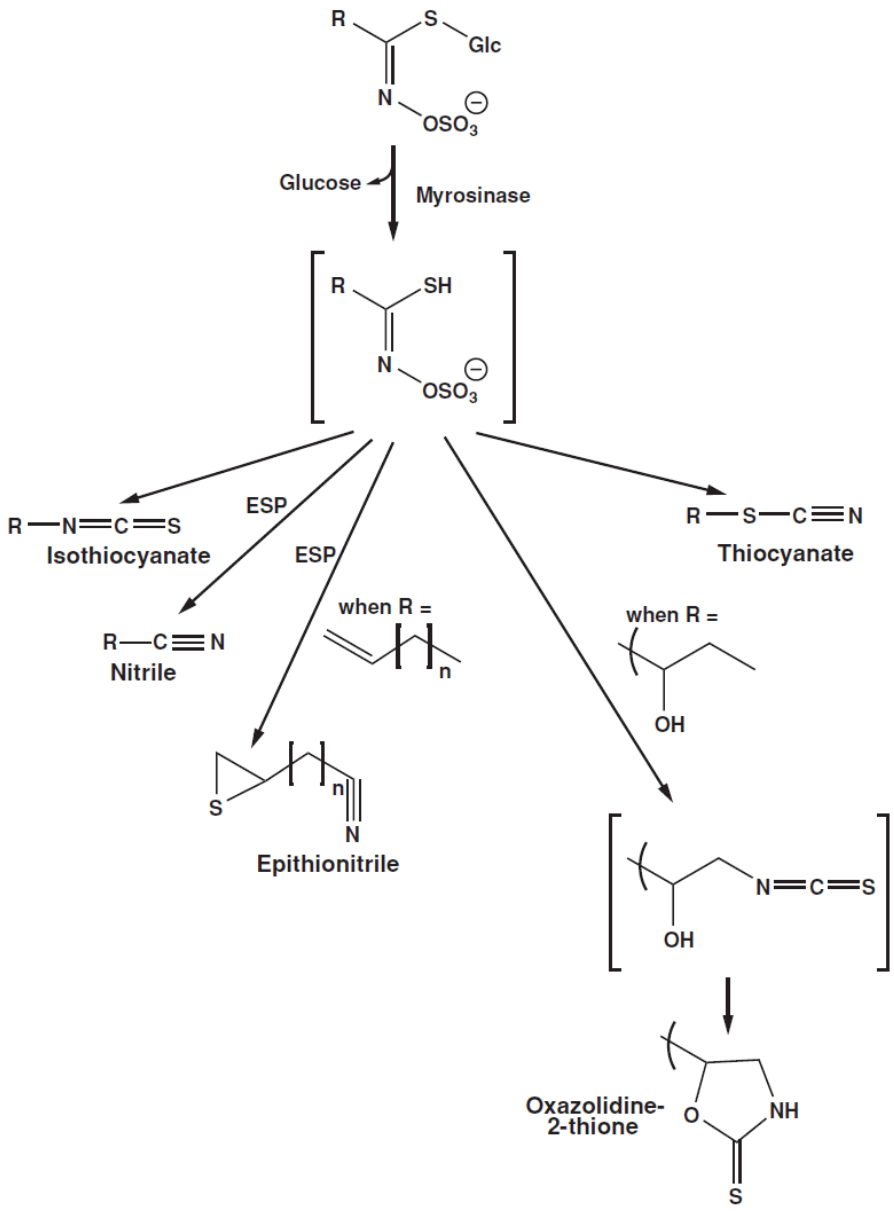


Figure 4. Scheme of glucosinolate hydrolysis (Halkier and Gershenzon, 2006)

Investigation of adverse effect of breakdown products in fish has been conducted. The results indicate that glucosinolates in rapeseed meal depressed growth and thyroid function in rainbow trout (Burel et al., 2000) and red sea bream (Glencross et al., 2004a; b). Reduced growth was mainly caused reduced feed intake due to the bitterness of some glucosinolates and their breakdown products. Progoitrin and sinigrin were considered to be responsible for the bitter taste (Fenwick et al., 1983), which is ascribed to a breakdown product of isothiocyanates (Mithen et al., 2000; van Doorn et al., 1998). In addition, thiocyanates, thiourea and oxazolidithione reduce iodine availability, reducing thyroid function (Wallig et al., 2002). Nitriles may also negatively influence the health of animals (Tanii et al., 2004). However, the adverse effects of glucosinolates are varied due to the different levels and compositions of glucosinolates in the diet and varied glucosinolate tolerance capabilities of different animal species. In addition to the adverse effects, one of the breakdown products, isothiocynates has a potential use in pharmacology due to its anti-tumour properties (Lund et al., 2003; van Poppel et al., 1999). The effects of glucosinolates in diets on growth performance, feed utilization and thyroid functions of fish has been investigated in a number of studies (Burel et al., 2000; 2001; Cheng et al., 2010; Glencross et al., 2004a; b). Those studies revealed that the tolerance levels of glucosinolates vary for different fish species. The upper limit level was lower than 1.4 mmol $kg^{-1}$ DM for rainbow trout, could be higher than 1.8 mmol $kg^{-1}$ DM for red seabream, and is lower than 1.6 mmol $kg^{-1}$ DM for Japanese seabass.

Phytate (myo-inositol hexaphosphate) is a common anti-nutritional factor in plant seeds. It is the principal storage form of phosphorus in many plant tissues. Phosphorus in this form is generally not bioavailable to animals that lack the phytase, required to hydrolyze phosphate from the phytate molecule. Phytates strongly bind to important cationic minerals such as calcium, magnesium, iron and zinc, and can therefore contribute to mineral deficiencies. Phytate also may form sparingly digestible phytate-protein complexes, which reduced availability of dietary protein in a study from Richardson et al. (1985). However, Denstadli et al. (2006) observed that phytate did not reduced the digestibility of dietary protein in Atlantic salmon. Rapeseed meal contains 50-75 g phytate $kg^{-1}$ (Francis et al., 2001).

Growth studies with commonly cultured fish species showed a negative effect in the diet containing phytate (reviewed by Francis et al., 2001). Sajjadi and Garter (2004) observed that the addition of phytate had no significant effect on feed intake or weight gain of Atlantic salmon, however, protein digestibility was significantly reduced. In addition, a study conducted by Reche and Garling (2004) showed that phytate does not reduce nitrogen retention in tilapia, and its removal from soybean meal may decrease nitrogen retention.

Denstadli et al. (2006) investigated the phytate dose-response on Atlantic salmon. They concluded that Atlantic salmon can grow well with phytate levels between 4.7 and 10.0 g $kg^{-1}$. Helland et al. (2006) investigated the effects of graded levels of phytate on skeletal development and mineral deposition in Atlantic salmon. They found that the level of phytate had no significant effect on the P content of either whole body or the vertebral column. However, negative effects of high phytate were found on whole body concentration of calcium, magnesium, the calcium to phosphorus ratio, and in the vertebral column concentration of zinc, and that high levels of dietary phytic acid introduced hyper dense vertebrae in the salmon.

### *3.2.4 Processing of rapeseed meal*

The use of rapeseed meal in fish feed is limited by the presence of glucosinolates, phytate, and a high fibre content. Rapeseed meal therefore probably requires processing to be optimally used in fish feeds. One processing methods is heat treatment. The beneficial effects of heat is ascribed to reduction of glucosinolates and inactivation of myrosinase (Newkirk and Classen, 2002), which catalyze the breakdown of glucosinolates to form secondary toxic compounds (Fig. 4). The extrusion process during oil extraction can reduce the activity of myrosinase (Fig. 2) but some active myrosinase remains in rapeseed meal after oil extraction. Fermentation is regarded as one process that has potential of degrading glucosinolates and phytate. The details of the effect of solid state fermentation on degradation of glucosinolates and phytate are discussed in Section 3.3.3.

## 3.3 Solid state fermentation

### *3.3.1 General information*

The fermentation processes can be divided into submerged liquid fermentation and solid state fermentations (SSF) based on the amount of free liquid in the substrate. In submerged liquid fermentation, the amount of solid substance rarely reaches more than 50 g $L^{-1}$, while in SSF the solids in the substrate normally varies between 20 and 70% of the total weight (Mitchell et al., 2002). SSF may be the oldest biotechnology to produce food in human history. Evidences show that the Egyptians were making bread using a fermentation process in 2600 BC (Krishna, 2005). Also in China, SSF has been used to produce brewed foods, for instance rice wine, soy sauce and vinegar, since ancient times (Chen, 1992). SSF has recently received renewed attention, particularly in areas such as solid waste treatment, biomass energy

conservation and for the production of high value-low volume products such as biologically active secondary metabolites (Singhania et al., 2009). This is mainly due to the advantages of SSF. These include high volume productivity, relatively higher concentration of products, lower effluent generation and simple fermentation equipment (Pandey et al., 2008).

Based on the strains used, SSF can be classified further into two major categories: pure culture SSF and natural/indigenous culture SSF (Pandey et al., 2008). Pure culture SSF is general used at an industry scale to produce value-added products (e.g. enzymes, organic acids), and individual strains or a mixed culture is used. Natural culture SSF is generally used to improve the quality of agro-industrial residues. The microbes used in SSF are mainly are fungi and yeasts, and due to the low moisture content, only a few bacteria can be used (Pandey et al., 2000; Raimbault, 1998). Fungi are the most adaptable microbes because their hyphae can grow on particle surfaces and penetrate into the interparticlular spaces and thereby colonizing solid substrates (dos Santos et al., 2004). Fig. 5 shows the microstructure of fungal SSF, which used a continuous gas phase and a minimum of water in the spaces between moist solid particles. The water may also existent on the surface of solid particles in the form of a thin film. The majority of the SSF processes involve filamentous fungi, although some involve bacteria and some involve yeasts.

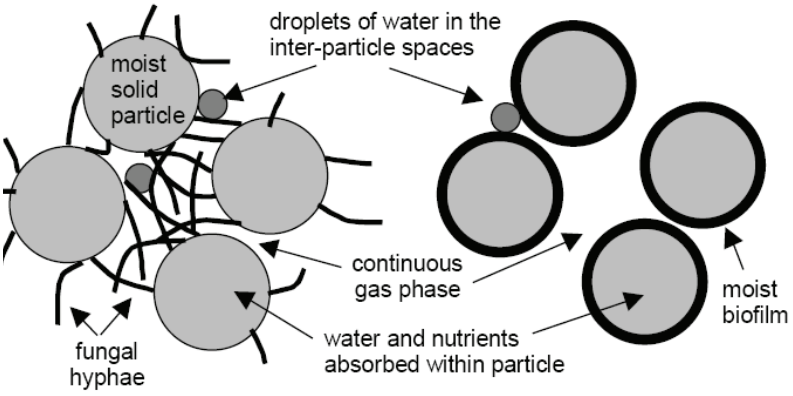


Figure 5. Micro structure of solid state fermentation with fungi (Mitchell et al., 2006).

### *3.3.2 SSF influences the nutritional value of the substrate*

SSF is used to produce enzymes, organic acids, secondary metabolites, polyunsaturated fatty acids, biofuels, single-cell protein, pigments and to enhance nutritional value of agricultural by-products (Bhargav et al., 2008; Krishna, 2005). Among these applications, enhancing nutritional value of agricultural by-products is increasingly interesting due to environmental

issues. The beneficial effects of SSF on nutrimental value may be ascribed to changes in nutrients and ANFs. The following will be limited to the effects of SSF on nutrition value of one type of agricultural product; plant protein sources (e.g. oil crop meals).

The production of microbes requires nutrients; including carbon and nitrogen, minerals, and vitamins or cofactors. Carbohydrates in the form of sugars, starch, cellulose, or hemi-cellulose are main carbon sources. Their hydrolytic breakdown into the monomeric or oligomeric constituents provides important fermentation substrates for many obligate and facultative anaerobes (Böck, 2009). Nitrogen sources include ammonium tartrate, oxalate, sulphate, nitrate, chloride, sodium nitrate, urea, peptones and amino acids. The oilseed meals are high in nutrients. Therefore, they are a good nutrient source for microbes during the SSF process. As a result, the nutritional value of oilseed meals is changed. The end products of amino acid fermentation include ammonium and organic acids, including formate, acetate, butyrate and propionate (Böck, 2009). The types of organic acids are determined by the species of microbe. The synthesis of amino acids and fatty acids, taking place by some microbes during the fermentation process, also leads to changes in the nutrition value of substrates. Synthesis of many amino acids during SSF process by adding supplemental nitrogen has been successfully performed (Reviewed by Kumagai, 2006). The synthesis of fatty acids during fermentation with *Aspergillus niger* has been reported by Singh (1991).

### *3.3.3 SSF influences ANFs of the substrate*

Plant protein sources usually contain a wide variety of ANFs (reviewed by Francis et al., 2001). SSF process is a tool that can degrade or eliminate ANFs. Many studies have been carried out to test the effect of SSF on ANFs. Results from some typical studies are given in Table 4, and showed that most of ANFs were degraded during SSF.

Table 4. An overview of the results of selected studies where fermentation was used to inactivate ANFs in plant protein sources

| Plant sources | ANFs reduced / Nutritional value improved | Microorganism used | Fermentation methods | Reference |
|---|---|---|---|---|
| Canola meal | Phytate content can be reduced by all microorganisms | *Rhizopus oligosporus* NRRL 2990, *Aspergillus niger* NRC 5765 and NRC 401 121, *Aspergillus ficuum* NRRL 3135 and a wild *Saccharomyces cerevisiae* strain | SSF | Nair and Duvnjak, 1991 |
| Rapeseed | 84% of carbohydrates, 30% of | *R. oligosporus* sp-T3 | SSF | Rozan et al., |

| | | | | |
|---|---|---|---|---|
| meal | lignin and other polyphenolic components indigestible by nonruminants, and 47% of total glucosinolates were degraded | | | 1996 |
| "Bomba" bean and "Opal" pea | A distinct decrease in raffinose, verbascose and stachyose contents up to 80-90% after fermentation | *Lactobacillus plantarum* | SSF | Czarnecka et al., 1998 |
| Rapeseed meal | Phytate content was reduced for all investigated parameters | *Aspergillus niger* | SSF | El-Batal and Karem, 2001 |
| Rapeseed meal | The contents of glucosinolates, thiooxazolidones, phytate and crude fibre declined by 43.1%, 34%, 42.4% and 25.5%, respectively | *R. oligosporus* | SSF | Vig and Walia, 2001 |
| Flour and whole beau seeds | In either fermented flour or whole beans, the alpha-galactosides and IP6 decreased (99-100%, 7-39% respectively); TIA levels and tannins content decreased (58-71% and 61-70%) by fermentation | Natural fermentation | SSF | Granito et al., 2002 |
| *Vigna sinensis* var. carilla flours | The levels of alpha-galactosides, inositol phosphates, trypsin inhibitor activity (TIA), soluble carbohydrates, starch (total and available), total available carbohydrates, thiamin, and riboflavin were reduced after fermentation | Natural fermentation and *Lactobacillus fermentum* or *L. plantarum* | | Doblado et al., 2003 |
| Teff (*Eragrostis teff*) and grass-pea (*Lathyrus sativus*), | Improve essential amino acid profile, and most of neurotoxin -N-oxalyl-alpha,beta-diamino-propionic acid (beta-ODAP) in grass-pea was removed when using the fungal strains | *L. plantarum, Aspergillus oryzae* and *R. oligosporus* | SSF | Yigzaw et al., 2004 |
| Black beans (*Phaseolus vulgaris*) | A significant diminution of the trypsin inhibitors and tannins was found | Natural lactic acid fermentation | SSF | Granito and Alvarez, 2006 |
| Cottonseed meal | Free gossypol was reduced in fermented cottonseed meal | *C tropicalis* ZD-3 with *A. niger* ZD-8 mixed | SSF | Zhang et al., 2006a |
| Cottonseed meal | Free gossypol was reduced, and the maximum detoxification efficiency of gossypol was achieved by employing the substrate, which consists of 70% of CSM, 20% of corn flour and 10% of wheat bran. | *Candida tropicalis* ZD-3 | SSF | Zhang et al., 2006b |
| Soybean protein | Neutral protease activity was the highest, having about 636 U/g at 54 h fermentation. The content of total free amino acid was almost 3-18 times higher than controls. | | SSF | Lee et al., 2007 |
| Cottonseed meal | CSM substrate supplemented with starch and sucrose enhanced detoxification of gossypol, but heat treatment and minerals were also effective in reducing FG levels during solid substrate fermentation of CSM. The detoxification effect with minerals was the best among treatments. | *Candida capsuligena* ZD-1, *C. tropicalis* ZD-3, *Saccharomyces cerevisae* ZD-5, *Aspergillus terricola* ZD-6, *A. oryzae* ZD-7, or *A. niger* ZD-8 | SSF | Zhang et al., 2007 |
| Soy protein | Fermentation can decrease soy | solid state of cracked | SSF, Liquid | Frias et al., |

| | | | | |
|---|---|---|---|---|
| | immunoreactivity and increase total amino acid. LSF has a better effect then SSF. | seeds inoculated with *A. oryzae*, *Rhizopus oryzae*, and *Bacillus subtilis* and in the liquid state of milled soybean flours fermented naturally by microorganisms present only in the seeds or by inoculation with *Lactobacillus plantarum* | state fermentation (LSF) | 2008 |
| Soybeans and soybean meal | Fermentation increased protein content, eliminated trypsin inhibitors, and reduced peptide size in soybeans and soybean meals. | *A. oryzae* GB-107 | SSF | Hong et al., 2004 |
| soybean (*Glycine max* Merr.), cowpea (*Vigna unguiculata* L. Walp) and groundbean (*Macrotyloma geocarpa* Harms) | A slight increase in trypsin inhibitor activity was observed during soybean fermentation. Phytate decreased during fermentation by 30.7%, 32.6% and 29.1% respectively in soybean, cowpea and groundbean | *R. oligosporus* | SSF | Egounlety and Aworh, 2003 |
| Rapeseed meal | A 24 h fermentation induced a degradation of 57.7% aliphatic glucosinolates, 97.3% indol glucosinolates and 73% ethanol-soluble sugars (alpha-galactosides, flatulence generator included) of rapeseed meal | *R. Oligosporus* Sp T-3 | SSF | Bau et al., 1994 |
| Brown mustard seed meal (*Brassica juncea*) | The complete degradation of glucosinolates occurred after 60-96 h fermentation at 30$^{o}$C | *Aspergillus sp* NR-4201 | SSF and LSF | Rakariyatham and Sakorn, 2002 |

Trypsin inhibitors occur widely in legume seeds and can be degraded by fermentation. The trypsin inhibitor level in *Vigna sinensis* var. carilla flours was decreased after natural fermentation and induced fermentation with *Lactobacillus fermentum* or *Lactobacillus plantarum* (Doblado, et al., 2003). Natural lactic acid fermentation significantly reduced the trypsin inhibitors in black beans (*Phaseolus vulgaris*). Hong, et al. (2004) fermented soybeans and soybean meals in a bed-packed solid fermentor for 48 hours and the results showed that the SSF eliminated most of the trypsin inhibitor activity from both soybeans and soybean meals.

Phytate is one of ANFs that also can be degraded by fermentation. Nair and Duvnjak (1991) carried out a SSF of Canola meal for the reduction of its phytate content using *R. oligosporus* NRRL 2990, *A. niger* NRC 5765 and NRC 401 121, *A. ficuum* NRRL 3135 and a wild *S. cerevisiae* strain. They reported that the phytate in Canola meal can be reduced by

these microorganisms. Afterwards, El-Batal and Karem (2001) investigated the effect of some fermentation parameters on the reduction of phytate content in rapeseed meal during SSF with *A. niger*, and confirmed that all investigated parameters reduced the phytate content. Similar results, observed by Vig and Walia (2001), showed that *R. oligosporus* reduced the phytate level by 42.4% during SSF of rapeseed meal. The same microbe strain later was employed in a fermentation study carried out by Egounlety and Aworh (2003). In this study the phytate content decreased by 30.7%, 32.6% and 29.1% respectively in soybean, cowpea and groundbean, due to the generation of phytase during fermentation (El-Batal and Karem, 2001; Papagianni et al., 1999).

Fermentation is a feasible method of reducing the glucosinolate content in Brassica. One fermentation method of improving rapeseed meal using *R. oligosporus* sp-T3 under 24 hour SSF induced a degradation of 57.7% aliphatic glucosinolates, 97.3% indol glucosinolates (Bau, et al., 1994). Another fermentation process with rapeseed meal using *R. oligosporus* and *Aspergillus sp.* under SSF reduced total glucosinolates by 43.1% (Vig and Walia, 2001). In addition, a complete degradation of glucosinolates occurred after 60-96 h fermentation of brown mustard seed meal at 30$^{o}$C (Rakariyatham and Sakorn, 2002). The reduction in glucosinolates and their metabolites during fermentation may be due to utilization of glucose and sulphur moieties of these compounds by microbial enzymes (Tripathi and Mishra, 2007).

Lectins are carbohydrate-binding proteins or glycoproteins which are highly specific for their sugar moieties. Plant lectins or phytohaemagglutinins are found in many legume seed. The common effects of lectins include disruption of the small intestinal metabolism and morphological damage to the villi. Lectins are inactivated by fermentation due to hydrolysis. Cuadrado et al. (2002) investigated the effect of natural fermentation on the degradation of lectins in the seeds of *Lens culinaris*. The observations confirmed the lectins almost disappeared after 72 h of natural fermentation under the optimum conditions of flour concentration and temperature.

Saponins are steroids or triterpenoids that exist in many plant-derived feed ingredients. Saponins are not destroyed by heat treatment. However fermentation appears to reduce the saponin content. Fowomola and Akindahunsi (2008) showed that fermentation reduced the content of saponins in sandbox (*Hura crepitans*) seed during fermentation.

Tannins are secondary compounds of various chemical structures widely occurring in the plant kingdom and are generally divided into hydrolysable and condensed tannins. Naturally occurring tannins are reported to interact with proteins (both enzyme and non-enzyme proteins) to form tannin-protein complexes, resulting in inactivation of digestive enzymes and protein insolubility. Fermentation has been considered to be an effective method

to remove tannins. Mukhopadhyay and Ray (1999) observed that fermentation with lactic acid bacteria (*Lactobacillus acidophilus*) resulted in reduction of the tannin content from 20 to 10 g $kg^{-1}$ in oilseed meal. Natural fermentation can also decrease tannins content 61-70% (Granito, et al., 2002). Besides, a reduction of tannin content in three local sorghum varieties after fermentation was reported in a study conducted by Osman (2004).

Gossypol, which is produced in the seeds of the cotton plant, is a naturally occurring toxin for animals. The negative effect of gossypol on animal health has been recognized. Therefore, several methods have been developed to remove gossypol from cottonseed. Fermentation can inactive gossypol. Zhang, et al. (2006a) succeed in reducing free gossypol in fermented cottonseed meal when *Candida tropicalis* ZD-3 and *A. niger* ZD-8 were mixed in a SSF. Further studies also investigated the effect of substrate on the reduction of gossypol in cottonseed meal, and determined the composition of substrate that can reduce the content of free gossypol greatly (Zhang, et al., 2007; Zhang, et al., 2006b).

Antigenic compounds (or allergens) are protein components that induce antigenic effects in animals. Efficient ways to reduce allergens include hot aqueous ethanol extraction, alkali treatment, moist extrusion at elevated temperatures and fermentation. The degradation of soybean allergens during fermentation by microbial proteolytic enzymes has been confirmed in several studies (Hong et al., 2004; Yamanishi et al., 1995). Frias et al. (2008) carried out SSF of cracked soybean seeds inoculated with *A. oryzae*, *Rhizopusoryzae*, and *Bacillus subtilis*, as well as in the liquid state of milled soybean flours fermented naturally by microorganisms present only in the seeds or by inoculation with *L. plantarum*. The results show that soy immunoreactivity was decreased in both SSF and liquid fermentation. Other microorganisms such as *L. plantarum*, *Bifidobacterium lactis*, *S. cereviseae* also have been proved to decrease allergens of soybean in both SSF and liquid state fermentation (Song et al., 2008).

Non-starch polysaccharides (NSPs) and oligosaccharides are carbohydrates that may be ANFs since they interfere with lipid digestion. Both are restricted for use in fish diets due to the lack of necessary enzymes, required for their hydrolysis. Fermentation can be utilized to reduce the levels of NSPs and oligosaccharides in plant protein. A distinct decrease in raffinose, verbascose and stachyose contents even up to 80-90% after fermentation was reported by Czarnecka, et al. (1998). Egounlety and Aworh (2003) observed that stachyose decreased during fermentation with a reduction of 83.9%, 91.5% and 85.5% respectively for soybean, cowpea and groundbean. Galactose, the predominant sugars, glucose, fructose, maltose and melibiose were also observed to decrease. Refstie et al. (2005) also reported that

lactic acid fermentation eliminated sucrose and reduced the level of raffinose in extracted (de-oiled) soybean white flakes.

### *3.3.4 Effect of fermented plant protein sources on fish growth and health*

Different fermented plant protein sources have been tested on different fish species (Table 5). Most of the results were positive and show that fermented plant protein sources were acceptable protein sources for fish. Effects of fermented soybean meal on fish growth or health performance have been widely investigated. Luo et al. (2004) used fermented soybean meal to feed grouper juveniles. They observed that the fish fed a diet containing 21% fermented soybean meal had higher growth, FE and PER than the fish fed the diet containing same amount of soybean meal. While Yang et al. (2005) concluded that the optimal dietary fermented soybean meal inclusion was 19.6% for the silver perch based on the growth performance. In Atlantic salmon, Refstie et al. (2005) reported that soybean white flakes fermented by *L. brevis* improved the digestibility of lipid and slightly reduced soybean meal-induced pathological changes in the intestine compared to the other soybean meals. Sesame seed meal (Mukhopadhyay and Ray, 1999), linseed meal protein (Mukhopadhyay and Ray, 2001) and black gram seed meal (Ramachandran and Ray, 2007) were fermented to improve quality, and all of these were well accepted by the Indian major carp rohu fingerlings in terms of its growth and feed utilization. Moreover, Skrede et al. (2002) fermented wheat and barley whole meal flours by using *Lactobacillus* in SSF. Those fermented products then were fed to Atlantic salmon, and resulted in the improvements in digestibility of fat and energy, as well as in increased absorption of sodium and zinc. Contrary to the positive effects of fermented plant protein sources on fish, negative effects have also occurred. Ng et al. (2002) reported reduced growth in red hybrid tilapia fed fermented palm kernel meal compared to the control group, which might have resulted from the presence of ANFs in fungal biomass.

Table 5. An overview of the results of selected studies where fermented plant protein sources were fed to fish

| Plant sources | Microorganism | Fish species | Fish performance | Reference |
|---|---|---|---|---|
| Black gram seed meal | A particular bacterial strain (*Bacillus sp*.) isolated from the intestine of adult common | Rohu, *Labeo rohita* (Hamilton), fingerlings | Growth and feed utilization efficiencies of diets containing fermented seed meal were superior to diets containing raw seed meal | Ramachandran and Ray, 2007 |

| | | | | |
|---|---|---|---|---|
| | carp | | | |
| Sesame seed meal | Lactic acid bacteria (*Lactobacillus acidophilus*) | Rohu fingerlings | Growth and feed utilization efficiencies of fish fed fermented sesame seed meal diets were superior to those fed raw oilseed meal diets. Carcass protein and lipid contents of fish fed fermented sesame seed meal diets increased with increasing level of incorporation | Mukhopadhyay and Ray, 1999 |
| linseed meal protein | lactic acid bacteria (*L. acidophilus*) | Rohu fingerlings | Rohu fingerlings can effectively utilize the supplemented amino acids and that linseed meal protein can replace up to 50% of the FM protein in rohu diets, if the linseed meal is properly fermented and supplemented with the lacking amino acids. | Mukhopadhyay and Ray, 2001 |
| Soybean white flakes | *Lactobacillus brevis* | Atlantic salmon | All soy products reduced the digestibility of lipid, but this effect was less severe when feeding the diets with fermented white flakes. Soybean meal-induced pathological changes in the intestine were less pronounced in fish fed fermented white flakes than in fish fed white flakes and soybean meal. | Refstie, et al., 2005 |
| Soybean meal | | Silver perch, | the optimal dietary fermented soybean meal inclusion was 19.6% for the suitable growth of silver perch | Yang, et al., 2005 |
| Soybean meal | | Grouper juveniles | Fish fed the diets containing 21 % fermented SBM had higher growth, FE and PER than fish fed the 20 % SBM diets, indicating that fermented SBM was a better protein source for fish growth than SBM. 10 % of white fish meal can be replaced by fermented SBM. | Luo, et al., 2004 |
| Wheat and barley whole meal flours | *Lactobacillus* | Atlantic salmon | Improvements in digestibility of fat and energy were obtained by fermenting the cereals. Fermentation resulted in improved Na absorption; from 68.8% to 73.2% for wheat diets and from 60.3% to 71.7% for | Skrede, et al., 2002 |

| | | | | |
|---|---|---|---|---|
| | | | barley diets. Fermentation caused a significant improvement in zinc absorption from 32.7% to 40.5% for wheat diets and from 33.2% to 43.5% for barley diets. | |
| Palm kernel meal | *Trichoderma koningii* (Oudemans) | Red hybrid tilapia | Hybrid tilapia fed fermeneted palm kernel meal-based diets at all dietary inclusions tested showed the poorest growth, and this might indicate the presence of ANFs in the resultant fungal biomass. | Ng, et al., 2002 |

# 4. MAIN RESULTS AND DISCUSSION

## 4.1 Effect of an organic acid salt blend (OAB) of sodium formate and sodium butyrate on digestibility and growth of rainbow trout and Nile tilapia

OAB added to plant-protein based diets before extrusion reduced the digestibility of macro nutrients (Fig. 6) and amino acids (Fig. 7) in rainbow trout (**Paper I**). The highest reduction in digestibility was found for cysteine, which is known to be the amino acid most sensitive to heat treatment (Storebakken et al., 2000). Improved digestibility of macro nutrients or amino acids have, however, been observed in other studies with rainbow trout (Morken et al., 2010) fed diets with sodium diformate added before extrusion and Atlantic salmon (Storebakken et al., 2010) fed diets with KDF added before extrusion. Adding OAB to plant-protein based diets after extrusion only affected digestibility of lipid and ash. One plausible explanation for the negative effect of adding the OAB to the plant-protein-based feed mix before extrusion is that the temperature in Sections 1-5 in the extruder and consequently the specific mechanical energy (SME) were lowered during extrusion. Lower temperature would result in lower efficiency in denaturation of protease inhibitors and, thus, decreased amino acid digestibility. Unfolding of seed proteins is also heat dependent, facilitating improved access of proteases to the protein.

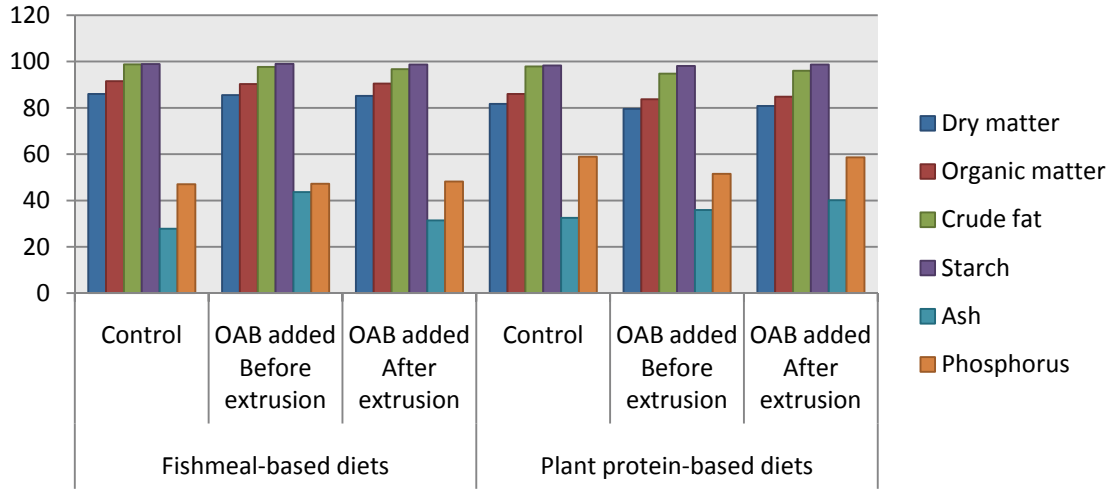


Figure 6. Apparent digestibility of main nutrients in the diets used in Experiment 1.

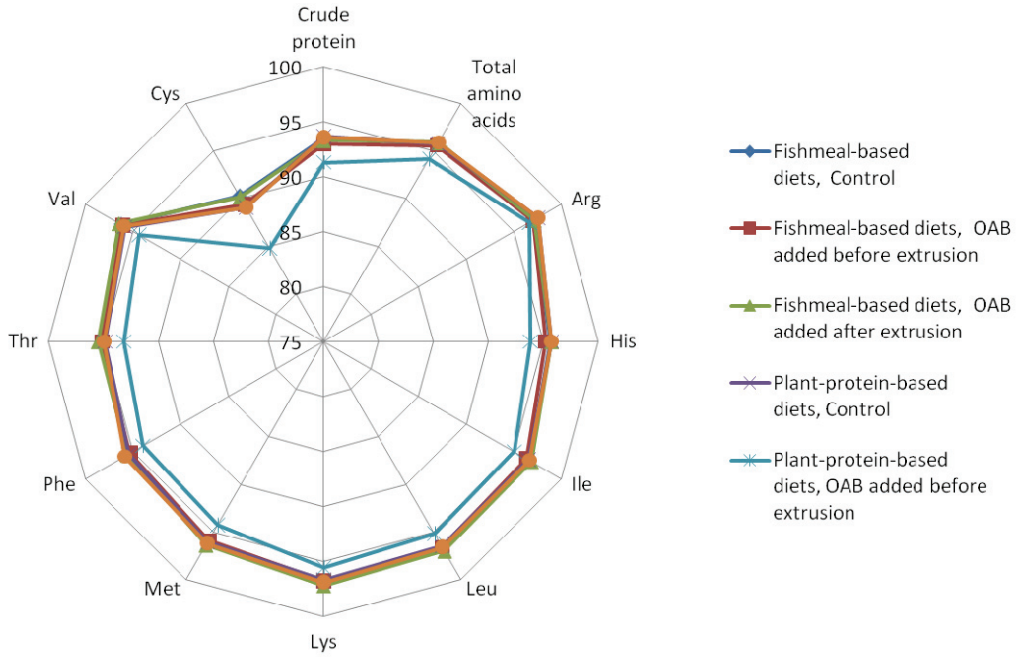


Figure 7. Apparent digestibility of crude protein, total amino acids, essential amino acids and cysteine in the diets used in Experiment 1.

OAB did not influence the digestibility of nitrogen, amino acids, phosphorus, zinc or magnesium, as well as nitrogen retention in Nile tilapia (**Paper III**). The same OAB to the extruded diet reduced the amino acid digestibility of rainbow trout (**Paper I**). The digestibility of phosphorus, zinc and magnesium, and phosphorus retention were not influenced. However, using organic acids in the diets for red sea bream exhibited an improvement on phosphorus utilization (Hossain et al., 2007). The reasons of the variant results between those studies may be ascribed to the type of organic acids or salts, feed preparation methods and fish species.

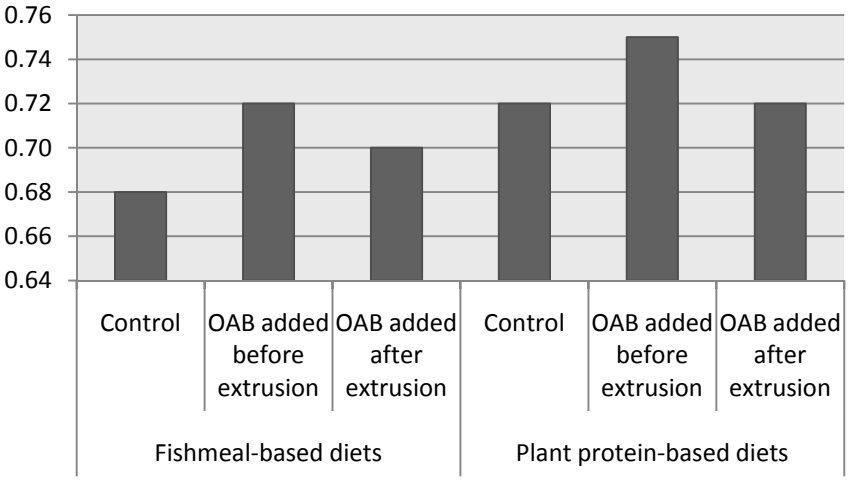


Figure 8. Feed conversation ratio in Experiment 1.

Higher feed conversion ratio (Fig. 8) when adding OAB to the diets before extrusion for rainbow trout (**Paper I**) is in keeping with previous findings in tilapia (Ng et al., 2009), but does not agree with our result with Nile tilapia (**Paper III)**. This effect is in contrast to the majority of published findings that demonstrate a positive effect of adding organic acids or their salts in fish feeds in Arctic charr (Ringø, 1991), Atlantic salmon (Lückstädt, 2008a; Ringø et al., 1994), rainbow trout (deWet, 2005), Red sea bream (Hossain et al., 2007; Sarker et al., 2005; 2007), and tilapia (Ramli et al., 2005; Zhou et al., 2009). The reasons for the conflicting results concerning weigh gain, feed intake and FCR of fish between some previous studies and the finding in the current work may be due to the type of organic acid or salt used, dietary inclusion level, diet composition, fish species and age, fish rearing conditions (deWet, 2005; Gislason et al., 1994; Ramli et al., 2005; Ringø, 1991; Sarker et al., 2007), and feed processing technology, as indicated in **Paper I**.

In the experiment with rainbow trout (**Paper I**), OAB added to the diets before extrusion, significantly increased middle intestine weight (Fig. 9) and tended to increase distal intestine weight. This indicates that the OAB may have stimulated intestinal growth, hypothesized as an effect of butyrate. The previous studies conducted by adding 0.17% (Galfi and Bokori, 1990) and 0.3% (Manzanilla et al., 2006) sodium butyrate to pig diets have confirmed the increased length of ileal microvilli and the depth of caecal or jejunum crypts. The need for heat processing of the OAB to obtain this effect may be rationalized by the fact that the butyrate used was coated (**Paper I-Table 1**). Coating is done both to protect additives against degradation during feed processing and to improve flow properties and mix ability with other feed ingredients. The results indicate that it was necessary to dissolve the coating material by extrusion, in order to liberate the butyrate so that it could be made available as an energy substrate for renewal of intestinal epithelium.

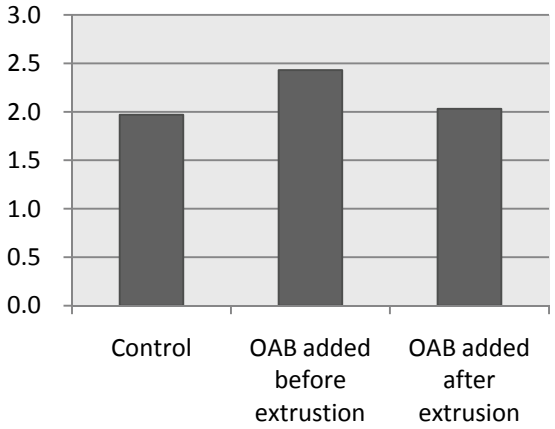


Figure 9. Ratio of middle intestine to whole body weight (g $kg^{-1}$).

The addition of OAB to the diets did not notably affect the morphological appearance of the distal intestine of rainbow trout. The morphological changes in the distal intestine were not as pronounced as observed in Atlantic salmon fed similar levels of SBM. Previous studies have also reported that rainbow trout were slower to develop distal intestine enteritis, and only do so at higher levels of SBM compared to Atlantic salmon (Refstie et al., 2000).

The stomach weight (% of body weight) of Nile tilapia fed the diets containing 1.8% OAB (0.38) was significantly higher than that of fish fed the diets without the OAB (0.34) (**Paper III**). This may be due to the sodium butyrate, which is known to increase gastric mucosa thickness by stimulating more cells to differentiate into enteroendocrine cells, and increasing the number of parental cells per gland in stomach of weaned pigs (Mazzoni et al., 2008). The different results between the experiments with Nile tilapia and rainbow trout may be ascribed to the different fish species and method of preparing the diets. Whole fish body composition of Nile tilapia was not changed by adding organic acid salts to the diets (**Paper III**), which is in agreement with the finding from Ng et al. (2009) except for dry matter content (from 30.2 to 28.0%). Using different type of organic acid salts between those two studies may be a reason.

### 4.2 Effect of SSF on nutritional quality of rapeseed meal (Paper II, III)

Rapeseed meal was fermented by using laboratory-scale facility for two times (**Paper II**) and medium-scale facility (**Paper III**). All SSFs changed the nutrient composition of rapeseed meal.

The fungal biomass in rapeseed meal increased from 3.86 to 11.99 and 22.05 g $kg^{-1}$ of dry matter after laboratory-scale SSF. However, the medium-scale SSF increased the fungal biomass only from 2.73 to 5.07 g $kg^{-1}$ of dry matter. Both SSF increased the contents of crude protein and crude fat, and reduced the content of starch in rapeseed meal, but laboratory-scale SSF resulted in greater change than medium-scale SSF (**Paper II-Table 1, Paper III-Table 1**). The laboratory-scale SSF slightly reduced the amount of amino acids in 100 g crude protein (**Paper II-Table 2**). In contrast to this, the medium-scale fermentation did not influence the amino acid profile obviously (**Paper III-Table 1**). Moreover, laboratory-scale SSF affected the size of peptide of protein in rapeseed meal (**Paper II-Figure 1**). The most peptides with size larger than 10 kDa were reduced. Laboratory-scale SSF also reduced the pH level of rapeseed meal (**Paper II-Table 1**)

ANFs in RSM were reduced by the SSF. The content of phytic acid (inositol hexaphosphate, IP6) in FRSM (10.5 and 19.0 g $(kg\ DM)^{-1}$) was lower than that in RSM (35.8

g (kg DM)$^{-1}$) (**Paper II-Table 1**). Also, virtually none of the glucosinolates detected in the RSM were detected in the FRSM, with the exception of trace amounts of progoitrin (**Paper II-Table 3**).

SSF resulted in changes in nutritional value of the RSM that were caused both by the fermentation itself and the hydro-thermal treatment employed for pasteurisation (laboratory-scale SSF) or only by the fermentation itself (medium-scale). The dry matter loss was 4.1 and 3.9% in laboratory-scale SSF. A similar magnitude of dry matter loss was observed by Penaloza et al. (1985) when subjecting coffee pulp to SSF with *A. niger*. Our recovery results indicate that *A. niger* mainly used carbohydrates as medium, as illustrated by starch recovery at 67 and 79 %, and recovery of the calculated analytical residue at 90 % during laboratory-scale SSF. This is in agreement with finding by Rozan et al. (1996). Sugars are the preferred energy substrate for *A.* niger, and hydrolysis of non-starch polysaccharides (NSP) occurs during fermentation (Rozan et al., 1996).

Recovery of crude protein was 100 % for laboratory-scale SSF, so nitrogen did not seem to be preferred substrate. The reduction in the proportion of both essential and non-essential amino acids relative to total nitrogen, however illustrates that a part of the amino acids in RSM was metabolised to other nitrogen-containing components during SSF. Muhammad and Oloyede (2009) concluded that the reduction in amino acid content was a result of nucleic acid synthesis during fermentation. Another reason may be that the germination of conidiospores in *A. niger* requires valine, leucine, cysteine and arginine (Abdelrahim and Arbab, 1985). Thus, in contrast to previously reported effects of SSF on other substrates (Frias et al., 2008; Hong et al., 2004; Yigzaw et al., 2004), SSF did not appear to increase protein content or improve the essential amino acid profile of RSM.

Lipid was the most notable fermentation product, with an increase of 26-27% in laboratory-scale SSF and 22% in medium-scale SSF. The fatty acid composition of RSM did not change by SSF (data not shown) and the fatty acid profiles were similar to those obtained during SSF with *A. niger* reported by Singh (1991).

The 47-71% reduction in phytic acid by laboratory-scale SSF with *A. niger* also was in agreement with previous findings (El-Batal and Karem, 2001), and was caused by the production of exogenous phytase during fermentation (El-Batal and Karem, 2001; Papagianni et al., 1999). In addition to rendering more than 1/3 of the phosphate bound in phytate in RSM available for digestion, SSF efficiently reduced the binding of divalent and trivalent cations. The ability of IP to bind essential kations such as zinc and magnesium is strongly reduced by decreasing the number of phosphates esterified to the inositol molecule (Persson et al., 1998). Thus, statistical power was low, and the only element that had its absorption significantly

improved by SSF was magnesium, probably due to reduced binding to IP6. That is in keeping with Usha and Chandra (1998) who found that the mineral availability was enhanced by fermentation. Also, the numerical improvement in apparent digestibility of phosphorus is in the range expected from the range of hydrolysis of phosphorus from IP6 in the RSM by SSF. $Zn^{++}$ has high affinity to both IP6 and oxalic acid (Vohra et al., 1965; Sayer and Gadd, 2001). Thus, it is probable that the lack of improvement in uptake of zinc was due to binding to residual IP6, eventually IP5 and oxalic acid in the FRSM. Furthermore, the amount of zinc in the diets was in excess of requirement (NRC, 1993). Zinc uptake is regulated at several levels, whereof uptake from the intestine is the first site of regulation (Hardy et al., 1987). Thus, improved availability of zinc does not automatically result in increased uptake.

The glucosinolates commonly found in RSM were efficiently reduced by SSF, in keeping with previous findings (Bau et al., 1994; Smits et al., 1993; Vig and Walia, 2001). The almost complete lack of glucosinolates after fermentation is partly due to the utilization of glucose and sulphur moieties in glucosinolates by *A. niger* (Verbiscar et al., 1981).The results can, however, not be interpreted as an indication that SSF rendered the glucosinolates in RSM harmless to the fish. Production of potent toxins such as isocyanate, thiocyanates goitrin, and nitrile is due to myrosinase activity (review by Bell, 1984). The analytical method used in this work only identified glucosinolates commonly found in RSM, and not metabolites thereof. Thus, additional analyses are required to determine if SSF reduced the potential of producing toxic components from glucosinolates in RSM, or if it in worst case introduced toxicity by fungal myrosinase activity.

Ng et al. (2002) observed that red tilapia fed a diet based on fermented palm kernel meal obtained poorer FCR than fish fed a diet based on unfermented palm kernel meal. They ascribed the difference to production of mycotoxins generated during fermentation (Lim et al., 2001). This emphasizes the need to carefully assess not only the fate of glucosinolates, but also the formation of other toxins if SSF will be developed into a tool to improve the nutritional value of RSM.

### 4.3 Fermented rapeseed meal in diets for tilapia (Paper II, III)

FRSM from 1$^{st}$ laboratory-scale SSF was used to produce a moist diet (soft pellet) , FRSM from 2$^{nd}$ laboratory-scale SSF was used to produce an extruded diets, and FRSM from medium-scale SSF was used to produce pelleted diets. The details of preparing the diets are described in the **Paper II** and **III**. Three Nile tilapia experiments were conducted to evaluate the moisture, extruded and pellet diets based on rapeseed meal and fermented rapeseed meals.

In the experiment with moist and pelleted diets (**Paper II and III**), the tilapia ingested more of the diet based RSM than the diet based on FRSM (**Paper II-Table 6; Paper III-Table 3 and 4**), possibly indicating reduced palatability caused by SSF. This might indicate that production of citric and oxalic acids and ethanol (Abouzied and Reddy, 1986; Nakamura et al., 1996) during SSF may have impaired the palatability in tilapia. However, citric acid has been shown to stimulate growth in hybrid tilapia (Pan et al., 2004). Thus, it is more likely that the lower feed intake may have been the result of improved nutritional characteristics, as demonstrated in rainbow trout by Bromley and Adkins (1984), who observed increased feed untake when the diet was diluted with a cellulose filler. In the experiment with extruded diets (**Paper II and III**), the intake of all diets was similar (**Paper II**-Table 8). The extrusion represented a hydro-thermal treatment of all extruded diets, not only the FRSM. Thus, it is probable that extrusion of the feeds participated to lowering compounds that impaired the feed intake, and increased the availability of nutrients to the digestive system.

The improved growth (Fig. 10) and feed conversion (Fig. 11) obtained by SSF of RSM in the experiment with moist diets may be related to a combination of several factors. Firstly, the reduction of ANFs such as phytic acid and glucosinolates are possible contributors. RSM has a total sugar content of more than 80 g $kg^{-1}$, indigestible polysaccharides like raffinose and stachyose account for nearly 20 %. The soluble NSPs content in RSM is 55 g $kg^{-1}$ (Knudsen, 1997), and it is expected that most of the oligosaccharides and a high proportion of the soluble NSPs was used as substrate. Raffinose, stachyose and soluble NSPs from soybeans are not limiting to digestibility and growth in salmonids (Kraugerud et al., 2007; Sørensen et al., 2011), in contrast to what is seen for poultry and swine (Choct et al., 2010). The reductions in nutrient digestibility in poultry and swine is associated with increased viscosity of the digesta (Choct et al., 2010), while in salmonids faecal dry matter is reduced in response to feeding SBM (Storebakken et al., 2000). The anatomy of the digestive system of the Nile tilapia and the adaptation to an omnivorous diet with high intake of plant materials makes it more similar to the pig than to the specialized carnivorous salmonids. However, results obtained by Leenhouwers et al. (2007) when feeding cereal grains with increasing viscosity, induced increased viscosity and reduced the dry matter content of digesta, but did not significantly affect nutrient digestibilities in Nile tilapia.

Thus, the improved feed conversion obtained by SSF of the RSM probably is due to a combination of reduced ANFs concentration and increased nutrient concentration, mainly achieved by converting indigestible carbohydrates into lipid.

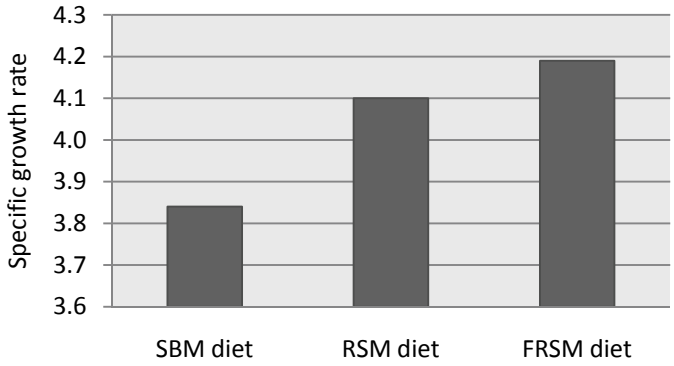


Figure 10. Specific growth rate of the tilapia fed the moist diets.

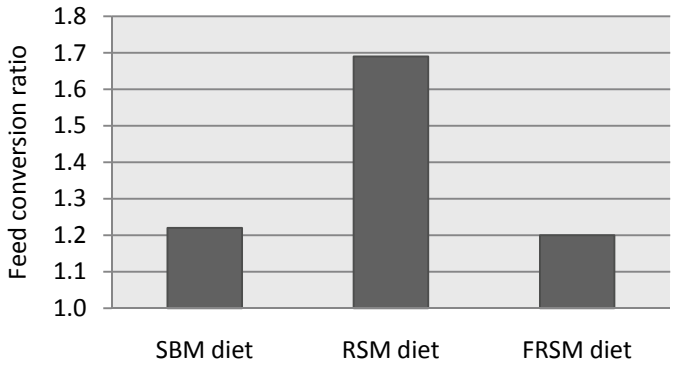


Figure 11. Feed conversion ratio of the tilapia fed the moist diets.

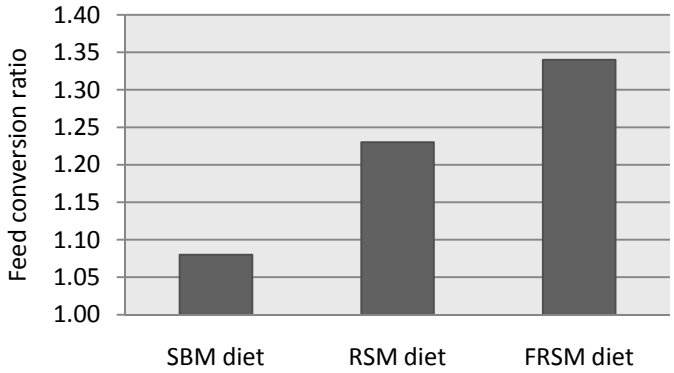


Figure 12. Feed conversion ratio of the tilapia fed the extruded diets.

The heat input for pasteurisation and drying was different for the two laboratory and one medium-scale SSFs (details in **Paper II and III**). Part of differences in nutritional

responses observed when feeding the moist diets to tilapia may be ascribed to differences in hydrothermal treatment. Hydrothermal treatment during SSF may have been of importance, since growth (**Paper II**-Table 8) and feed conversion (Fig. 12) of the tilapia fed the SBM diet became more efficient than for the fish fed the FRSM diet when the diets were extruded. In this scenario SSF also resulted in poorer growth (**Paper II**-Table 8) and feed conversion (Fig. 12) that in the fish fed RSM. Under the current design it is not possible to separate the effects of extrusion from the differences seen in the fermentations.

The crude protein contents of the diets were higher than the recommendation for tilapia from NRC (1993). This indicates that protein was in excess of requirement. The fact that more than half of all EAA were metabolized after absorption, confirms that dietary protein was in excess. Thus, the differences in growth and feed conversion seen in the current work can't be ascribed to essential amino acid deficiency.

### 4.4 Hydro-thermal (steam) treatment does not improve nutritional value of fermented RSM in diets for Nile tilapia (Paper IV)

The previous results (**Paper II**) implied that thermal-hydro treatment for pasteurisation may influence the nutrient value of FRSM in diets for Nile tilapia. Thus two pelleted diets based on steam treated FRSM and non-treated FRSM were prepared, and fed the Nile tilapia.

Steam treatment did not result in notable changes in the proximate concentration of the diets (**Paper IV-Table 1**). A small loss of amino acids (0.5-3%) was, however, observed, and is also reflected in the amino acid composition of the diet. Increased concentration of amino acids in crude protein is expected if volatile nitrogen components had been flushed off by the steam heating of the FRSM. This indicates that volatile nitrogen production was not significant during SSF of RSM.

Steam treatment did not result in significant differences in feed intake, growth rates, or in the chemical composition of the tilapias (**Paper IV-Tables 2 and 3**). Over the course of the whole experiment, the apparent feed intake, however, tended ($P=0.071$) to be higher for the tilapia fed the diet with steam treated FRSM. Because this was not reflected in improved growth, FCR was significantly higher for the fish fed the diet with steam treated FRSM than what was observed in the tilapia fed the diet with FRSM that had not been subject to steam treatment.

This poorer feed conversion can't be rationalized by steam processing leading to heat-induced reduction in the nutritional value of the protein (Opstvedt et al., 1984; 2003), because digestibility of crude protein, essential or total amino acids (**Paper IV-Tables 5 and 6**) did

not differ significantly between dietary treatments. The small reduction in amino acid concentration caused by the steam treatment can not be considered an important factor in explaining the differences in feed conversion, because neither total nor digestible nitrogen retentions were significantly affected by dietary treatments (**Paper IV-Tables 5 and 6**). This is further supported by the intake of all digestible essential amino acids being well above the requirement for Nile tilapia (**Paper II**).

The results clearly show that 5 min steam treatment to increase the temperature of FRSM to 70$^{o}$C did not induce effects that explain the improved nutritional value of FRSM that had been pasteurised, when compared with RSM (**Paper II**). The current result, however, do not rule out the possibility that more excessive hydrothermic treatment may have an effect on nutritional value of the FRSM, because the biomass was pasteurised for as long as 24 h at 70$^{o}$C in the previous experiment (**Paper II**).

# 5. CONCLUSIONS

- Replacing 36% fishmeal with soybean meal and pea protein concentrate (ratio 2:1 on crude protein moiety weight basis) negatively influenced the digestibility of nutrients and feed utilization in rainbow trout.
- Supplementing diets with 10 g acid moiety $kg^{-1}$ of a sodium formate and butyrate blend (ratio 2:1 on acid moiety weight basis) did not improve growth rate or feed utilization of rainbow trout. Adding an organic acid salt blend to plant protein-based diets before extrusion increased weight of the intestine and reduced the digestibility of macro nutrient and amino acids, but not when added after extrusion. Supplementing diets with 18 g $kg^{-1}$ of the organic acid salt blend did not impact the feed utilization, growth rate, whole fish body composition, and apparent digestibility of nitrogen and minerals, nitrogen retention in Nile tilapia.
- Laboratory-scale solid state fermentation reduced contents of glucosinolates and phytic acid as well as amino acids in rapeseed meal. Solid state fermentation of rapeseed meal improved the feed utilization when used in a moist diet, but it impaired the protein retention and feed utilization when used in an extruded diet.
- The influence of medium-scale solid state fermentation treatment of rapeseed meal on growth rate, FCR, and whole body composition of Nile tilapia was less than of laboratory-scale solid state fermentation.
- Short-term thermal-hydro treatment (steam) for pasteurisation did not improve the nutrient value of fermented rapeseed meal in diets for Nile tilapia.

# 6. FUTURE PERSPECTIVES

Based on the results presented in this thesis, several questions relevant to replacing fish meal by plant protein sources in the diets for rainbow trout, supplementing sodium formate and butyrate formate blend in the diets for rainbow trout, solid state fermentation treatment of rapeseed meal, and the potential for use of fermented rapeseed meal in different type of diets from Nile tilapia have been clarified. A method of solid state fermentation has been established. However, several questions were emerged when we carried out this work, and those questions need to be further investigated. The main questions that have emerged are pointed out as following.

- The results from this work were based on the ratio of sodium formate and sodium butyrate as 2:1, therefore, the investigation of other ratio of sodium formate and sodium butyrate in the diets is needed.
- The blend of sodium formate and sodium butyrate was only evaluated in the diets for rainbow trout and Nile tilapia in this work. Further studies should be with other fish species.
- Although the method of solid state fermentation has been established in this work, further improvement of the method by using upgraded facilities are necessary to obtain fermented rapeseed meal with stable quality. The conditions of solid state fermentation subsequently need to be optimized for large-scale processing.
- The reduction of glucosinolates in the rapeseed meal detected after solid state fermentation, indicated generation of breakdown products. This may affect the fish growth and health, and impairment of fish growth and FCR were observed when the extruded diet based on fermented rapeseed meal was used in this work. Further studies should determine the breakdown products after solid state fermentation.
- This work mainly focused on the effect of rapeseed meal and fermented rapeseed meal on fish growth and digestibility. Based on our knowledge, the gut morphology and microflora in the fish may be affected by the different protein sources. Therefore, the evaluation of gut morphology and microflora are required to better understand the properties of rapeseed meal and fermented rapeseed meal.

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# PAPER I

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# Supplementation of fishmeal and plant protein-based diets for rainbow trout with a mixture of sodium formate and butyrate

Youling Gao [a], Trond Storebakken [a], Karl D. Shearer [a], Michael Penn [b], Margareth Øverland [a,*]

[a] Aquaculture Protein Centre, CoE, Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, P. O. Box 5003, NO-1432 Ås, Norway
[b] Aquaculture Protein Centre, CoE, Department of Basic Science and Aquatic Medicine, Norwegian School of Veterinary Science, P. O. Box 8146 Dep, NO-0033 Oslo, Norway

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ABSTRACT

Two experiments were conducted to evaluate the effects of adding an organic acid salt blend (OAB, 10 g acid moiety $kg^{-1}$ of a mixture of sodium formate and butyrate, ratio 2:1) and plant proteins (soybean meal and pea protein concentrate) to diets for rainbow trout (*Oncorhynchus mykiss*) reared in freshwater. The experiments were designed according to a 2×3 factorial design, where the factors were: two protein sources (fish or plant meals) and three methods of applying OAB to the diets (none, before, or after extrusion). In Exp. 1, duplicate groups of 0.95-kg trout were used to assess the effect of OAB on digestibility of nutrients. The supplementation of OAB before extrusion in the plant protein-based diet significantly reduced the digestibility of dry matter, organic matter, crude fat, and most amino acids. Supplementation of OAB after extrusion reduced the digestibility of crude fat both in the fishmeal and plant protein-based diets. Partly replacing fishmeal with plant proteins reduced the digestibility of dry matter, organic matter, crude fat, phosphorous and several amino acids. In Exp. 2, triplicate groups of 0.23-kg trout were used to evaluate the effect of OAB on growth performance and gut health. Supplementation of OAB in both fishmeal and plant protein-based diets before extrusion significantly increased feed conversion ratio, and middle intestine to body weight ratio. Partly replacing fishmeal with plant proteins significantly increased feed conversion ratio. There was no effect of dietary treatment on morphology in the distal intestine. In conclusion, supplementing diets with a sodium formate and butyrate blend did not improve growth rate or feed utilization of rainbow trout. Replacing 36% fishmeal with a combination of soybean meal and pea protein concentrate negatively influenced the nutrient digestibility and feed conversion ratio, but did not affect the growth rate of rainbow trout.



## 1. Introduction

There is a current strong focus on natural components that can stimulate health and growth in all animal productions. Organic acids are widely distributed in nature as constituents of plant and animal tissue. Their salts (Na, K or Ca) appear to have the potential to improve growth performance of some animals. Their positive effects on growth performance and animal health, especially when added in sufficient amounts in diets for pigs, have been well documented (Partanen and Mroz, 1999; Jongbloed et al., 2000; Øverland et al., 2000, 2008; Canibe et al., 2001).

Several studies have also addressed the effects of organic acids and their salts on growth performance and health of fish. These include Arctic charr (Ringø, 1991), Atlantic salmon (Ringø et al., 1994; Lückstädt, 2008b), rainbow trout (Rungruangsak and Utne, 1981; deWet, 2005; Pandey and Satoh, 2008), tilapia (Ramli et al. 2005; Ng et al., 2009; Zhou et al., 2009), catfish (Owen et al., 2006), red sea bream (Sarker et al. 2005, 2007; Hossain et al., 2007) and rohu (Baruah et al. 2007a,b). Results indicate that growth performance, nutrient digestibility and gut health, can be improved by some organic acids and their salts, particularly formic acid salts in some fish species (Reviewed by Lückstädt, 2008a). The beneficial effects of formic acid and acid salts result mainly from strong antimicrobial properties and growth promotion (Jongbloed et al., 2000; Øverland et al., 2000; Eisemann and van Heugten, 2007). There is a high energy requirement for renewal of the intestinal epithelium. Butyric acid and butyrates are efficient at providing energy for epithelial growth (Topping and Clifon, 2001). Moreover, butyric acid and butyrates influence a wide array of cellular functions relevant to gut health (Hamer et al., 2008). Thus, butyric acid and butyrates may exert a positive effect on gut health. Except for the studies by Bjerkeng et al. (1999) and Owen et al. (2006), literature concerning butyric acid or its salts in fish feed is scarce. Therefore, the primary aim of the present study was to investigate the effects of an organic acid salt blend (OAB) of sodium formate and sodium butyrate in diets on digestibility of nutrients, growth performance, and intestinal morphology of rainbow trout.

Organic acids or their salts may affect the chemical and nutritional properties of the feed during feed manufacturing (Storebakken et al.,

* Corresponding author. Tel.: +47 64 96 52 06; fax: +47 64 96 51 01.
*E-mail address:* margareth.overland@umb.no (M. Øverland).

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2010). Heat treatment during feed manufacturing (conditioning, extrusion and drying) may be influenced by organic acids or their salts. Morken et al. (2010) demonstrated that supplementation of sodium diformate in the diet together with increasing extrusion temperature improved nutrient digestibility of barley protein concentrate-based diets for rainbow trout. The second aim was therefore to compare two different methods of adding OAB to the extruded diets, before and after extrusion.

The use of soybean meal in feed for salmonids is limited due to reduced digestibility of nutrients and soy enteritis (Baeverfjord and Krogdahl, 1996). Pea protein concentrate, however, is a suitable protein source in aquaculture feed due to high protein content, highly digestible nutrients, lack of growth reduction when compared to high quality fish meal, and lack of negative effects on the intestine when included in amounts up to 200 g $kg^{-1}$ feed (Øverland et al., 2009). Other studies have showed that up to 27% pea protein concentrate in the diet produced acceptable weight gain, feed intake, and feed conversion ratio in Atlantic salmon (Carter and Hauler, 2000) and rainbow trout (Thiessen et al., 2003). Hence, the third aim was to investigate the effect of a plant protein-based diet with soybean meal and pea protein concentrate on nutrient digestibility, growth performance and gut health of rainbow trout.

## 2. Materials and methods

Two experiments were conducted with rainbow trout in the fish nutrition laboratory at The Norwegian University of Life Sciences (UMB). The first experiment focused on the digestibility of macro nutrients and amino acids, and the second focused on growth performance and the intestinal morphology of the distal intestine.

### 2.1. Diets

Two different basic diets (Table 1) were prepared, one was based on high quality fishmeal and one was based on plant protein and fishmeal, where soybean meal and pea protein concentrate (ratio 2:1 on a crude protein weight basis) replaced 36% of fishmeal. In addition to being used as is, these diets were supplemented with an organic acid salt blend (OAB) to produce four treatment diets. The OAB contained a mixture of sodium formate and sodium butyrate (ratio 2:1 on acid moiety weight basis, total amount 10 g acid moiety $kg^{-1}$). The OAB was either mixed with dry mash and added to the diets before extrusion, or dispersed in fish oil and added by vacuum coating after extrusion. The diets were formulated to contain similar amounts of crude protein. Inclusion of fish oil and vitamin and mineral mixture were kept constant for all diets. Yttrium oxide ($Y_2O_3$) was added as an inert marker (Austreng et al., 2000). The inclusion level was 0.01% on a dry matter basis.

The diets were produced at The Centre for Feed Technology at UMB. All dry ingredients were milled in a hammer mill with a 1-mm screen, mixed in a twin shaft mixer, conditioned (90–95 °C) and extruded in a five-section twin-screw extruder with a 3-mm die. Conditioning and extrusion parameters are presented in Table 2. The pellets were dried until the bulk density was 445 to 450 g $l^{-1}$ in a fluidized bed dryer before they were oil coated in a vacuum coater. Details on the equipment used for feed processing are given by Øverland et al. (2009).

### 2.2. Fish, facilities and management

#### 2.2.1. Digestibility experiment

Each diet was fed to two groups of 20 fish (0.94 kg mean initial weight) over a period of 24 days. A commercial diet was fed prior to the experiment. The fish were kept in fibre-glass tanks with a water volume of 250 l, covered by a transparent lid and subject to continuous illumination (5 W). The tanks were supplied with fresh water at a rate of 8 l $min^{-1}$, a temperature of 12.2–12.6 °C and average dissolved oxygen level, measured daily inside the tank at 5.9 mg $l^{-1}$.

The fish were fed two times per day (12:00–13:15 pm and 00:00–01:15 am) by using electrically driven feeders. Feed intake was monitored according to the method developed by Helland et al. (1996), and the feeding rate was planned for 20% overfeeding (Helland et al., 1996). Stripping of faeces was done three times, on days 11, 18 and 24 as described by Austreng (1978). Faeces from each tank at each stripping were pooled and freeze-dried before analysis.

#### 2.2.2. Growth experiment

Experiment 2 used the same facilities as Experiment 1. Each diet was fed to three groups of 15 trout for 50 days. The temperature varied from 11.0 to 11.4 °C, the water flow was 8 l $min^{-1}$, and the average dissolved oxygen level was 7.8 mg $l^{-1}$. Feeding was 3 times per day (07:30–08:20 am, 13:00–13:40 pm, and 19:00–19:50 pm). Feed intake was assessed according to Helland et al. (1996), and the overfeeding rate ranged from 13.7 to 18.7% in individual tanks over the experimental period.

The fish were bulk-weighted by tank at the start of the experiment. At the end of the experiment, 5 fish from each tank were anaesthetized, and then weight and length were measured. The weights of liver, middle and distal intestines without content were taken. Samples for histology of approximately 5 mm were taken from distal intestine sections. The histological samples were fixed in buffered formalin (pH 7.4) over 24 h, and then kept in 70% ethanol solution until histological evaluation. The contents of stomach, pyloric caeca, middle intestine, and distal intestine were transferred to 3 ml Eppendorf tubes, and the pH in intestinal contents was measured. Three additional fish from each tank were anaesthetized, and their stomach and gut contents were removed before the fish were saved for whole body composition analysis. The remaining fish in each tank then were bulk-weighted.

### 2.3. Chemical analysis

The pH value of the diets was determined based on a modified method of Pandey and Satoh (2008). One gram of each diet was dissolved in 10 ml of de-ionized water and agitated for 1 min. Then the pH was measured with a pH meter. The dry matter and ash content of diets and freeze-dried faeces were determined by heating at 105 °C until stable weight and 500 °C in a muffle furnace, respectively. Crude protein (Kjeldahl N × 6.25) was determined using a Kjeltec auto 1035/1038 system (Tecator, Sweden). Crude lipid was determined using an Accelerated Solvent Extractor (ASE 200) from Dionex (Danvers, MA, USA). Starch was analyzed as glucose after starch hydrolysis with a heat tolerant amylo-glucosidase in accordance with the procedure of McCleary et al. (1994). For total phosphorus in diets and faeces analysis, the sample was first burned to ash at 550 °C, then dissolved in 1 M HCl, and analyzed on a spectrophotometer (Bourke and Yanagawa, 1993). Amino acids, were analysed according to EC (1998) using a Biochrom 30 amino acid analyser (Biochrom Ltd., Cambridge, UK). Tryptophan was not analyzed in the present study. Methionine and cyst(e)in were oxidized before analysis for optimal recovery. Yttrium oxide content in diets and faeces was determined by inductively coupled plasma mass spectroscopy (ICP-MS) after complete digestion of the homogenised and dried samples in $HNO_3$ and cooking in a micro-wave oven for 1 h. Formate and butyrate analysis were conducted at Eurofin, Moss, Norway. The samples were prepared by milling, homogenization, dilution and mixing with water. The prepared sample was filtered at 4 μm and analysed by HPLC on a cation exchange column designed for separation of organic acids (Nulceogel Ion 300 OA, Macherey-Nagel, Düren, Germany) at 50 °C. Formate was detected at 205 nm and butyrate by a RI-detector.

**Table 1**
Formulation and analysed chemical composition of diets.

| Diet | Fishmeal-based diets | | | Plant protein-based diets | | |
|---|---|---|---|---|---|---|
| | Control | OAB added | | Control | OAB added | |
| | | Before extrusion | After extrusion | | Before extrusion | After extrusion |
| *Ingredients, g kg$^{-1}$* | | | | | | |
| Fish meal[a] | 551.8 | 532.2 | 543.0 | 354.1 | 337.0 | 348.5 |
| Pea protein concentrate[b] | 0 | 0 | 0 | 103.7 | 100.1 | 102.1 |
| Soybean meal[c] | 0 | 0 | 0 | 194.9 | 195.2 | 191.8 |
| Wheat[d] | 175.6 | 173.4 | 172.8 | 81.9 | 81.0 | 80.6 |
| Fish oil[e] | 267.6 | 267.3 | 257.5 | 260.3 | 259.8 | 250.6 |
| Vitamin and mineral mixture[f] | 4.99 | 4.99 | 4.91 | 4.99 | 4.99 | 4.91 |
| Na-butyrate[g] | 0 | 12.62 | 12.42 | 0 | 12.52 | 12.32 |
| Na-formate[h] | 0 | 9.38 | 9.23 | 0 | 9.30 | 9.16 |
| Inert marker[i] | 0.094 | 0.093 | 0.092 | 0.093 | 0.092 | 0.091 |
| *Chemical composition, kg$^{-1}$* | | | | | | |
| Dry matter (DM), g | 951 | 963 | 948 | 950 | 952 | 949 |
| *In DM* | | | | | | |
| Crude protein, g | 454 | 424 | 447 | 450 | 410 | 444 |
| Crude fat, g | 285 | 288 | 277 | 260 | 267 | 280 |
| Starch, g | 114 | 110 | 107 | 70 | 70 | 73 |
| Ash, g | 86 | 103 | 90 | 79 | 89 | 89 |
| Total phosphorous, g | 11.3 | 11.7 | 11.7 | 10.4 | 9.9 | 10.8 |
| Formate, g | <0.1 | 5.8 | 5.6 | <0.1 | 6.6 | 5.5 |
| Butyrate, g | <0.1 | 3.2 | 3.4 | <0.1 | 3.2 | 3.5 |
| *Amino acid profile, g 16 g$^{-1}$ N* | | | | | | |
| Total amino acids | 74.9 | 77.9 | 76.2 | 75.7 | 73.7 | 77.8 |
| Essential amino acids | | | | | | |
| Arg | 4.88 | 5.08 | 4.97 | 5.33 | 5.11 | 5.44 |
| His | 1.74 | 1.81 | 1.76 | 1.84 | 1.79 | 1.88 |
| Ile | 3.47 | 3.55 | 3.45 | 3.53 | 3.4 | 3.57 |
| Leu | 6.01 | 6.24 | 6.11 | 6.07 | 5.88 | 6.21 |
| Lys | 6.17 | 6.45 | 6.33 | 6.16 | 5.92 | 6.32 |
| Met | 2.18 | 2.26 | 2.25 | 1.89 | 1.83 | 1.97 |
| Phe | 3.31 | 3.43 | 3.35 | 3.49 | 3.39 | 3.62 |
| Thr | 3.43 | 3.68 | 3.52 | 3.42 | 3.39 | 3.53 |
| Val | 3.88 | 4.10 | 3.90 | 3.86 | 3.82 | 3.96 |
| Non-essential amino acids | | | | | | |
| Asp | 7.56 | 8.06 | 7.67 | 8.15 | 8.03 | 8.38 |
| Cys | 0.86 | 0.82 | 0.86 | 0.87 | 0.81 | 0.88 |
| Glu | 13.12 | 12.97 | 13.35 | 13.05 | 12.59 | 13.41 |
| Gly | 4.12 | 4.73 | 4.30 | 3.95 | 4.11 | 4.05 |
| Ala | 4.39 | 4.71 | 4.52 | 4.17 | 4.10 | 4.29 |
| Pro | 3.79 | 3.79 | 3.82 | 3.63 | 3.56 | 3.77 |
| Ser | 3.64 | 3.82 | 3.74 | 3.79 | 3.72 | 3.92 |
| Tyr | 2.29 | 2.36 | 2.34 | 2.45 | 2.29 | 2.59 |
| pH | 6.31 | 6.13 | 6.32 | 6.38 | 6.20 | 6.37 |

[a] NorsECO-LT, Norsildmel, Egersund, Norway.
[b] AgriMarin AS, Stavanger, Norway; 50% crude protein content.
[c] Denosoy, extracted and toasted soybean meal with hulls, Denofa, Fredrikstad, Norway.
[d] Toro AS, Bergen, Norway.
[e] NorSalmOil, Norsildmel, Egersund, Norway.
[f] Supplied per kg feed: Vitamin A 2500 IU; Vitamin $D_3$ 2400 IU; Vitamin E 0.2 IU; Vitamin $K_3$ 40.0 mg; Thiamine 15.0 mg; Riboflavin 25.0 mg; d-Ca-Pantothenate 40.0 mg; Niacin 150.0 mg; Biotin 3.0 mg; Cyanocobalamine 20.0 μg; Folic acid 5.0 mg; Pyridoxine 15.0 mg; Vitamin C: 0.098 g (Stay-C 35, ascorbic acid phosphate, DSM Nutritional Products, Basel, Switzerland); Cu: 12.0 mg; Zn: 90.0 mg; Mn: 35.0 mg; I: 2.0 mg; Se: 0.2 mg; Cd ≤ 3.0 μg; Pb ≤ 28.0 μg; total Ca: 0.915 g; total K 1.38 g; total Na 0.001 g; total Cl 1.252 g; Trouw Nutrition LA, Putten, The Netherlands.
[g] Adimix® 30% Coated; Sodium butyrate 30 ± 2%; Butyric acid 21.7-26.6%; pH 10-11; Water ≤ 2.5%; Nutri-Ad International N.V., Kasterlee, Belgium; The coating consists of vegetable fat and fatty acids.
[h] Contents: Sodium formate ≥ 97% (App. 66% formic acid); Water ≤ 0.5; Organic substances ≤ 3.0; Perstorp Specialty Chemicals AB, Perstorp, Sweden.
[i] Metal Rare Earth Limited, Shenzhen, China.

### 2.4. Histological evaluation

Tissues were processed at the Pathohistological Laboratory of the Norwegian School of Veterinary Science (Oslo, Norway). Tissues were routinely dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin according to standard histological techniques. The tissue samples were sectioned longitudinally (i.e. perpendicular to the macroscopically visible circular folds; approximately 5 μm thick) and stained with haematoxylin and eosin. A blind examination was performed using light microscopy. Tissue morphology was evaluated according to the descriptions of Amin et al. (1992) and Baeverfjord and Krogdahl (1996). Nine fish from each diet group (3 fish × 3 replicate tanks per diet) were examined.

### 2.5. Calculation and statistical analyses

Apparent digestibility was assessed by the indirect method (Austreng, 1978) with $Y_2O_3$ as an inert marker (Austreng, et al., 2000), and calculated as follows: $100 \times (1 - (Di/Fi \times Fn/Dn))$. Where Di represents concentration of inert marker $Y_2O_3$ in diet, Fi is concentration of inert marker in faeces, Dn represents nutrient concentration in diets, and Fn is nutrient concentration in faeces.

**Table 2**
Processing parameters during production of the experimental diets.

| | Fishmeal-based diets | | Plant protein-based diets | |
|---|---|---|---|---|
| | Control and OAB added after extrusion | OAB added before extrusion | Control and OAB added after extrusion | OAB added before extrusion |
| *Conditioning* | | | | |
| Temperature, °C | 95 | 90 | 95 | 92 |
| Water, kg $h^{-1}$ | 14–21 | 9–14 | 15–23 | 15–23 |
| Steam, kg $h^{-1}$ | 8–12 | 10–12 | 9–12 | 9–13 |
| *Extrusion temperature, °C* | | | | |
| Section 1 | 77 | 80 | 82 | 80 |
| Section 2 | 95 | 107 | 97 | 101 |
| Section 3 | 128 | 132 | 126 | 122 |
| Section 4 | 121 | 123 | 125 | 120 |
| Section 5 | 141 | 144 | 141 | 132 |
| *Extruder steam into extruder* | | | | |
| Injection | Off | On | On | On |
| Pressure, bar | 0.9 | 1 | 1.4 | 1 |
| Die resistant pressure, bar | 35 | 35 | 33 | 29 |
| Die temperature, °C | 123 | 134 | 132 | 116 |
| SME, W h $kg^{-1}$ | 57 | 55 | 49 | 42 |
| Revolution of screws, RPM | 325 | 300 | 300 | 300 |
| Revolution of knife, RPM | 2600 | 2600 | 2700 | 2700 |
| Water in extrusion, kg $h^{-1}$ | 4–6 | 3–5 | 7–11 | 6–9 |
| Density, g $l^{-1}$ | 450 | 450 | 450 | 445 |
| Diameter, mm | 3.8 | 3.8 | 3.6 | 3.6 |

The results were analyzed using GLM (SAS, 1999). Main effects of protein sources and OAB treatments were analyzed by two-way ANOVA. The proportion of total variation in the main effects was calculated by percent-wise contribution of Type I SS to the sum of squares. Significant ($P<0.05$) interactions were ranked by least square means and PDIFF. Duncan's multiple range test was used to rank significant differences among six diets due to a high number of significant interactions ($P>0.01$). Tendency of difference was defined as $0.05 \le P<0.1$. The results are presented as least square means and pooled standard errors of the means (S.E.M.).

## 3. Results

### 3.1. Characteristics of experiment diets

Chemical composition of the experimental diets did not differ significantly (Table 1), except that crude protein and total amino acids in the fishmeal-based and plant protein-based diets with OAB added before extrusion were slightly lower than that in the other four diets. Crude fat contents in the six diets ranged from 260 to 288 g (kg DM)$^{-1}$. Starch contents in the three fishmeal-based diets were higher than those in the three plant protein-based diets. Ash content in the six diets was similar except for the fishmeal-based diet with OAB added before extrusion, which was slightly higher. Total phosphorus content in all diets varied from 10 to 12 g (kg DM)$^{-1}$. Amino acid compositions in all diets were similar, except for lower content of methionine in the plant protein-based diets than in the fishmeal-based diets.

The formic and butyric acid contents in both the fishmeal-based and plant protein-based diets were similar and close to the calculated content (acid moiety recovery rate: 89.5–97.3%). The pH levels of the six diets were also similar (6.13–6.38), except that the OAB added to the diets before extrusion resulted in a slight reduction in pH. The processing parameters of uncoated diets during production were similar (Table 2), but the fishmeal-based diets had larger diameter than plant protein-based diets.

**Table 3**
Two-way ANOVA results of digestibility for dietary treatment effects with different protein sources and OAB treatments[a].

| | Protein sources (P) | OAB treatments (O)[b] | Interaction (P×O) | Error |
|---|---|---|---|---|
| Dry matter | 90.9[c] | 4.6[d] | 2.3 | 2.2 |
| Organic matter | 92.8[c] | 5.3[c] | 0.7 | 1.2 |
| Crude fat | 28.0[c] | 48.9[c] | 12.2 | 10.9 |
| Starch | 18.0 | 1.0 | 8.2 | 72.8 |
| Ash | 2.9 | 48.5[c] | 37.9[d] | 10.7 |
| Phosphorous | 65.1[c] | 10.9 | 9.1 | 15.0 |
| Crude protein | 6.7[e] | 56.9[c] | 29.3[c] | 7.1 |
| Total amino acids | 13.4[e] | 43.8[d] | 25.0[e] | 17.9 |
| *Essential amino acids* | | | | |
| Arg | 7.1 | 42.5[d] | 31.4[e] | 19.0 |
| His | 8.6 | 53.9[c] | 23.0[e] | 14.5 |
| Ile | 16.7[e] | 44.3[d] | 16.8 | 22.3 |
| Leu | 25.1[d] | 36.3[e] | 17.5 | 21.2 |
| Lys | 28.6[d] | 40.0[d] | 16.9[e] | 14.4 |
| Met | 15.0[d] | 48.6[c] | 25.4[d] | 11.1 |
| Phe | 5.1 | 43.2[d] | 26.8 | 24.9 |
| Thr | 31.5[d] | 30.5[d] | 20.6[e] | 17.3 |
| Val | 31.3[d] | 33.5[d] | 16.5 | 18.7 |
| *Non-essential amino acids* | | | | |
| Asp | 0.0 | 29.6[e] | 45.9[d] | 24.6 |
| Cys | 34.6[c] | 39.8[c] | 17.5[d] | 8.2 |
| Glu | 9.6 | 62.8[c] | 12.2 | 15.4 |
| Gly | 23.0[d] | 23.6[e] | 36.9[d] | 16.6 |
| Ala | 24.8[d] | 27.4 | 23.8 | 24.0 |
| Pro | 27.1[c] | 45.3[c] | 16.2[e] | 11.5 |
| Ser | 17.0[e] | 24.2[e] | 39.0[d] | 19.7 |
| Tyr | 0.6 | 65.3[c] | 24.0[d] | 10.1 |

[a] Percent-wise contribution of Type I SS of each effects and their interaction to the sum of squares.
[b] The treatments of OAB absence, OAB added before extrusion via mixing with other dry ingredients, and after extrusion via vacuum coating were included.
[c] $P<0.01$.
[d] $0.01 \le P<0.05$.
[e] $0.05 \le P<0.1$.

### 3.2. Digestibility

Two-way ANOVA results (Table 3) showed that digestibility of dry matter, organic matter, crude fat, and phosphorous were higher in the fishmeal-based diets ($P<0.01$). Fishmeal-based diets tended ($P=0.055$) to have higher digestibility of crude protein than plant protein-based diets (Table 3). Protein source significantly affected the digestibility of total amino acids and some individual amino acids (Table 3). Adding OAB to diets before extrusion resulted in significantly lower digestibility of dry matter, organic matter and crude fat in the plant protein-based diet, and significantly higher digestibility of ash in the fishmeal-based diet (Table 4). No significant differences were seen in the digestibility of dry matter and organic matter between the plant protein-based diets where OAB was added before and after extrusion via vacuum coating. Digestibility of crude fat in the plant protein-based control diet was significantly higher than it in both plant protein-based diets with OAB, and digestibility of crude fat in the fishmeal-based control diet was significantly higher than it in the fishmeal-based diet with OAB added after extrusion. There was no effect of the method of applying the OAB on digestibility of crude fat in neither the fishmeal-based diets nor the plant protein-based diets. Adding OAB did not affect the digestibility of crude protein in fishmeal-based diets. In plant protein-based diets, however, adding OAB before extrusion resulted in a significant reduction in crude protein digestibility compared to the control diet or the diet where OAB was added after extrusion (Table 5). Similar digestibility values were obtained between the plant protein-based control diet and the plant protein-based diet with OAB added after extrusion. Adding OAB to the plant protein-based diet before extrusion significantly reduced the digestibility of all amino acids (Table 5), but not in fishmeal-based diets

**Table 4**
Apparent digestibility of main nutrients of six diets.

| Diet | Fishmeal-based diets | | | Plant protein-based diets | | | Pooled S.E.M. |
|---|---|---|---|---|---|---|---|
| | Control | OAB added | | Control | OAB added | | |
| | | Before extrusion | After extrusion | | Before extrusion | After extrusion | |
| *Apparent digestibility, %* | | | | | | | |
| Dry matter | 86.0[a] | 85.5[a] | 85.2[a] | 81.7[b] | 79.6[c] | 80.8[bc] | 0.38 |
| Organic matter | 91.5[a] | 90.3[a] | 90.5[a] | 86.0[b] | 83.7[c] | 84.8[bc] | 0.34 |
| Crude fat | 98.8[a] | 97.7[ab] | 96.7[bc] | 97.9[ab] | 94.8[d] | 96.0[cd] | 0.47 |
| Starch | 98.9 | 99.0 | 98.7 | 98.3 | 98.1 | 98.7 | 0.52 |
| Ash | 27.8[d] | 43.6[a] | 31.4[cd] | 32.5[cd] | 35.9[bc] | 40.1[ab] | 1.85 |
| Phosphorous | 47.0[b] | 47.2[b] | 48.2[b] | 58.9[a] | 51.5[ab] | 58.6[a] | 2.12 |

[a,b,c,d] different superscript letters indicate significant ($P<0.05$) difference among six diets.

(only tyrosine was significantly reduced), whereas, adding OAB after extrusion via vacuum coating did not significantly affect these digestibilities.

### 3.3. Growth, feed conversion ratio and organ weights

One fish fed plant protein-based diet with OAB added before extrusion died during the experiment. There were no significant differences in feed intake among fishmeal-based diets and among plant protein-based diets. Results of fish growth performance did not reveal significant differences among treatments (Tables 6 and 7). The FCR of the fish fed the fishmeal-based diet with OAB added before extrusion was higher compared to the fishmeal-based control diet ($P=0.0155$) and the FCR of the plant protein-based diet with OAB added before extrusion tended to be higher compared to the plant protein-based control diet ($P=0.075$).

Liver to whole body weight ratio was significantly affected by protein sources (Table 8). The liver index was higher in the fishmeal-based diet compared with the plant protein-based diets (Table 9), while distal and middle intestine weights were not significantly affected by protein sources. The middle intestine weight was, however, affected by adding OAB (Table 9). Adding OAB to the diets before extrusion resulted in significantly higher middle intestine weight compared with the control diets and the diets with OAB added after extrusion by vacuum coating. The distal intestine also tended to be affected by adding OAB before extrusion ($P=0.082$).

**Table 5**
Apparent digestibility of crude protein and amino acids of six diets.

| Diet | Fishmeal-based diets | | | Plant protein-based diets | | | Pooled S.E.M |
|---|---|---|---|---|---|---|---|
| | Control | OAB added | | Control | OAB added | | |
| | | Before extrusion | After extrusion | | Before extrusion | After extrusion | |
| Crude protein | 93.5[a] | 93.0[a] | 93.3[a] | 93.7[a] | 91.3[b] | 93.6[a] | 0.24 |
| Total amino acids | 95.8[a] | 95.6[a] | 95.9[a] | 95.8[a] | 94.2[b] | 95.9[a] | 0.27 |
| *Essential amino acids* | | | | | | | |
| Arg | 97.0[ab] | 97.0[ab] | 97.1[ab] | 97.5[a] | 96.6[b] | 97.5[a] | 0.16 |
| His | 95.6[a] | 95.2[a] | 95.7[a] | 95.7[a] | 93.8[b] | 95.7[a] | 0.27 |
| Ile | 96.6[a] | 96.3[a] | 96.8[a] | 96.5[a] | 95.0[b] | 96.6[a] | 0.31 |
| Leu | 96.7[a] | 96.6[a] | 97.0[a] | 96.5[a] | 95.2[b] | 96.6[a] | 0.30 |
| Lys | 97.0[a] | 96.8[a] | 97.2[a] | 96.7[a] | 95.6[b] | 96.9[a] | 0.21 |
| Met | 96.1[a] | 95.9[a] | 96.4[a] | 96.1[a] | 94.3[b] | 96.2[a] | 0.25 |
| Phe | 95.4[a] | 95.3[a] | 95.6[a] | 95.5[a] | 94.0[b] | 95.9[a] | 0.35 |
| Thr | 95.2[a] | 95.2[a] | 95.5[a] | 94.9[a] | 93.2[b] | 95.0[a] | 0.35 |
| Val | 96.3[a] | 96.1[a] | 96.5[a] | 95.9[a] | 94.4[b] | 96.0[a] | 0.32 |
| *Non-essential amino acids* | | | | | | | |
| Asp | 92.2[ab] | 92.4[a] | 92.2[ab] | 92.9[a] | 90.8[b] | 93.1[a] | 0.42 |
| Cys | 90.3[a] | 89.4[a] | 90.2[a] | 89.1[a] | 84.8[b] | 89.1[a] | 0.56 |
| Glu | 97.4[a] | 97.0[a] | 97.5[a] | 97.3[a] | 96.2[b] | 97.4[a] | 0.20 |
| Gly | 93.7[a] | 93.9[a] | 93.6[a] | 93.6[a] | 91.7[b] | 93.6[a] | 0.34 |
| Ala | 96.3[a] | 96.3[a] | 96.4[a] | 96.0[a] | 94.9[b] | 96.2[a] | 0.29 |
| Pro | 95.8[a] | 95.3[a] | 95.7[a] | 95.3[a] | 93.6[b] | 95.5[a] | 0.27 |
| Ser | 95.0[a] | 95.2[a] | 95.1[a] | 95.0[a] | 93.6[b] | 95.2[a] | 0.28 |
| Tyr | 96.1[a] | 95.6[b] | 96.0[ab] | 96.8[a] | 94.6[c] | 96.6[a] | 0.24 |

[a,b,c] different superscript letters indicate significant ($P<0.05$) difference among six diets.

### 3.4. Stomach and gut pH level

The pH level in stomach, pyloric caeca, middle and distal intestines were not significantly affected by the different dietary treatments. pH ranged from 4.68 to 5.14 (pooled S.E.M., ±0.18) in the stomach, 7.52 to 7.65 (±0.05) in pyloric caeca, 7.82 to 7.96 (±0.07) in the middle intestine and 7.90 to 8.08 (±0.08) in the distal intestine.

### 3.5. Intestinal morphology

Fish fed the fishmeal-based diets had mucosal folds that appeared moderately tall to tall with a thin lamina propria and submucosa. Low to moderate numbers of intraepithelial leukocytes were noted. Enterocytes were highly vacuolated (supranuclear absorptive vacuoles) with basally located nuclei. Samples from fish fed the plant protein-based control diet presented similar to fish fed fishmeal-based diets for most characteristics. However, slight to moderate reduction in enterocyte vacuolization was observed in a few fish. The most consistent finding was an increase in enterocyte vacuole size in fish fed plant protein-based diets, regardless of inclusion of organic acids. The presence of OAB in the diets did not significantly alter the morphology of the distal intestine.

## 4. Discussion

The high and similar levels of formate and butyrate recovered in diets independent of the method of adding the OAB, indicated low loss during the extrusion process. The main reason for lower digestibility of dry matter and organic matter in the plant protein-based than in

**Table 6**
Two-way ANOVA results of growth, feed intake, and FCR in the growth experiment.[a]

| | Protein sources (P) | OAB treatments (O)[b] | Interaction (P×O) | Error |
|---|---|---|---|---|
| Body weight, g | | | | |
| Day 0 | 0.3 | 2.0 | 18.7 | 79.0 |
| Day 51 | 0.0 | 20.0 | 14.9 | 65.0 |
| Weight gain | 0.0 | 20.8 | 15.1 | 64.1 |
| Feed intake, g DM (fish day)$^{-1}$ | 6.9 | 14.4 | 19.5 | 59.3 |
| FCR | 31.6[c] | 32.5[d] | 2.8 | 33.1 |
| Condition factor | 3.8 | 3.0 | 14.7 | 78.5 |

[a] Percent-wise contribution of Type I SS of each effects and their interaction to the sum of squares.
[b] The treatments of OAB absence, OAB added before extrusion via mixing with other dry ingredients, and after extrusion via vacuum coating were included.
[c] $P<0.01$.
[d] $0.01 \cdot P<0.05$

**Table 7**
Fish weight, weight gain, feed conversion ratio, condition factor and overfeeding rate of six diets in the growth experiment.

| Diet | Fishmeal-based diets | | | Plant protein-based diets | | | Pooled S.E.M |
|---|---|---|---|---|---|---|---|
| | Control | OAB added | | Control | OAB added | | |
| | | Before extrusion | After extrusion | | Before extrusion | After extrusion | |
| Body weight, g | | | | | | | |
| Day 0 | 233.77 | 233.50 | 232.70 | 232.91 | 232.87 | 234.65 | 0.92 |
| Day 51 | 510.69 | 514.91 | 493.47 | 530.44 | 492.35 | 499.09 | 12.97 |
| Weight gain, g | 276.92 | 281.41 | 260.76 | 297.53 | 259.48 | 264.44 | 12.74 |
| Feed intake, g DM (fish day)$^{-1}$ | 3.74$^{ab}$ | 4.06$^{ab}$ | 3.64$^{b}$ | 4.28$^{a}$ | 3.88$^{ab}$ | 3.81$^{ab}$ | 0.18 |
| FCR | 0.68$^{c}$ | 0.72$^{ab}$ | 0.70$^{bc}$ | 0.72$^{ab}$ | 0.75$^{a}$ | 0.72$^{ab}$ | 0.0002 |
| Condition factor | 1.40 | 1.39 | 1.34 | 1.38 | 1.39 | 1.41 | 0.03 |
| Overfeeding rate, % | 18.21$^{ab}$ | 16.68$^{ab}$ | 18.72$^{a}$ | 16.03$^{ab}$ | 17.51$^{ab}$ | 13.71$^{b}$ | 1.44 |

$^{a,b,c}$ different superscript letters indicate significant ($P<0.05$) difference among six diets.

**Table 8**
Two-way ANOVA results of organ to whole body weight ratios in the growth experiment[a].

| | Protein sources (P) | OAB treatments (O)[b] | Interaction (P×O) | Error |
|---|---|---|---|---|
| Liver | 47.3[c] | 1.6 | 3.5 | 47.6 |
| Distal intestine | 2.7 | 26.4[d] | 19.9 | 51.0 |
| Middle intestine | 1.2 | 44.9[c] | 18.0[d] | 35.9 |
| Middle + distal | 0.7 | 41.5[c] | 24.5[e] | 33.3 |

[a] Percent-wise contribution of Type I SS of each effects and their interaction to the sum of squares.
[b] The treatments of OAB absence, OAB added before extrusion via mixing with other dry ingredients, and after extrusion via vacuum coating were included.
[c] $P<0.01$.
[d] $0.05 \le P<0.1$.
[e] $0.01 \le P<0.05$.

the fishmeal-based diets is that soybean meal contains high amounts of indigestible sugars and non-starch polysaccharides (Storebakken et al., 2000), while the concentration of indigestible carbohydrates is lower in pea protein concentrate than starch, but higher than in fishmeal (Øverland et al., 2009). Reduced lipid digestibility in the plant protein-based diets is largely ascribed to the soybean meal (Olli and Krogdahl, 1994; Olli et al., 1994; Refstie et al., 1998, 1999, 2000; Øverland et al., 2009), and seems to be a result of physiological changes in secretion of bile acid (Romarheim et al., 2006; Yamamoto et al., 2007), caused by the alcohol-soluble substances of soybean meal (Yamamoto et al., 2008).

The mechanism of reduced digestibility by adding OAB to the diets is unclear. Addition of 0.1–0.2% potassium diformate (KDF) or an unspecified OAB did not have the same effects when incorporated in diets for red hybrid tilapia (Ng et al., 2009), but their highest dietary dose was around 5 to 10-fold lower than the dose given in the present experiment and, thus not directly comparable. Similarly, Bjerkeng et al. (1999) did not observe any effects of a short-chain fatty acid blend (sodium salts of acetic, propionic and butyric acid, 5:5:2 w/w, 5 and 20 g kg$^{-1}$ feed) on growth, organ indices or nutrient digestibilities in Atlantic salmon. Morken et al. (2010), however, observed improved lipid digestibility when 1.06% sodium diformate was incorporated in a barley protein concentrate-based diet for rainbow trout, indicating that the reduced lipid digestibility in the present study may be associated with the butyrate rather than the formate moiety of the OAB. Adding OAB to the fishmeal-based diet before extrusion and the plant protein-based diet after extrusion significantly increased the digestibility of ash. This may be a result of the high bioavailability of the sodium in both the formate and the butyrate leading to an increase in ash digestibility. Alternatively, the formate may have increased the ash digestibility as reported by Pallauf and Hüter (1993).

Digestibility of phosphorous was significantly lower in the fishmeal-based diets compared to the plant protein-based diets. It is known that the concentration and availability of phosphorous in the diet are the two most important factors affecting the utilization of phosphorous in feeds by the fish (Sugiura et al., 1999). In the present study, the fishmeal-based diets contained higher total phosphorous levels than the plant protein-based diets. Higher phosphorous may contribute to the reduced fractional absorption of phosphorous (Sugiura et al., 1998b).

In the present study, we used organic acid salts that do not contain acid groups. Thus, neither the pH of the digestive system nor the utilization of dietary phosphorus was affected. In previous studies improved phosphorus utilization has been obtained both in red sea bream (Sarker et al., 2005, 2007; Hossain et al., 2007) and rainbow trout (Vielma and Lall, 1997; Sugiura et al., 1998a) fed diets supplemented with organic acids. In these studies the improved availability of phosphorus has been ascribed to the lowered pH, resulting in a higher dissociation of mineral compounds and formation of chelated mineral complexes that can be easily absorbed (Partanen and Mroz, 1999).

The similar digestibility of crude protein and total amino acids between the fishmeal-based and the plant protein-based diets are in keeping with previous observations (Storebakken et al., 2000; Øverland et al., 2009). The soybean meal was toasted, and the high digestibility values for all essential amino acids, and Cys indicates that toasting had not been excessive (Storebakken et al., 2000). The pea protein concentrate was not heat treated before extrusion of the feed. Pea contains the Bowman–Birk type protease inhibitor, but inhibitor activity is approximately ten-fold lower that in soy (Pisulewska and Pisulewski, 2000). Thus, the plant protein-based diets should only require moderate heat treatment to reach levels of inhibition that can be acceptable to salmonids (Storebakken et al., 2000).

**Table 9**
Organ to whole body weight ratios of two main effects in the growth experiment.

| | Protein sources (P) | | OAB treatments (O) | | | Pooled S.E.M. (P) | Pooled S.E.M. (O) |
|---|---|---|---|---|---|---|---|
| | Fishmeal-based diets (n = 9) | Plant protein-based diets (n = 9) | None (n = 6) | Before extrusion (n = 6) | After extrusion (n = 6) | | |
| Tissue: whole body weight, g kg$^{-1}$ | | | | | | | |
| Liver | 13.18$^{A}$ | 11.56$^{B}$ | 12.16 | 12.49 | 12.46 | 0.33 | 0.41 |
| Distal intestine | 6.29 | 6.10 | 5.97 | 6.63 | 5.98 | 0.17 | 0.21 |
| Middle intestine | 2.11 | 2.18 | 1.97$^{b}$ | 2.43$^{a}$ | 2.03$^{b}$ | 0.08 | 0.09 |

$^{A,B}$ different superscript letters indicate significant ($P<0.05$) difference between protein sources; $^{a,b}$ different superscript letters indicate significant ($P<0.05$) difference among OAB treatments.

OAB added to plant protein-based diets before extrusion reduced the digestibility of macro nutrients and amino acids. The highest reduction in digestibility was found for Cys, which is known to be the amino acid most sensitive to heat treatment (Storebakken et al., 2000). Improved digestibility of macro nutrients or amino acids have, however, been observed in other studies with rainbow trout (Morken et al., 2010) fed diets with sodium diformate added before extrusion and Atlantic salmon (Storebakken et al., 2010) fed diets with potassium diformate added before extrusion. Adding OAB to plant protein-based diets after extrusion only affected digestibility of lipid and ash. One plausible explanation for the negative effect of adding the OAB to the plant protein-based feed mix before extrusion is that the temperature in Sections 1–5 in the extruder and consequently the specific mechanical energy (SME) were lowered during extrusion. Lower temperature would result in lower efficiency in denaturation of protease inhibitors and, thus, decreased amino acid digestibility. Unfolding of seed proteins is also heat dependent, facilitating improved access of proteases to the protein. Protease inhibitor activity was not measured in this experiment, so the data do not rule out the possibility that bindings were made between the plant proteins and the OAB, reducing digestibility of the protein. This is, however, not likely in view of the high recovery of the OAB, and the similar recovery from all supplemented groups.

Higher FCR when using organic acid salts is in keeping with previous findings in tilapia (Ng et al., 2009). This effect along with the lack of effect of dietary inclusion of OAB on fish growth are however in contrast to the majority of published findings that demonstrate a positive effect of adding organic acids or their salts in fish feeds in Arctic charr (Ringø, 1991), Atlantic salmon (Ringø et al., 1994; Lückstädt, 2008b), rainbow trout (deWet, 2005), Red sea bream (Sarker, et al. 2005, 2007; Hossain, et al., 2007), and tilapia (Ramli et al., 2005; Zhou et al., 2009). The reasons for the conflicting results concerning nutrient digestibility, weigh gain, feed intake and FCR of fish between some previous studies and the present study may be due to the type of organic acid or salt used, dietary inclusion level, diet composition, fish species and age, fish rearing conditions (Ringø, 1991; Gislason et al., 1994; deWet, 2005; Ramli et al., 2005; Sarker et al., 2007), and feed processing technology, as indicated by the present study.

The trout fed the fishmeal-based diet had higher liver weights than those fed the plant protein-based diets. The fishmeal-based diets contained higher levels of starch than the plant protein-based diets. Diets with a high content of digestible carbohydrates increase liver glycogen in salmonids, thus increasing relative liver weight (Krogdahl et al., 2005). In the present study, dietary protein source did not influence the weight of middle and distal intestines. This is in contrast to previous observations that distal intestinal size was decreased in trout fed soybean meal based diets (Romarheim et al., 2006), probably due to the shortening of mucosal folds caused by soybean meal-induced enteritis (Ingh et al., 1981).

OAB added to the diets before extrusion, however, significantly increased middle intestine weight and tended to increase distal intestine weight. This indicates that the OAB may have stimulated intestinal growth, hypothesized as an effect of butyrate. The previous studies conducted by adding 0.17% (Galfi and Bokori, 1990) and 0.3% (Manzanilla et al., 2006) sodium butyrate to pig diets have confirmed the increased length of ileal microvilli and the depth of caecal or jejunum crypts. The need for heat processing of the OAB to obtain this effect may be rationalized by the fact that the butyrate used was coated (Table 1). Coating is done both to protect additives against degradation during feed processing and to improve flow properties and mixability with other feed ingredients. The results indicate that it was necessary to dissolve the coating material by extrusion, in order to liberate the butyrate so that it could be made available as an energy substrate for renewal of intestinal epithelium.

The addition of sodium butyrate or sodium formate to the diets did not affect the morphological appearance of the distal intestine in the present experiments. The morphological changes in the distal intestine were not as pronounced as observed in Atlantic salmon fed similar levels of soybean meal. Previous studies have also reported that rainbow trout were slower to develop distal intestine enteritis, and only do so at higher levels of soybean meal compared to Atlantic salmon (Refstie et al., 2000). Thus, rainbow trout seems to be a less useful model species for studies of factors affecting soybean meal-induced enteritis than Atlantic salmon. Soybean meal is expected to be the predominant cause of the enteritis observed in the present experiment, since no effect on morphology in the distal intestine was observed by Øverland et al. (2009) when feeding diets with 20% pea protein concentrate to Atlantic salmon.

In conclusion, the results implied that supplementing diets with 10 g acid moiety $kg^{-1}$ of a sodium formate and butyrate blend (ratio 2:1 on acid moiety weight basis) did not improve growth rate or feed utilization of rainbow trout. Adding an organic acid salt blend to plant protein-based diets before extrusion increased weight of the intestine and reduced the digestibility of macro nutrient and amino acids, but not when added after extrusion. Replacing 36% fishmeal with soybean meal pea protein concentrate (ratio 2:1 on crude protein moiety weight basis) negatively influence the digestibility and feed conversion ratio of rainbow trout.

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# PAPER II

# Solid state fermentation of *Aspergillus niger* improves the nutritional value of rapeseed meal as a feed ingredient for Nile tilapia (*Oreochromis niloticus*)

Youling Gao[1, 3], Mary Margaret Nabulime[1], Jon Fredrik Hanssen[2], Liv Torunn Mydland [1], Vegard Denstadli[1], Hans Magnus Gjøen[1], Trond Storebakken[1*]

[1]Aquaculture Protein Centre, CoE, Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences (UMB), NO-1432 Ås, Norway.

[2]Department of Chemistry, Biotechnology and Food Science, UMB, NO-1432 Ås, Norway.

[3] College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo, Zhejiang, P.R. China.

[*]Corresponding author: Address: Department of Animal and Aquacultural Sciences, UMB, P.O. Box 5003, NO-1432 Ås (Norway) e-mail: trond.storebakken@umb.no

## Abstract

The aims of the present study were to determine the potential of aerobic solid state fermentation (SSF) to increase the nutritional quality of rapeseed meal, and to evaluate the potential for use of untreated (RSM) and fermented rapeseed meal (FRSM) in diets for Nile tilapia. *Aspergillus niger* strain ATCC 10577 was used in SSF to produce two batches of fermented rapeseed meal. In Exp. 1, three moist diets were produced: a diet based on fish meal and soybean meal (SBM), and two diets in which soybean meal was replaced by RSM or FRSM. The diets were fed to triplicate groups of tilapia to apparent satiation twice daily for 37 d. In Exp. 2, three extruded diets were produced with

the same formulation as used in Exp. 1. Each diet was fed to triplicate groups of 54.6 g tilapia, in 3 meals a day for 43 d.

The content of crude protein in rapeseed meal was increased from 385 to 402-403 g $kg^{-1}$ DM, total amino acids were reduced from 76.3 to 70.7-70.8 g (100 g CP)$^{-1}$, phytic acid was reduced from 35.8 to 10.5 -19.0 g $kg^{-1}$ DM, and virtually none of the glucosinolates from RSM were found in the material treated by SSF. In Exp.1, the specific growth rate (SGR) of fish fed the SBM diet was 3.8, which was significantly lower than that of tilapias fed the RSM (4.1) and FRSM (4.2) diets. Feed conversion ratio (FCR) of fish fed the RSM diet (1.69) was significantly poorer than that of fish fed the SBM (1.22) and FRSM (1.20) diets. Fish fed the RSM diet had significantly higher whole body protein than fish fed the SBM diet. In Exp. 2, the SGR showed no significant difference among the dietary treatments, while FCR differed among all 3 dietary treatments (SBM, 1.08; RSM, 1.23; FRSM, 1.34). The nitrogen retention of fish fed the SBM diet was significantly higher than that of fish fed the RSM diet, which was higher than that for FRSM. The digestibility of nitrogen in fish fed the SBM diet was higher than that in fish fed the RSM and FRSM diets. The digestibility of magnesium was significantly higher in fish fed the SBM and FRSM diets than that in tilapias fed RSM. The differences in nutritional value in the two experiments are rationalized by differences in fermentation of the rapeseed meal, and by the two methods of feed production used.

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*Keywords***:** Solid state fermentation; *Aspergillus niger*; rapeseed meal; Nile tilapia; Specific growth rate; Feed conversion ratio; Digestibility

45 **1. Introduction**

46 Tilapia is the aquaculture species with relatively high production worldwide. There is high demand
47 both from the high end fillet market, and as a major animal protein sources for the poor in
48 developing countries. World tilapia production reached 2.4 m tons in 2006 (Fitzsimmons, 2008), and
49 more than 22 tilapia species are being cultured. Nile tilapia (*Oreochromis niloticus* L.) is the most
50 cultured species. In 2006 a total of 2 million tons of this species was farmed (FAO, 2006). The
51 growth potential of this fish is continuously improved by selective breeding (Eknath et al., 2007),
52 and genetically improved tilapias are available to the fish farmers both in East and South-East Asia.
53 In traditional extensive farming of tilapia, natural pond organisms are the main source of nutrients.
54 As tilapia farming is intensified, the need for low-cost and high-quality feeds increases.
55
56 Rapeseed meal (RSM) is the second largest oil-seed meal production after soybean meal (SBM) in
57 the world. World production of rapeseed and RSM were 58.4 and 34.1 million tons annually in
58 2010/2011 (USDA, 2011). It has high accessibility in the tropical and subtropical markets, with a
59 moderately high content of protein. RSM production is increasing internationally. Rapeseed protein
60 is less expensive than protein from SBM, and has limited application in human food. Furthermore, it
61 has lower activity of heat labile antinutrients such as protease inhibitors and lectins (Francis et al.,
62 2001), rendering the need for heat treatment lower than that for SBM. Other antinutrients, mainly
63 glucosinolates and phytic acid (IP6), however, limit the nutritional value of RSM for fish (Francis et
64 al., 2001). Glucosinolates in rapeseed meal have been shown to impair growth and thyroid function
65 in rainbow trout (Burel et al., 2000b), and red sea bream (Glencross et al., 2004 a; b). In order to
66 become toxic, the glucosinolates must be hydrolysed by myrosinase enzyme that is present in the
67 rapeseed and is also produced by intestinal microflora, to release a range of negative products,
68 including isothiocynates, nitriles, thiocyanates or oxazolidithione (Duncan, 1991; Cheng et al., 2004;
69 Fenwick et al., 1982; Mithen et al., 2000). IP6 consists of phosphorus that is not digestible by

70 monogastric animals, and strongly binds essential elements such as zinc, calcium, magnesium and
71 iron (Denstadli et al., 2006; 2010). A high dietary concentration of IP6, thus, may contribute to
72 mineral deficiencies. Tilapia are known to contain some phytase activity in their digestive system
73 (Ellestad et al., 2002), probably of bacterial origin.
74
75 Solid state fermentation (SSF) is an ancient technology that has been reported to improve the
76 nutritional value of RSM by breakdown of antinutrients through production of exogenous enzymes,
77 as well as using the antinutrients as substrate for microbial growth (Bau et al., 1994; El-Batal and
78 Karem, 2001; Nair and Duvnjak, 1991; Rozan et al., 1996; Vig and Walia, 2001). In addition, SSF
79 can increase protein content (Frias et al., 2008; Hong et al., 2004) and improve the essential amino
80 acid profile of the substrate (Yigzaw et al., 2004).
81
82 RSM has been evaluated as protein source in feed for Chinook salmon (Higgs et al., 1982), tilapia
83 (Davies et al., 1990), and rainbow trout (Burel et al., 2000a; Gomes et al., 1995; Shafaeipour et al.,
84 2008). Nevertheless, a paucity of information is available concerning the use of fermentation to
85 improve the nutritional value of RSM and replacement soybean meal with rapeseed meal for Nile
86 tilapia. The aims of present study, thus, were to determine if aerobic SSF was useful to increase the
87 nutritional quality of RSM, and to determine if untreated or fermented RSM could replace SBM in
88 diets for Nile tilapia.
89
90 **2. Materials and Methods**
91 *2.1 SSF of rapeseed meal*
92 Two separate aerobic SSF of rapeseed meal were conducted. Each of the fermentations was followed
93 by a growth trial with Nile tilapia. *Aspergillus niger* ATCC 10577 was obtained from the Department
94 of Chemistry, Biotechnology and Food Science (IKBM), Norwegian University of Life Sciences

95 (UMB). The strain was grown and maintained on potato-dextrose-agar (PDA) medium for 7 days at
96 20 ºC before the spores were transferred to a 100 ml vial containing seed medium (a blend with 20 g
97 rapeseed meal (RSM) and 20 ml water), which had been autoclaved at 121ºC for 15 min. The vial
98 then was incubated at 30 ºC for 72 h while a sterilized cotton plug was used as air filter to allow the
99 exchange of air between outside and inside the vial. The incubated medium with mycelium and
100 spores then was transferred to a 1,000 ml vial containing seed medium (a blend with 200 g rapeseed
101 meal and 200 ml water), which had been autoclaved at 121 ºC for 15 min. This medium then was
102 incubated at 30 ºC for 72 h, and a sterilized cotton plug was inserted.
103
104 A blend of RSM and water, with a ratio of 1:1, was sealed into a sterile gas-permeable bag (37×56
105 $cm^2$; Saco2, Eke, Belgium). The inoculation was conducted by transferring the medium with growing
106 mycelium and spores in a 1,000 ml vial into the bag, and the inoculation size was 10%. Incubation
107 was conducted at 30ºC for 48 h. Pasteurization at 70 ºC for 24 h was applied after incubation. This
108 fermented and pasteurized biomass was mainly used for production of moist feed. The same
109 fermentation procedure was applied to the biomass that was used for production of extruded feed,
110 and the biomass was pasteurized at 70 ºC for 6 h, frozen at -20$^{o}$C and freeze dried. The procedure
111 was repeated until sufficient amount of biomass for feed production was produced. Before the
112 biomass was used for production of feed, the biomass was dried for 30 min in a 40 l twin shaft
113 paddle mixer (IDE-CON AS, Porsgrunn, Norway) for 30 min at 70 ºC. The moisture content after
114 drying was approximately 250 g $kg^{-1}$.
115
116 *2.2 Feed production*
117 Three moist diets were formulated (Table 4): a diet based on SBM and two diets in which SBM was
118 replaced by RSM or fermented RSM (FRSM). The diets were formulated to contain the same
119 amounts of crude protein and lipid based on prior analysis. Production of the diets was carried out at

120 the Department of Animal Sciences, Norwegian University of Life Sciences (UMB). All the
121 ingredients were mixed (Kenwood Patisseir MX 270, Kenwood Ltd., Hampshire, UK) prior to
122 adding water and lipid. The diets then shaped into soft pellets using a meat mincer (Braun Type
123 4175, Frankfurt, Germany) and were kept frozen until feeding. The diets were transferred to room
124 temperature for 10 to 20 min before feeding.
125
126 Three extruded diets were produced in accordance with the formulation used for the moist diets. The
127 rapeseed meals were homogeneously mixed for 30 min at 70 ºC. The diets were produced at The
128 Centre for Feed Technology (FORTEK), UMB. All dry ingredients were milled in a Münch Hammer
129 mill (HM 21.115, Wuppertal, Germany) with a 1-mm screen. The ingredients then were mixed in a
130 small scale Dinnisen twin shaft mixer (Pegasus Menger 400 1, Sevenum, Holland) for 2 min, and
131 transferred to a feed mash delivery system that was connected to a motor frequency converter to
132 maintain the feed flour flow rate at 40 kg $h^{-1}$. The feed mash was extruded in a five-section Bühler
133 twin-screw extruder with 3-mm dies. The Specific mechanical energy (SME) created by the
134 extrusion was 1,233 J $kg^{-1}$ for the SBM diet, 395 J $kg^{-1}$ for the RSM diet, and 557 J $kg^{-1}$ for the
135 FRSM diet.
136
137 *2.3 Fish experiments*
138 2.3.1 Fish, facilities and management
139 The fish experiments were conducted at the Fish Nutrition Laboratory at UMB. Nine fibre-glass
140 tanks (0.5 m diameter, 0.7 m height, volume 0.11 $m^3$) were used. The tanks were covered by a
141 transparent lid and had continues light (24 h). The Nile tilapia were hatched from the Genomar
142 Supreme Tilapia strain (GST, Genomar, Oslo, Norway) raised at UMB. The GST originates from the
143 Genetically Improved Farmed Tilapia (GIFT) project (Eknath et al., 2007). The fish in this study

144 came from the 16th generation of selection for growth, survival, fertility and rough handling
145 resistance. Before the experiments, all the fish were fed a diet produced at FORTEK.
146
147 2.3.2 Experiment with moist diets
148 The experiment was designed according to a randomized block model, with three diets and three size
149 classes of fish (5.6, 7.2, and 10.7 g). Each size class of fish was randomly distributed to three tanks
150 (n = 33-34 per tank for the small size groups, n=39-40 for the middle size groups, n=24-25 for the
151 large size groups). The fish were 6 wks old at the start of the experiment. The feeding period lasted
152 for 35 d. The tanks were supplied with fresh water with a temperature at 25ºC and average dissolved
153 oxygen level was 6.0 mg $l^{-1}$. The water flow was kept at 8 l $min^{-1}$. The diets were assigned randomly
154 to fish within each size class. Fish were fed by hand to apparent satiety twice daily (09:30-11:00 h
155 and 14:00–15:30 h). Apparent feed consumption in each tank was recorded visually for each feeding.
156 Water temperature and dissolved oxygen level were measured daily after the last feeding.
157
158 Fish were weighed at the start of the experiment, on days 13, 23 and at the end of the experiment
159 (day 37). The fish were fasted for 24 h before weighing at day 37. The fish were anaesthetized with
160 MS 222 (0.2 g $l^{-1}$ in freshwater; Apotekproduksjon AS, Oslo, Norway), weighed individually before
161 the abdominal cavity of 3 fish was cut open, and weight of liver, gut and stomach were taken. Five
162 fish were taken randomly from each tank for analyses of whole body composition.
163
164 2.3.3 Experiment with extruded diets
165 The experiment was designed according to a completely randomized model. Fish with average initial
166 body weigh 56.4 g were randomly distributed to 9 tanks (n = 25 per tank). The fish were 16 wks old
167 at the start of the experiment. The experiments lasted for 43 d. The tanks were the same those used in
168 with the moist diets, and were supplied with fresh water at 26 ºC and average dissolved oxygen level

169 at 3.8 mg $l^{-1}$. Water flow was 8 l $min^{-1}$. The diets were assigned randomly. Fish were fed by hand to
170 apparent satiety three times per day (08:00-09:30 h, 14:00–15:30 h and 20:00-21:30 h). Apparent
171 feed consumption in each tank was visually recorded for each feeding. The floating uneaten feed was
172 collected, weighted and stored at -20ºC until subsequent dry matter determination. Water
173 temperature and dissolved oxygen level were measured daily after the last daily feeding.
174
175 Fish were weighted at the start of the experiment, on day 21 and at the end of the experiment. Three
176 samples of 5 fish, were randomly taken from the holding tank for initial whole body composition. At
177 the end of experiment, the fish were euthanized with MS 222 (0.8 g $l^{-1}$), then weighed and measured
178 individually before the abdominal cavity of fish was opened. The weight of liver, gut and stomach
179 were taken. Five randomly selected fish were taken from each tank for whole body composition (the
180 gut content was removed). The content of the last part of the distal intestine (5 cm to anus) was
181 collected as faeces from the rest of fish. Apparent digestibility of nitrogen, amino acids, phosphorus
182 and zinc were assessed by the indirect method with $Y_2O_3$ as an inert marker (Austreng, et al., 2000).
183
184 *2.5 Sample analysis*
185 2.5.1 Sample preparation
186 Fermented rapeseed meal and moist diets were freeze dried and stored at 4 ºC before conducting
187 chemical analysis. The pellets of extruded diets were ground using an A11 basic Analytical mill
188 (IKA, Wilmington, USA) and stored at 4 ºC until analysis. Faeces were stored frozen until being
189 freeze dried and ground prior to chemical analysis. Frozen whole fish were homogenised using a
190 Braun Multisystem 3-in-1 kitchen machine (Braun GmbH, Frankfurt, Germany), freeze dried, and
191 ground once more with $CO_2$ ice on an A11 basic Analytical mill before chemical analysis.
192
193 2.5.2 pH and nutrient analysis

194 The pH of the diets was determined by dissolving 1 g of each diet in 10 ml of de-ionized water and
195 stirred for 1 min before measurement with an electrode. Dry matter and ash in diets and whole fish
196 body were determined by drying at 105 and 550ºC until stable weight. Crude protein (Kjeldahl
197 N×6.25) in feed and whole fish body was determined by using a Kjeltec auto 1035/1038 system
198 (Tecator, Sweden). Crude lipid in feed and whole fish body were determined using Accelerated
199 Solvent Extraction (ASE 200, Dionex, Denvers, MA, USA). Starch in feed was analyzed as glucose
200 after starch hydrolysis with a heat tolerant amylo-glucosidase according to McCleary et al. (1994).
201 Nitrogen in faeces was determined by the Dumas method (Kirsten and Hesselius, 1983) using an EA
202 1108 element analyzer system (Fison, Waltham, USA). Amino acids in feed and whole fish body
203 were analysed according to EC (1998) by using a Biochrom 30 Amino Acid Analyzer (Biochrom
204 Ltd., Cambridge, UK). Yttrium, potassium, magnesium, phosphorous, and zinc in diets and faeces
205 was determined by inductively coupled plasma mass spectroscopy (ICP-MS) after complete
206 digestion of the homogenized and dried samples in $HNO_3$ and cooking in a micro-wave oven for 1 h.
207 Due to the small amount of faecal material available for analysis, down scaling of the analytical
208 procedure for amino acids was necessary. The EC procedure (1998) was followed; however the
209 sample amounts, chemicals and solutions were all down-scaled by a factor of 10. A closed hydrolysis
210 was performed in Schott Duran® GL18 test tubes with PTFE (Teflon) lined screw caps in a
211 Labtherm heating block (Liebisch Labortechnik GmbH, Bielefeld, Germany) at 110 °C for 23 h.
212
213 2.5.3 Phytic acid and glucosinolates
214 Phytic acid was determined according to the method described by Carlsson et al. (2001). The
215 samples (0.5 g) were extracted with 10 ml 0.5 M HCl for 3 h, followed by centrifugation at 2000 g
216 for 10 min. The supernatants were filtered in 30 kDa Vectraspin filters (Whatman, Banbury, UK),
217 and transferred to glass vials. The samples were injected and conveyed by a Ultimate 3000 HPLC

pump (Dionex, Sunnyvale, CA, USA) equipped with a HPIC Carbopac PA-100 column (Dionex). A post column reaction with 0.1% $Fe(NO_3)_3*9H_2O$ dissolved in a 2% $HClO_4$ was serviced by a Shimadzu HPLC pump (CC-10AT, Duisburg, Germany), and the inositol phosphates were detected by UV detection (290 nm) integrated in the Ultimate 3000 system (Dionex). Glucosinolates were analyzed by Nofima Food (Ås, Norway) according to Volden et al (2008).

2.5.4 Fungal biomass quantification

Fungal biomass was estimated by analyzing the ergosterol content. The extraction followed the method used by Afanou (2008), with small modifications made. In brief, 0.3 g was placed into a test tube in parallels. The samples were extracted with 1ml cyclohexane for 45 min followed by saponification with 4 ml 4% KOH in methanol (w/v) for 45 min at room temperature, and then for 90 min at 70 °C in water bath. After cooling to room temperature, one ml MQ water and 2 ml cyclohexane were added to separate the organic and aqueous phases. The sample was mixed with a vortex apparatus, and centrifuged for 5 min at 2,000 g. The upper phase (hexane phase) was transferred to a glass tube and another 2 ml cyclohexane was added to the remainder, vortexed, centrifuged with the same conditions before collection of the hexane phase. The collected hexane phases were mixed, and evaporated at 40°C on a heating block with $N_2$ flow. The dried extracts were kept at -20 °C until HPLC analysis. Fifty mg crystalline ergosterol (Sigma Aldrich, St. Louis, USA) was dissolved in 100 ml methanol for stock solution and the following concentrations were prepared: 0.01, 0.05, 0.1, 0.2, and 0.4 mg $ml^{-1}$. Methanol served as the blank. A recovery test was performed to determine the recovery of the extraction procedure in the initial step by spiking the samples(0.3 g) with different known amounts of ergosterol dissolved in methanol (0.01, 0.1, 0.2 and 0.4 mg $ml^{-1}$, 0.3 ml of each concentration). Ergosterol was then extracted and eluted as described above. The recovery rate ranged from 70.4 to 91.5%.

243 The dried extracts were dissolved in 2 ml methanol, incubated at 40$^{o}$C in a heating block for 5 min,
244 then vortexed and centrifuged at 2,000 g for 5 min before further analysis. Ergosterol was analyzed
245 on a HPLC system consisting of a Gilson 321 (Middleton, USA) high-pressure pump, a Gilson
246 UV/VIS-152 detector, a Gilson 234 auto-injector, and a Supelcosil LC-18-DB (2.1×250 mm, 5 µm)
247 analytical column with column guard (Supelguard LC-18-DB; 2.1×20 mm, 5 µm). The system was
248 run with 100% methanol (HPLC grade) as mobile phase, 10 µl of injection volume, and 2 ml min$^{-1}$
249 of flow. The running time was set to be 10 min, and the samples were monitored at 282 nm.
250
251 2.5.5 Electrophoresis
252 Proteins in RSM and FRSM were extracted by a modified method based on Aluko and McIntosh
253 (2001). Samples were finely ground with the A11 basic Analytical mill. Ground samples (125 mg)
254 were mixed with 2.5 ml of 50 mM Tris-HCl (pH 8.0) containing 1% SDS, 5 mM DTT and 5 µg ml$^{-1}$
255 protease inhibitor. Samples were then incubated on ice for 3 h, followed by 8 consecutive
256 ultrasonications with ice, each lasting 1 min. Samples were kept on ice during ultrasonication. The
257 samples were thereafter transferred to 1.5 ml microtube containing glass beads (Qiagen, Hilden,
258 Germany) and further disrupted in a FastPrep machine (FP120, BIO101, Savant) at 6,500 g for 45
259 sec, 5 time repeats with 1 min interval, on ice. The samples were then treated in an ultrasonication
260 water bath (2 cycles of 5 min) prior to centrifugation at 16,000 g for 30 min at 4°C (Centrifuge 5415
261 R, Eppendorf, Hamburg, Germany). The supernatants were transferred to Eppendorf tubes and kept
262 at -20°C overnight. The samples were concentrated from 380 to 40 µl using Nanosep® Centrifugal
263 Devices (Pall Life Sciences, Ann Arbor, USA) before mixing with 40 µl SDS-PAGE loading buffer,
264 and incubation at 65 °C for 5 min (Thermomixer comfort, Eppendorf, Hamburg, Germany). Mixed
265 samples were then centrifuged at 16,000 g for 15 min at 4°C.
266

SDS-PAGE was conducted in a Bio-Rad Electrophoresis system (Bio-Rad, Hercules, CA) with 12% polyacrylamide separating gels in 20x dilution of XT MOPS running buffer. One µl of RSM sample, 2 µl of FRSM1 sample, and 2 µl of FRSM2 sample were loaded respectively and run at 120 V for 1.5 h. After electrophoresis, the gel was fixed in a solution of 50% methanol, 15% acetic acid and 35% water with shaking (Heidolph Unimax 2010, Heidolph, Schwabach, Germany). The gel was further stained for 1h and destained until the background was clean before taking photos.

*2.6 Calculations and statistical analyses*

Apparent digestibility coefficients were calculated as $100 \times (1-(D_i/F_i \times F_n/D_n))$, where $D_i$ represents concentration of inert marker ($Y_2O_3$) in diet. $F_i$ is concentration of inert marker in faeces. $D_n$ represents nutrient concentration in diets. $F_n$ is nutrient concentration in faeces. Nutrient retentions were calculated as $100 \text{ x } ((W_e \text{ x } N_e) - (W_0 \text{ x } N_0)) (D_n \text{ x } F_i)^{-1}$, where $W_0$ and $W_e$ represent initial and final fish weight, $N_0$ and $N_e$ are nutrient concentration in fish, and $F_i$ is feed intake. Condition factor was calculated as: $100\times$ body weight (g) $\times$ (body length (cm))$^{-3}$.

The data were analysed using the GLM procedure in the SAS software (SAS, 1999). Results are presented as the least square mean (LSMEANS) for each diet. Variance is expressed as pooled standard error of the mean (SEM). Duncan's multiple range test was used to rank significant differences ($P<0.05$) among diets. The amounts of freeze dried faeces for analysis was small (down to 200 mg). Consequently, there was not enough sample available for re-analysis when analytical error was identified, and outlier results were omitted when satisfying criteria for "Gross errors; rejection of extreme observations" given by Snedecor and Cochran (1967).

## 3. Results

*3.1 Solid state fermentation*

292 The characteristics of untreated and fermented rapeseed meals are given in Table 1. The fungal
293 biomass in RSM was estimated to be 3.9 g $kg^{-1}$. After the fermentation, the fungal biomass in FRSM
294 was increased to 12.0 and 22.1 g $kg^{-1}$. The dry mater recovery rates of rapeseed meal were 96.0%
295 and 96.2% after fermentation. Chemical composition of rapeseed meal also was changed by the
296 fermentation. The crude protein content was increased from 385 to 403 and 402 g $(kg\ DM)^{-1}$,
297 whereas the fat content was increased from 43.7 to 58.0 and 57.6 g $(kg\ DM)^{-1}$. However, the starch
298 content was decreased from 27.8 to 19.3 and 22.9 g $(kg\ DM)^{-1}$. The fermentation slightly reduced the
299 amount of both essential and non-essential amino acids (Table 2). Moreover, fermentation affected
300 the sizes of the peptides in rapeseed meal (Fig. 1). Most of the peptides larger than 10 kDa were
301 reduced by fermentation, and the peptides were more degraded more during the 1$^{st}$ fermentation for
302 moist diets than during the 2$^{nd}$ fermentation for extruded diets.
303
304 Antinutrients in RSM were reduced by the fermentation. The content of phytic acid (inositol
305 hexaphosphate, IP6) in FRSM (10.5 and 19.0 g $(kg\ DM)^{-1}$) was lower than that in RSM (35.8 g (kg
306 $DM)^{-1}$) (Table 1). Small amounts of IP 6 were found in the RSM, and reduced in the FRSM. IP 2 was
307 the main component accumulated in FRSM. SSF resulted in a total release of phosphate of 38%
308 during the 1$^{st}$ SSF, and 33% during the 2$^{nd}$.
309
310 Also, virtually none of the glucosinolates detected in the RSM were detected in the FRSM, with the
311 exception of trace amounts of progoitrin (Table 3). However, unidentified peaks, not detected in the
312 RSM and not co-eluting with any of the standards in the HPLC analysis, were observed in the
313 chromatograms for the FRSM samples.
314
315 *3.2 Characteristics of moist and extruded diets*

316 The chemical composition of both moist and extruded diets was close to the planned level (Table 4).
317 The crude protein content in both moist SBM diet and extruded SBM diet were lower than that in the
318 RSM and FRSM diets. The lipid content in the moist RSM and FRSM diets was slightly higher than
319 in the other diets. Starch content showed a different pattern. The highest content was in moist and
320 extruded SBM diets. pH was lower in moist and extruded FRSM diets than that in other diets. The
321 content of amino acids in SBM diet was higher than those in RSM (except cysteine, methionine and
322 glycine) and FRSM (except cysteine, methionine, threonine, proline, glycine and valine) diets (Table
323 5). In addition, the content of amino acids in the FRSM diet was higher than those in RSM diet
324 (except aspartic acid, lysine and arginine).
325
326 *3.3 Experiment with moist diets*
327 All the diets were accepted by the fish, and RSM diet was the most attractive diet for tilapia among
328 the three diets. The intake of feed with RSM was higher than that of tilapia fed the SBM and FRSM
329 diets (Table 6). The mean weight of fish fed SBM diet at day 13 was significantly higher compared
330 with that of fish fed RSM and FRSM diets. The mean weights at day 23 were not significantly
331 different. At day 37 the fish fed the FRSM diet had caught up with SBM, and were significantly
332 larger. The specific growth rate (SGR) over the whole experimental period was significantly lower in
333 the fish fed SBM diet compared to tilapia fed the other two diets.
334
335 Feed conversion ratio (FCR) of fish fed RSM diet was significantly higher than that in fish fed SBM
336 and FRSM diets, while there was no significant difference in FCR between fish fed the SBM and
337 FRSM diets. Fish fed the SBM diet had significantly higher intestine and stomach weights compared
338 with tilapia fed the other two diets. A significantly lower intestine weight was observed in tilapia fed
339 the RSM diet compared with the FRSM diet.

340 Dry matter content of fish fed the SBM diet was significantly lower than that of fish fed RSM and
341 FRSM diets (Table 7). The crude protein content of fish fed RSM diet was significantly higher than
342 that in fish fed SBM diet. No significant differences were observed in fat content. Ash content
343 differed significantly among the three treatments; the lowest values were found in tilapia fed the
344 SBM diets and highest as was observed in fish fed the FRSM diet.
345
346 *3.4 Experiment with extruded diets*
347 Feed intake (Table 8) did not differ significantly among the three extruded diets. The mean weight of
348 fish fed SBM diet at day 21 was significantly higher than that of tilapia fed the RSM and FRSM diets
349 (Table 8). SGR was significantly higher for the SBM diet than for FRSM diet during the first 21 days,
350 but was significantly lower than that observed for the RSM and FRSM diets over the whole 37 day
351 feeding period. Furthermore, FCR in fish fed RSM was significantly lower than that fish fed FRSM
352 during the whole experimental period. Fish fed FRSM diet had significantly higher liver and stomach
353 weights compared with fish fed other two diets.
354
355 The chemical composition of the fish did not differ among dietary treatments (Table 9). However,
356 whole-body dry matter, protein and fat were higher at the end of the experiment than at the start. The
357 amino acid profiles of whole tilapia bodies were similar for all dietary treatments.
358
359 The nitrogen retention of fish fed SBM diet was significantly higher than that of fish fed RSM and
360 FRSM diets (Table 10). In addition, fish fed FRSM diet had significantly lower nitrogen retention
361 than that of fish fed RSM diet. The digestibility of nitrogen in fish fed SBM diet was marginally
362 higher than that in fish fed RSM and FRSM diets. Moreover, the digestibility of Mg was
363 significantly higher in fish fed SBM and FRSM diets than that in fish fed RSM diet. The digestibility
364 of K, P, and Zn was in marginal difference among three dietary treatments. Apparent digestibility

365 coefficients of amino acid in Nile tilapia fed extruded diets are listed in Table 11. The results showed
366 that the fish fed SBM diet obtained highest digestibility of amino acids among three dietary
367 treatments, while the lowest was in the fish fed FRSM diet.
368
369 **4. Discussion**
370 SSF resulted in changes in nutritional value of the RSM that were caused both by the fermentation
371 itself and the hydro-thermal treatment employed for pasteurisation. The dry matter loss was 4.1%
372 during the 1st fermentation (for moist diets) and 3.9% for the 2nd fermentation (for extruded diets). A
373 similar magnitude of dry matter loss was observed by Penaloza et al. (1985) when subjecting coffee
374 pulp to SSF with *A. niger*. Our recovery results indicate that *A. niger* mainly used carbohydrates as
375 medium, as illustrated by starch recovery at 67 and 79 %, and recovery of the calculated analytical
376 residue at 90 % during both fermentations. This is in agreement with finding by Rozan et al. (1996).
377 Sugars are the preferred energy substrate for *A.* Niger, and hydrolysis of non-starch polysaccharides
378 (NSP) occurs during fermentation (Rozan et al., 1996). The analytical residue includes organic acids
379 in addition to carbohydrates, as illustrated by the reduction in pH obtained by SSF. *A. Niger*
380 produces especially high amounts of citric and oxalic acids (Van de Merbel et al., 1994; Schrickx et
381 al., 1995; Cameselle et al., 1998; Krishna, 2005).
382
383 Recovery of crude protein was 100 % for both fermentations, so protein was not a preferred substrate.
384 However, Singh et al. (1989) reported crude protein contents up to 22% of *A. niger* biomass
385 produced by SSF. Based on our estimates of fungal biomass, this represents up to 0.7-1.2 % of the
386 nitrogen presented in FRSM. The reduction in the proportion of both essential and non-essential
387 amino acids relative to total nitrogen also illustrates that a part of the amino acids in RSM was
388 metabolised to other nitrogen-containing components during SSF. Muhammad and Oloyede (2009)
389 concluded that the reduction in amino acid content was a result of nucleic acid synthesis during

390 fermentation. Another reason may be that the germination of conidiospores in *A. niger* requires
391 valine, leucine, cysteine and arginine (Abdelrahim and Arbab, 1985). Thus, in contrast to previously
392 reported effects of SSF on other substrates (Frias et al., 2008; Hong et al., 2004; Yigzaw et al., 2004),
393 SSF did not appear to increase protein content nor improve the essential amino acid profile of RSM.
394 Lipid was the most notable fermentation product, with an increase of 26-27%. The fatty acid
395 composition of RSM did not change by SSF (data not shown) and the fatty acid profiles were similar
396 to those obtained during SSF with *A. niger* reported by Singh (1991).
397
398 The 47-71% reduction in phytic acid by SSF with *A. niger* also was in agreement with previous
399 findings (El-Batal and Karem, 2001), and was caused by the production of exogenous phytase during
400 fermentation (El-Batal and Karem, 2001; Papagianni et al., 1999). In addition to rendering more than
401 1/3 of the phosphate bound in phytate in RSM available for digestion, SSF efficiently reduced the
402 binding of divalent and trivalent cations. The ability of IP to bind essential kations such as zinc and
403 magnesium is strongly reduced by decreasing the number of phosphates esterified to the inositol
404 molecule (Persson et al., 1998). The amounts of faeces available for analyses of digestibility were
405 small, limiting the opportunity for analytical replication. Thus, statistical power was low, and the
406 only element that had its absorption significantly improved by SSF was Mg, probably due to reduced
407 binding to IP6. That is in keeping with Usha and Chandra (1998) who found that the mineral
408 availability was enhanced by fermentation. Also, the numerical improvement in apparent
409 digestibility of P is in the range expected from the range of hydrolysis of P from IP6 in the RSM by
410 SSF. $Zn^{++}$ has high affinity to both IP6 and oxalic acid (Vohra et al., 1965; Sayer and Gadd, 2001).
411 Thus, it is probable that the lack of improvement in uptake of Zn was due to binding to residual IP6,
412 eventually IP5 and oxalic acid in the FRSM. Furthermore, the amount of Zn in the diets was in
413 excess of requirement (NRC, 1993). Zn uptake is regulated at several levels, whereof uptake from

414 the intestine is the first site of regulation (Hardy et al., 1987). Thus, improved availability of Zn does
415 not automatically result in increased uptake.
416
417 The glucosinolates commonly found in RSM were efficiently reduced by SSF, in keeping with
418 previous findings (Bau et al., 1994; Smits et al., 1993; Vig and Walia, 2001). The almost complete
419 lack of glucosinolates after fermentation is partly due to the utilization of glucose and sulphur
420 moieties in glucosinolates by *A. niger* (Verbiscar et al., 1981). However, the occurrence of new
421 peaks eluting close to the glucosinolates in the HPLC analysis may also indicate that the fungi just
422 slightly modified some of the glucosinolates.The results can, however, not be interpreted as an
423 indication that SSF rendered the glucosinolates in RSM harmless to the fish. Production of potent
424 toxins such as isocyanate, thiocyanates goitrin, and nitrile is due to myrosinase activity (review by
425 Bell, 1984). Typically, progoitrin may be metabolised to goitrin, a main metabolite from
426 glucosinolates affecting thyroid activity, by microorganisms in the rumen (Cheeke, 1998). The
427 analytical method used in this study only identified glucosinolates commonly found in RSM, and not
428 metabolites thereof. Thus, additional analyses are required to determine if SSF reduced the potential
429 of producing toxic components from glucosinolates in RSM, or if it in worst case introduced toxicity
430 by fungal myrosinase activity.
431
432 Ng et al. (2002) observed that red tilapia fed a diet based on fermented palm kernel meal obtained
433 poorer FCR than fish fed a diet based on unfermented palm kernel meal. They ascribed the
434 difference to production of mycotoxins generated during fermentation (Lim et al., 2001). This
435 emphasizes the need to carefully assess not only the fate of glucosinolates, but also the formation of
436 other toxins if SSF will be developed into a tool to improve the nutritional value of RSM.
437

438 Compared to the 1st SSF, the 2nd fermentation resulted in higher recovery of dry matter, less efficient
439 hydrolysis of phytic acid, less efficient reduction of peptides, and slightly higher pH on one hand
440 indicating less complete fermentation. These differences point to the fact that it may be challenging
441 to obtain stable and predictable improvement in the nutritional quality of RSM by SSF, and that
442 more research is needed to determine which factors must be controlled to obtain stable results.
443
444 Feed processing with relevance for nutritional quality of the diets also differed both within and
445 between experiments. During extrusion SBM strongly increased the viscosity of the extrudate,
446 resulting in 3.1 times higher SME than when extruding the SBM diet and 2.2 times higher than for
447 the FRSM diet. This increase was largely related to the non-starch polysaccharide content of the
448 SBM, resulting in an extruded pellet with improved durability (Sørensen et al., 2009). A moderate
449 increase in energy input, during extrusion of feeds with plant seed proteins, firstly results in
450 improved protein digestibility due to mild denaturing (Morken et al., 2011), while excessive heat
451 results in reduced protein digestibility (Opstvedt et al., 1984).
452
453 The heat input for pasteurisation and drying was different for the two SSFs. The reason was that the
454 material from the 1st fermentation was used in moist feed, and that fermentation in the finished feed
455 was not desirable. Thus, 24 h heat treatment at 70°C was employed, while drying prior to inclusion in
456 the feed mix was not necessary. During the 2nd fermentation the feed was extruded and dried,
457 allowing a lesser heat treatment when fermentation was completed (6 h), while partial drying of the
458 fermented RSM was needed in order to successfully extrude the feed. Thus, the SBM and RSM used
459 in the moist diets were not hydrothermally treated, while the FRSM was subject to such treatment.
460 Extrusion is known to improve nutrient digestibilities both from SBM (Romarheim et al., 2005) and
461 RSM (Lichovnikova et al., 2004), both due to inactivation of heat labile antinutrients, especially
462 abundant in SBM (Francis et al., 2001), and by unfolding tightly packed seed proteins (Morken et al.,

463 2011). Correspondingly, part of differences in nutritional responses observed when feeding the moist
464 diets to tilapia may be ascribed to differences in hydrothermal treatment.
465
466 The diets used in this experiment contained considerably more fish meal than what is commonly
467 accepted to produce a commercially viable tilapia feed. The reason for the high fish meal use was to
468 avoid confounding effects of antinutrients, indigestible oligosaccharides and NSP from other crude
469 plant protein sources than RSM.
470
471 The digestibility of crude protein obtained by dissecting the contents from the last part of the distal
472 intestine in the tilapias fed extruded feeds were in the same range as previous results in tilapias (De
473 Silva et al., 1990; Shiau and Liang, 1995; Fagbenro, 1998; Goddard and McLean, 2001; Maina et al.,
474 2002; Ng and Chong, 2002; Köprücü and Özdemir, 2005; Leenhouwers et a., 2007; Ghazalah et al.,
475 2010). These results were obtained by several faecal collection methods (rectal dissection (Fagbenro,
476 1998; De Silva et al., 1990); stripping from the hindgut (Maina et al., 2002); siphoning of intact
477 faecal strands shortly after defecation (Shiau and Liang, 1995; Goddard and McLean, 2001; Ng and
478 Chong, 2002; Leenhouwers et a., 2007; Ghazalah et al., 2010); or by the use of a settling column
479 ( Köprücü and Özdemir, 2005; Leenhouwers et a., 2007)). These similarities in the level of nitrogen
480 digestibility indicate that leaching due to washing-out of the faecal strand before it is released from
481 the tilapia, and subsequent leaching of nutrients during settling can be minimized if the collection
482 method targets minimal loss of nutrient from the faeces. The observation that EAA and total amino
483 acids were more efficiently digested in SBM compared to FRSM, and the indication that SSF did not
484 seem to improve digestibility of amino acids from RSM, implies that slightly more protein from
485 RSM is needed to satisfy the EAA requirement of Nile tilapia when RSM products are used as
486 dietary source of protein.
487

In the experiment with moist diets, the tilapia ingested more of the RSM diet than of the other two diets, possibly indicating reduced palatability caused by SSF. This might indicate that production of citric and oxalic acids and ethanol (Abouzied and Reddy, 1986; Nakamura et al., 1996) during SSF may have impaired the palatability in tilapia. However, citric acid has been shown to stimulate growth in hybrid tilapia (*O. Niloticus x O. aureus*) (Pan et al., 2004). Thus, it is more likely that the lower feed intake may have been the result of improved nutritional characteristics, as demonstrated in rainbow trout by Bromley and Adkins (1984), who observed increased feed untake when the diet was diluted with a cellulose filler.

In the experiment with extruded diets, the feed intake of all three diets was similar. Larger fish were utilized in the experiment with extruded diets, indicating that smaller fish are more sensitive to the selection of dietary ingredients than larger fish. The extrusion also represented a hydro-thermal treatment of all diets, not only the FRSM, as discussed previously. Thus, it is probable that extrusion of the feeds participated to lowering compounds that impaired the feed intake, and increased the availability of nutrients to the digestive system.

For tilapias fed the SBM diet growth was faster (SGR at 4.5-3.8 for 8-g fish and 3.5-2.7 for 56-g fish) and the feed conversion of the same magnitude (1.1-1.2 for small tilapia and 1.0-1.1 for the larger ones) as in experiments with Nile tilapia fed diets with comparable composition (Fontaínheras-Fernandes et al., 1999; Leenhouwers et al., 2007). One reason for the improved growth rates is that the growth potential of Nile tilapia is continously improved through combined family-penotype selection programs (Eknath et al., 2007), and that genetic improvement for growth rate in the range of 10-15% per generation can be expected (Gjedrem and Thodesen, 2005). The body composition of the Nile tilapia fed the SBM diet was similar to previous observations (Azaza et al., 2009).

513 There is no published information available on the nutritional effects of SSF RSM in feed for fish,
514 but results obtained with chickens show improved growth and gut health (Chiang et al., 2010). Thus,
515 the improved growth and feed conversion obtained by SSF of RSM in the experiment with moist
516 diets may be related to a combination of several factors. Firstly, the reduction of antinutrients such as
517 phytic acid and glucosinolates are possible contributors.
518
519 RSM has a total sugar content of more than 80 g $kg^{-1}$, indigestible polysaccharides like raffinose and
520 stachyose account for nearly 20 %. The soluble non-starch polysaccharide (NSP) content is 55 g $kg^{-1}$
521 (Knudsen, 1997), and it is expected that most of the oligosaccharides and a high proportion of the
522 soluble NSB was used as substrate. Raffinose, stachyose and soluble NSP from soybeans are not
523 limiting to digestibility and growth in salmonids (Kraugerud et al., 2007; Sørensen et al., 2011), in
524 contrast to what is seen for poultry and swine (Choct et al., 2010). The reductions in nutrient
525 digestibility in poultry and swine is associated with increased viscosity of the digesta (Choct et al.,
526 2010), while in salmonids faecal dry matter is reduced in response to feeding SBM (Storebakken et
527 al., 2000). The anatomy of the digestive system of the Nile tilapia and the adaptation to an
528 omnivorous diet with high intake of plant materials makes it more similar to the pig than to the
529 specialized carnivorous salmonids. However, results obtained by Leenhouwers et al. (2007) when
530 feeding cereal grains with increasing viscosity, induced increased viscosity and reduced the dry
531 matter content of digesta, but did not significantly affect nutrient digestibilities in Nile tilapia. Thus,
532 the improved feed conversion obtained by SSF of the RSM probably is due to a combination of
533 reduced antinutrient concentrations and increased nutrient concentration, mainly achieved by
534 converting indigestible carbohydrates into lipid. In contrast, Wang et al. (2008) obtained improved
535 growth and feed conversion, accompanied by improved intestinal protease activity when applying an
536 NSPase to feed for hybrid tilapia. They, however, did not quantify changes in carbohydrate
537 composition, did not report information on the purity of the enzyme preparation, and did not provide

information on nutrient digestibilities. This makes it difficult to judge if their response was due to a reduction in NSP content or increased nutrient concentration in the feed due to the hydrolysis of indigestible polysaccharides. A possibility is also that there was improved protein digestibility due to a contaminating protease in the NSPase used, similar to that reported by Wu et al. (2004), who found decreased viscosity in the digesta of broiler chickens fed a diet with digesta, and ascribed this to a contaminating NSPase in the phytase product.

Hydrothermal treatment during SSF may have been of importance, since growth and feed conversion of the tilapia fed the SBM diet became more efficient than for the fish fed the FRSM diet when the diets were extruded. In this scenario SSF also resulted in poorer growth and feed conversion that in the fish fed RSM. Under the current design it is not possible to separate the effects of extrusion from the differences seen in the two fermentations.

The crude protein contents of the experimental diets of 386-406 g $(kg\ DM)^{-1}$ (331-350 g digestible protein $(kg\ DM)^{-1}$) were higher than the 330 g crude protein and 280 g digestible protein $(kg\ feed)^{-1}$ recommended for tilapia feed by NRC (1993). This indicates that protein was in excess of requirement, as illustrated by the protein retentions ranging from 31 to 40%. These retention figures may, however, have been under estimated, since feed intake was only quantified by observation and not by quantification of uneaten feed (Helland et al., 1996). The higher efficiency of nitrogen retention of the extruded SBM diet than the ones with RSM and FRSM is rationalized by a combination of amino acid digestibility and EAA profile. When comparing the EAA profiles in tilapia with those in feed and digested, methionine was the first limiting in the SBM diet, followed by lysine and threonine (Table 12). Lysine was first limiting in RSM and FRSM, followed by methionine. All diets contained excess of digestible cysteine compared to the contents in the body, meaning that conversion of methionine to cysteine probably was down-regulated. The retention of

563 EAA into growth, in spite of lacking opportunity to carry out statistical analysis, supports that
564 methionine was first limiting EAA in the SBM diet, and lysine was most efficiently used for growth
565 in the in the RSM and FRSM diets. The fact that more than half of all EAA were metabolized after
566 absorption, confirms that dietary protein was in excess. Thus, the differences in growth and feed
567 conversion seen in the present experiment can't be ascribed to essential amino acid deficiency.
568
569 Feed intake seemed to be a main explanation for the differences in intestinal weights in the
570 experiment with moist diets. Intestinal weight was inversely related to feed intake (IW=84.5-1.05 FI,
571 $R^2$=0.68; IW, intestinal weight g $kg^{-1}$ fish; FI, feed intake, g $fish^{-1}$). No clear effect of feed intake
572 was seen for stomach size. Thus, it is probable that the smaller stomach in tilapia fed the moist SBM
573 diet than RSM and FRSM diets reflects dietary composition rather than quantitative feed intake. The
574 underlying cause for these effects needs further investigation, since no histological analyses were
575 carried out on these fish.
576
577 In conclusion, solid state fermentation reduced contents of glucosinolates and phytic acid, as well as
578 amino acids in rapeseed meal. Solid state fermentation of rapeseed meal improved the feed
579 conversion ratio when used in a moist diet, while it impaired the protein retention and feed
580 conversion ratio when used in an extruded diet. The differences in nutritional value in the two
581 experiments with Nile tilapia may partly be rationalized by differences in fermentation of the
582 rapeseed meal, and partly by the two methods of feed production used.
583

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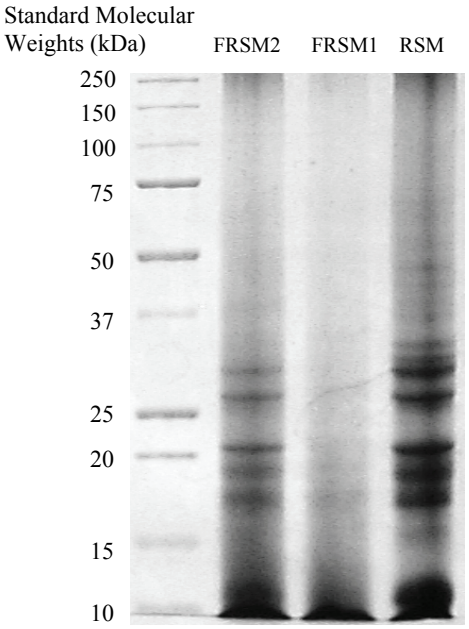


Figure 1.

SDS-PAGE of untreated rapeseed meal, first (FRSM1) and second (FRSM2) fermented rapeseed meals.

Table 1.

Characterization of untreated and fermented rapeseed meals

| | Rapeseed meal | | |
|---|---|---|---|
| | Untreated | 1st fermentation | 2nd fermentation |
| Dry matter (DM), g $kg^{-1}$ | 905 | 450 | 750 |
| DM recovery rate[1], *%* | | 96.0 | 96.2 |
| *In DM, $kg^{-1}$* | | | |
| Fat, g | 43.7 | 58.0 | 57.6 |
| Starch, g | 27.8 | 19.3 | 22.9 |
| Crude protein, g | 385.0 | 402.8 | 401.8 |
| Ash, g | 72.2 | 75.2 | 75.1 |
| Residue[2], g | 471.3 | 449.3 | 450.4 |
| Phytic acid (IP6), g | 35.8 | 10.5 | 19.2 |
| Phosporus from | | | |
| IP6, g | 9.1 | 2.7 | 5.0 |
| IP5, g | 0.7 | 0.3 | 0.2 |
| IP4, g | n.d. | 0.2 | 0.7 |
| IP3, g | n.d. | n.d. | n.d. |
| IP2, g | n.d. | 3.0 | 1.0 |
| Ergosterol, mg | 21.2 | 65.9 | 121.3 |
| Fungal biomass[3], g | 3.9 | 12.0 | 22.1 |
| pH | 5.81 | 4.52 | 4.84 |

[1]The calculation is based on the principle that the total ash amount is constant during solid state fermentation (RuizTeran and Owens, 1996).

[2](1000-(fat+starch+crude protein+ash))

[3] Estimated based on an ergosterol content of 5.5 mg $g^{-1}$ fungal biomass (Charcosset and Chauvet, 2001; Gessner and Chauvet, 1993).

n.d., below detection limit

853 Table 2.

854 Essential amino acids, cyst(e)ine, non-essential and total amino acids in untreated and fermented rapeseed

855 meals[1]

| | Rapeseed meal | | |
|---|---|---|---|
| | Untreated | 1st fermentation | 2nd fermentation |
| *g (100g CP)$^{-1}$* | | | |
| Arg | 5.23 | 4.38 | 4.58 |
| His | 2.32 | 2.07 | 2.16 |
| Ile | 3.52 | 3.38 | 3.34 |
| Leu | 5.84 | 5.45 | 5.47 |
| Lys | 4.66 | 3.99 | 4.26 |
| Met | 1.67 | 1.52 | 1.54 |
| Phe | 3.44 | 3.17 | 3.20 |
| Thr | 3.87 | 3.66 | 3.66 |
| Val | 4.35 | 4.20 | 4.16 |
| Cys | 1.90 | 1.82 | 1.73 |
| Total non-essential amino acids | 41.4 | 38.9 | 38.5 |
| Total amino acids[2] | 76.3 | 70.7 | 70.8 |

856 [1]Presented in dehydrated form.

857 [2]Without tryptophan.

858 Table 3

859 Content of glucosinolates in untreated and fermented rapeseed meals

| | Rapeseed | | |
|---|---|---|---|
| | Untreated | 1st fermentation | 2nd fermentation |
| *µg (g DM)*$^{-1}$ Progoitrin | 919.0 | t.a. | t.a. |
| Epi-progoitrin | 54.9 | n.d. | n.d. |
| Gluconapin, | 288.8 | n.d. | n.d. |
| Gluconapoleiferin | 58.6 | n.d. | n.d. |
| 4-OH-Glucobrassicin | 270.1 | n.d. | n.d. |
| Glucobrassicanapin | 79.5 | n.d. | n.d. |
| Glucobrassicin | 80.21 | n.d. | n.d. |
| Gluconasturtiin | 78.7 | n.d. | n.d. |

860 t.a., trace amounts; n.d., below detection limit,

Table 4.

Diet formulation and chemical composition

| | Moist diets | | | Extruded diets | | |
|---|---|---|---|---|---|---|
| | SBM diet | RSM diet | FRSM diet | SBM diet | RSM diet | FRSM diet |
| Dry matter (DM), g $kg^{-1}$ | 572.9 | 583.7 | 572.3 | 909.1 | 938.5 | 942.5 |
| *Ingredients, g (kg DM)$^{-1}$* | | | | | | |
| Fish meal [a] | 261.9 | 261.9 | 267.4 | 270.8 | 270.8 | 278.5 |
| Soybean meal [b] | 373.2 | | | 385.9 | | |
| Rapeseed meal [c] | | 501.8 | | | 518.9 | |
| Fermented rapeseed meal[d] | | | 491.4 | | | 505.2 |
| Gelatinised potato starch [e] | 256.3 | 147.0 | 150.1 | | | |
| Native potato starch [f] | | | | 265.0 | 152.0 | 156.3 |
| Alginate [g] | 33.0 | 32.8 | 33.5 | | | |
| Soy oil [h] | 70.1 | 50.8 | 51.8 | 72.5 | 52.5 | 54.0 |
| Vitamin and mineral premix [i] | 5.493 | 5.469 | 5.584 | 5.680 | 5.655 | 5.815 |
| Yttrium oxide [j] | 0.1099 | 0.1094 | 0.1117 | 0.1136 | 0.1131 | 0.1163 |
| *Chemical composition, g (kg DM)$^{-1}$* | | | | | | |
| Crude protein | 384.3 | 407.1 | 405.5 | 385.9 | 406.2 | 406.3 |
| Lipid | 89.9 | 94.1 | 95.1 | 90.0 | 90.5 | 90.1 |
| Starch | 270.0 | 158.1 | 155.7 | 294.8 | 167.2 | 173.4 |
| Ash | 68.7 | 79.8 | 83.9 | 62.3 | 78.4 | 79.0 |
| Phosphorus (P) | | | | 8.7 | 12.0 | 12.9 |
| Magnesium (Mg) | | | | 2.0 | 3.5 | 3.6 |
| Zinc (Zn) | | | | 0.18 | 0.19 | 0.20 |
| pH | 6.4 | 6.1 | 5.1 | 6.5 | 6.1 | 5.2 |

[a] NorsECO-LT, Norsildmel, Egersund, Norway.

[b] Denosoy, extracted and toasted soybean meal, with hulls, Denofa, Fredrikstad, Norway.

[c] ExPro-00E produced from double-low rapeseed in Sweden.

[d] Fermented rapeseed used for moist diet was from the first-time fermentation; 87.8% fermented rapeseed meal used for extruded diet was from the second-time fermentation and the rest was from the first-time fermentation. [e] Gelatinised potato starch, Lygel F 60, (moisture ≤ 90 g $kg^{-1}$; fat 1 g $kg^{-1}$; protein 1 g $kg^{-1}$; viscosity 1000 cP at 5% concentration), Lyckeby Culinar, Fjälkinge, Sweden.

[f] Native potato starch, Lyckeby Culinar, Fjälkinge, Sweden.

[g] Protanal® LF 20 alginate, FMC BioPolymer A/S, Drammen, Norway.

[h] Egersund sildoljefabrikk, Egersund, Norway.

873 [i] Contents per kg: Vitamin A 2500.0 IU; Vitamin $D_3$ 2400.0 IU; Vitamin E 0.2 IU; Vitamin $K_3$ 40.0 mg;
874 Thiamine 15.0 mg; Riboflavin 25.0 mg; d-Ca-Pantothenate 40.0 mg; Niacin 150.0 mg; Biotin 3.0 mg;
875 Cyanocobalamine 20.0 μg; Folic acid 5.0 mg; Pyridoxine 15.0 mg; Vitamin C: 0.098 g (Stay-C 35, ascorbic
876 acid phosphate, DSM Nutritional Products, Basel, Switzerland); Cu: 12.0 mg; Zn: 90.0 mg; Mn: 35.0 mg; I:
877 2.0 mg; Se: 0.2 mg; Cd ≤ 3.0 μg; Pb ≤ 28.0 μg; total Ca: 0.915 g; total K 1.38 g; total Na 0.001 g; total Cl
878 1.252 g; Trouw Nutrition, LA Putten, The Netherlands.
879 [j] Metal Rare Earth Limited, Shenzhen, China.

880 Table 5

881 Amino acid profiles of the extruded diets[1]

| | SBM diet | RSM diet | FRSM diet |
|---|---|---|---|
| *g (100 g CP)$^{-1}$* | | | |
| Essential amino acids | | | |
| Arg | 6.06 | 5.15 | 5.01 |
| His | 2.32 | 2.13 | 2.15 |
| Ile | 4.14 | 3.53 | 3.66 |
| Leu | 6.86 | 6.02 | 6.19 |
| Lys | 6.31 | 5.62 | 5.40 |
| Met | 1.88 | 2.04 | 2.07 |
| Phe | 4.04 | 3.27 | 3.36 |
| Thr | 3.83 | 3.75 | 3.85 |
| Val | 4.36 | 4.17 | 4.36 |
| Non-essential amino acids | | | |
| Cys | 1.02 | 1.28 | 1.37 |
| Total non-essential amino acids | 43.9 | 38.4 | 39.9 |
| Total amino acids[2] | 84.7 | 75.3 | 77.3 |

882 [1]Presented in dehydrated form.

883 [2]Without tryptophan.

884 Table 6.

885 Growth performance of Nile tilapia fed moist diets

| | SBM diet | RSM diet | FRSM diet | Pooled s.e.m | P-value |
|---|---|---|---|---|---|
| Feed intake, g fish-$^{1}$ | | | | | |
| 0-13 days | 6.39$^{b}$ | 12.14$^{a}$ | 9.11$^{b}$ | 0.75 | 0.014 |
| 0-23 days | 15.06$^{b}$ | 27.29$^{a}$ | 20.16$^{b}$ | 1.45 | 0.010 |
| 0-37 days | 28.16$^{b}$ | 46.66$^{a}$ | 34.52$^{b}$ | 2.35 | 0.012 |
| Mean weight, g fish$^{-1}$ | | | | | |
| Day 0 | 7.58 | 7.97 | 7.93 | 0.21 | 0.43 |
| Day 13 | 13.32$^{b}$ | 16.56$^{a}$ | 16.36$^{a}$ | 0.7 | 0.052 |
| Day 23 | 20.85 | 25.65 | 25.24 | 1.25 | 0.094 |
| Day 37 | 30.67 | 35.74 | 36.66 | 1.35 | 0.067 |
| Mean weight increase, g fish$^{-1}$ | | | | | |
| 0-13 days | 5.74$^{b}$ | 8.59$^{a}$ | 8.43$^{a}$ | 0.53 | 0.033 |
| 0-23 days | 13.27 | 17.69 | 17.31 | 1.11 | 0.084 |
| 0-37 days | 23.09 | 27.78 | 28.73 | 1.19 | 0.056 |
| Specific growth rate (SGR) | | | | | |
| 0-13 days | 4.46$^{b}$ | 5.70$^{a}$ | 5.63$^{a}$ | 0.13 | 0.0041 |
| 0-23 days | 4.50 | 5.13 | 5.08 | 0.14 | 0.053 |
| 0-37 days | 3.84$^{b}$ | 4.10$^{a}$ | 4.19$^{a}$ | 0.06 | 0.036 |
| Feed conversion ratio, g DM intake (g body weight gain)$^{-1}$ | | | | | |
| 0-13 days | 1.10$^{b}$ | 1.41$^{a}$ | 1.09$^{b}$ | 0.06 | 0.044 |
| 0-23 days | 1.13$^{b}$ | 1.55$^{a}$ | 1.18$^{b}$ | 0.08 | 0.030 |
| 0-37 days | 1.22$^{b}$ | 1.69$^{a}$ | 1.20$^{b}$ | 0.07 | 0.016 |
| Tissue:whole body weight, g kg$^{-1}$ | | | | | |
| Liver | 30.9 | 26.1 | 24.1 | 1.8 | 0.12 |
| Intestine | 54.7$^{a}$ | 36.9$^{c}$ | 44.6$^{b}$ | 2.31 | <0.0001 |
| Stomach | 10.1$^{a}$ | 6.5$^{b}$ | 7.2$^{b}$ | 0.43 | <0.0001 |

886

887 Table 7.

888 Whole body composition of Nile tilapia fed moist diets

| | SBM diet | RSM diet | FRSM diet | Pooled s.e.m | P-value |
|---|---|---|---|---|---|
| Dry matter (DM), g $kg^{-1}$ | 282.2[b] | 306.4[a] | 305.6[a] | 2.92 | 0.016 |
| Crude protein, g $kg^{-1}$ | 148.2 | 153.3 | 151.1 | 1.1 | 0.10 |
| Fat, g $kg^{-1}$ | 98.5 | 113.8 | 112.5 | 3.83 | 0.12 |
| Ash, g $kg^{-1}$ | 26.9[c] | 30.1[b] | 33.2[a] | 0.56 | 0.010 |

889

890 Table 8.

891 Growth performance of Nile tilapia fed extruded diets

| | SBM diet | RSM diet | FRSM diet | Pooled S.E.M | P-value |
|---|---|---|---|---|---|
| Feed intake, g fish$^{-1}$ | | | | | |
| 0-21 days | 61.4 | 63.5 | 65.7 | 2.17 | 0.42 |
| 0-43 days | 130.9 | 142.3 | 134.4 | 7.19 | 0.55 |
| Mean weight, g fish$^{-1}$ | | | | | |
| Day 0 | 56.4 | 56.3 | 56.3 | 0.11 | 0.87 |
| Day 21 | 118.2$^{a}$ | 111.4$^{b}$ | 107.3$^{b}$ | 1.93 | 0.020 |
| Day 43 | 177.8 | 172.5 | 156.7 | 7.01 | 0.17 |
| Mean weight increase, g fish$^{-1}$ | | | | | |
| 0-21 days | 61.9$^{a}$ | 55.1$^{b}$ | 51.0$^{b}$ | 1.90 | 0.019 |
| 0-43 days | 121.4 | 116.1 | 100.3 | 6.96 | 0.17 |
| Specific growth rate (SGR) | | | | | |
| 0-21 days | 3.52$^{a}$ | 3.24$^{ab}$ | 3.07$^{b}$ | 0.08 | 0.020 |
| 0-43 days | 2.66 | 2.60 | 2.38 | 0.09 | 0.15 |
| Feed conversion ratio, g DM intake (g body weight gain)$^{-1}$ | | | | | |
| 0-21 days | 0.99$^{c}$ | 1.15$^{b}$ | 1.29$^{a}$ | 0.02 | 0.0001 |
| 0-43 days | 1.08$^{c}$ | 1.23$^{b}$ | 1.34$^{a}$ | 0.02 | 0.0005 |
| Tissue:whole body weight, g kg$^{-1}$ | | | | | |
| Liver | 26.8 | 27.8 | 22.4 | 10.0 | 0.72 |
| Intestine | 46.1 | 38.9 | 42.7 | 7.1 | 0.78 |
| Stomach | 4.2$^{a}$ | 4.4$^{b}$ | 4.4$^{b}$ | 0.2 | 0.020 |
| Condition factor | | | | | |
| Day 43 | 2.19 | 2.20 | 2.17 | 0.03 | 0.70 |

892

893 Table 9.

894 Whole body composition of Nile tilapia fed extruded diets

| | Initial | SBM diet | RSM diet | FRSM diet | Pooled S.E.M | P-value |
|---|---|---|---|---|---|---|
| *g kg$^{-1}$* | | | | | | |
| Dry matter | 258.9$^{b}$ | 350.7$^{a}$ | 348.7$^{a}$ | 346.05$^{a}$ | 3.46 | <0.0001 |
| Fat | 69.2$^{b}$ | 146.1$^{a}$ | 145.6$^{a}$ | 141.2$^{a}$ | 5.00 | <0.0001 |
| Ash | 33.8 | 33.6 | 34.0 | 35.3 | 0.83 | 0.50 |
| Crude protein | 148.8$^{b}$ | 160.6$^{a}$ | 162. 6$^{a}$ | 160.7$^{a}$ | 2.68 | 0.024 |
| *g (100 g CP)$^{-1}$* | | | | | | |
| Essential amino acids[1,2] | | | | | | |
| Arg | 5.52 | 5.44 | 5.46 | 5.51 | | |
| His | 2.07 | 2.23 | 2.24 | 2.25 | | |
| Ile | 3.33 | 3.31 | 3.31 | 3.35 | | |
| Leu | 5.73 | 5.75 | 5.8 | 5.79 | | |
| Lys | 6.31 | 6.35 | 6.46 | 6.5 | | |
| Met | 2.1 | 2.13 | 2.15 | 2.15 | | |
| Phe | 3.12 | 3.09 | 3.11 | 3.13 | | |
| Thr | 3.72 | 3.69 | 3.74 | 3.75 | | |
| Val | 3.81 | 3.8 | 3.82 | 3.84 | | |
| Cys | 0.81 | 0.79 | 0.81 | 0.8 | | |
| Total AA[2] | 77.7 | 77.2 | 77.6 | 78.2 | | |

895 [1]Triplicate samples were pooled.

896 [2]Trp was not analysed.

897 Table 10.

898 Apparent digestibility of mineral elements and nitrogen, and retention of nitrogen in Nile tilapia fed the

899 extruded diets

| | SBM diet | RSM diet | FRSM diet | Pooled S.E.M. | P value |
|---|---|---|---|---|---|
| *Apparent digestibility coefficient, %* | | | | | |
| P | 61.5 | 49.2 | 60.3 | 3.72 | 0.11 |
| Mg | 70.2[b] | 37.6[a] | 61.6[b] | 4.06 | 0.0033 |
| Zn | 26.7 | 17.6 | 18.3 | 2.81 | 0.11 |
| Nitrogen | 90.6 | 84.5 | 81.6 | 2.54 | 0.11 |
| *Nitrogen retention, %* | | | | | |
| of dietary N | 40.1[a] | 34.2[b] | 31.0[c] | 0.64 | 0.0002 |
| of digested N | 44.2 | 40.5 | 38.2 | 1.83 | 0.14 |

900

901 Table 11.

902 Apparent digestibility coefficients of essential amino acids, cyst(e)ine and total amino acids in Nile tilapia fed

903 the extruded diets

| | SBM diet[1] | FRSM diet[2] | Pooled S.E.M | P value | RSM diet[3] |
|---|---|---|---|---|---|
| Arg | 95.1 [b] | 90.9 [a] | 1.06 | 0.004 | 91.7 |
| His | 91.9 [b] | 87.7 [a] | 1.17 | 0.039 | 87.8 |
| Ile | 92.2 [b] | 87.2 [a] | 1.34 | 0.028 | 86.2 |
| Leu | 91.1 [b] | 87.0 [a] | 1.12 | 0.041 | 85.4 |
| Lys | 92.9 [b] | 88.7 [a] | 1.11 | 0.02 | 89 |
| Met | 91.9 | 90.3 | 0.65 | 0.27 | 89.9 |
| Phe | 91.7 [b] | 86.4 [a] | 1.39 | 0.021 | 85.5 |
| Thr | 89.6 [b] | 84.6 [a] | 1.39 | 0.037 | 82.9 |
| Val | 91.2 [b] | 86.9 [a] | 1.19 | 0.043 | 85.6 |
| Cys | 87.1 | 83.1 | 1.34 | 0.15 | 82.2 |
| Total AA[4] | 92.1 [b] | 87.8 [a] | 1.16 | 0.031 | 87.2 |

904 [1] Means of triplicate samples.

905 [2] Means of duplicate samples.

906 [3] Result of one sample pooled from three replicate tanks, not included in the analysis of variance.

907 [4] Not including tryptophan.

908 Table 12.

909 Individual essential amino acids[1] (EAA) in percent of total EAA[1] in published requirements, in whole tilapia
910 body, experimental diets, mean values for apparently digested EAA by the fish, and retention of apparently
911 digested EAA in Nile tilapia

| EAA | Arg | His | Ile | Leu | Lys | Met | Phe | Thr | Val |
|---|---|---|---|---|---|---|---|---|---|
| Reference EAA source | | | | | | | | | |
| NRC 1993, % of total EAA | 13.8 | 5.6 | 10.2 | 11.1 | 16.8 | 8.8 | 12.3 | 12.3 | 9.2 |
| Tilapia whole body EAA, % of total EAA | 15.3 | 6.1 | 9.2 | 16.1 | 17.8 | 6.1 | 8.6 | 10.3 | 10.6 |
| Dietary EAA, % of total EAA | | | | | | | | | |
| SBM diet | 15.2 | 5.8 | 10.4 | 17.2 | 15.9 | 4.7 | 10.2 | 9.6 | 11.0 |
| RSM diet | 14.4 | 6.0 | 9.9 | 16.9 | 15.8 | 5.7 | 9.2 | 10.5 | 11.7 |
| FRSM diet | 13.9 | 6.0 | 10.2 | 17.2 | 15.0 | 5.7 | 9.3 | 10.7 | 12.1 |
| Apparently digested EAA in tilapia fed, % of total EAA | | | | | | | | | |
| SBM diet | 15.7 | 5.8 | 10.4 | 17.1 | 16.0 | 4.7 | 10.1 | 9.4 | 10.9 |
| RSM diet | 15.2 | 6.0 | 9.8 | 16.5 | 16.1 | 5.9 | 9.0 | 10.0 | 11.5 |
| FRSM diet | 14.4 | 6.0 | 10.1 | 17.0 | 15.1 | 5.9 | 9.2 | 10.3 | 12.0 |
| Retention of EAA in tilapia, % of apparently digested EAA intake | | | | | | | | | |
| SBM diet | 37.4 | 42.9 | 34.5 | 36.8 | 43.3 | 48.5 | 33.1 | 42.7 | 38.0 |
| RSM diet | 39.0 | 41.9 | 36.8 | 38.4 | 44.2 | 39.3 | 37.6 | 40.9 | 36.3 |
| FRSM diet | 37.2 | 38.1 | 32.4 | 32.4 | 42.3 | 35.0 | 33.2 | 35.5 | 31.3 |

912 [1]tryptophan not included

# PAPER III

# Fermentation of rapeseed meal, and supplementation with sodium butyrate and formate in diets for Nile tilapia (*Oreochromis niloticus*)

Youling Gao[1, 3], Dilip Chowdhury[1], Jon Fredrik Hanssen[2], Hans Magnus Gjøen[1], Liv Torunn Mydland[1], Margareth Øverland[1], Trond Storebakken[1*]

[1] Aquaculture Protein Centre, CoE, Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences (UMB), NO-1432 Ås (Norway).

[2] Department of Chemistry, Biotechnology and Food Science, UMB, NO-1432 Ås (Norway)

[3] College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo, Zhejiang, P.R. China

[*] Corresponding author: Address: Department of Animal and Aquacultural Sciences, UMB, P.O. Box 5003, NO-1432 Ås (Norway) e-mail: trond.storebakken@umb.no

**Abstract**

An experiment with Nile tilapia was conducted to evaluate the effects of supplementing an organic acid salt blend (18 g $kg^{-1}$ of a 2:1 blend of sodium formate and butyrate) to the diets, and solid state fermentation (SSF) of rapeseed meal, on apparent nutrient digestibilities, growth performance, and whole body composition. Four diets were prepared in accordance with a 2×2×2 factorial design, the factors being without and with supplementation of organic acid salts, without or with solid state fermentation of rapeseed meal, and small (100 g start weight) vs. large (224 g) tilapia. Each diet was fed to apparent satiation to two tanks of each fish size group for 45 d. The results showed that supplementing diets with organic acid salts did not have any effects except increasing the stomach weight in percentage of body weight. SSF of rapeseed meal significantly reduced the feed intake and

growth, liver weight in percentage of body weight, increased stomach weight, and increased feed conversion ratio. SSF also significantly reduced dry matter, crude fat and increased ash contents of whole fish body, but did not significantly influence the digestibility of nutrients or retention of nitrogen and phosphorus.

*Key words:* Nile tilapia; Solid state fermentation; Sodium formate and butyrate; Rapeseed meal; Growth; Apparent digestibility.

## 1. Introduction

Rapeseed meal (RSM) has potential to be used as source of protein in diets for fish without compromising performance (Higgs et al., 1982; Davies et al., 1990; Burel et al., 2000; 2001; Glencross et al., 2004; Shafaeipour et al., 2008). However, the use of RSM is limited by its content of glucosinolates and phytic acid (IP6). Glucosinolates may be hydrolyzed by the enzyme myrosinase which is present both in rapeseed and in the intestinal microflora, releasing toxic metabolites, including isothiocynates, nitriles, thiocyanates or oxazolidithione (Cheng et al., 2004; Fenwick et al., 1982; Mithen et al., 2000). These metabolites are known to affect the growth and thyroid function of fish (Burel et al., 2000; Glencross et al., 2004). Phosphorus (P) in IP6 is not digestible by monogastric animals, and strongly binds essential cations such as zinc, calcium, magnesium and iron. High dietary concentration of IP6, thus, may contribute to deficiencies in P and trace elements, and eutrofication of freshwater due to high faecal excretion of P.

Solid state fermentation (SSF) may improve in the nutritional value of RSM by breakdown of anti-nutrients through microbial metabolism (Bau et al., 1994; El-Batal and Karem, 2001; Nair and Duvnjak, 1991; Rozan et al., 1996; Vig and Walia, 2001). Our previous experiment also showed that

50 the contents of glucosinolates and phytic acid in rapeseed meal were reduced by SSF (Gao et al.,
51 manuscript).
52
53 Our previous results (Gao et al., manuscript) also showed that the pH of RSM was reduced by SSF.
54 It is well known that the fungus *Aspergillus niger* used in our SFF produces significant amounts of
55 organic acids, especially citric and oxalic acids (Van de Merbel et al., 1994; Schrickx et al., 1995;
56 Cameselle et al., 1998; Krishna, 2005). Dietary citric acid has resulted in improved growth in hybrid
57 tilapia (*O. Niloticus x O. aureus*) (Pan et al., 2004). The effects of organic acids and their salts in fish
58 nutrition can be summarized into several main categories (Reviewed by Lückstädt, 2008a). Organic
59 acids may reduce the pH in the digestive system and thereby affect the composition of the microflora
60 (reviewed by Freitag, 2008) and improve the availability of essential mineral elements such as
61 phosphorus (Hossain et al., 2007; Sarker et al., 2007). Essential cationic elements such as zinc,
62 however, binds strongly to oxalic acid (Sayer and Gadd, 2001), and this metabolite from SSF by *A.*
63 *Niger* may contradict positive effects of reduced pH on mineral uptake. Dietary organic acid salts
64 may improve nutrient digestibilities by acting in hydrothermic feed processing such as extrusion
65 (Morken et al., 2011). Especially organic acid salts which can dissociate have antimicrobial effects in
66 the digestive tract (Reviewed by Partanen and Mroz, 1999). Organic acids such as butyrate are also
67 preferred energy substrate for renewal of intestinal epithelium (Topping and Clifton, 2001), and our
68 previous results (Gao et al., 2011) have shown that intestinal weights were enlarged when rainbow
69 trout were fed diets with a blend of sodium formate and butyrate. Furthermore, we observed a similar
70 increase in intestinal weight when Nile tilapia was fed a diet with fermented RSM compared to
71 untreated RSM (Gao et al., manuscript). Thus, the present experiment aimed to determine the effect
72 of salts of organic acids in diets containing untreated or fermented RSM on growth performance,
73 nutrient digestibility and whole body composition.
74

## 2. Materials and methods

### *2.1 Solid sate fermentation*

SSF of RSM with *Aspergillus niger* ATCC 10577 was initiated by the production of a seed culture with 2 kg RSM in 2 l water as described by Gao et al. (manuscript). The SSF was carried out in 40 l twin shaft paddle mixer (IDE-CON AS, Porsgrunn, Norway). An amount of 25 kg RSM and 25 l tap water were mixed homogenously before the seed was added. The medium was then incubated at 30 ºC for 48 hours, and stirred frequently during incubation. Before being used in diets, the fermented rapeseed meal (FRSM) was stored at -20 ºC.

### *2.2 Diet preparation*

Four diets were prepared based on a 2×2×2 factorial design where the factors were: Organic acid salts; SSF (Table 2); and fish size groups. Diet 1 was prepared with untreated RSM; Diet 2 was made with RSM with a 2:1 mixture of sodium formate and butyrate (organic acid salt blend, OAB) added; Diet 3 was prepared with FRSM; Diet 4 was based on FRSM with the OAB added.

All diets were formulated to be iso-nitrogenous and contain the same amount of lipids based on prior analyses of RSM and FRSM. Sodium alginate was used as the binder. All dry ingredients were mixed in the IDE-CON mixer prior to adding water and lipid. The diets then were shaped using a Pressa P35 A pasta extruder (Italgi Srl, Genova, Italia), equipped with a cutter to produce moist pellet with a length of 3-4 mm, and dried at 65 $^{\circ}$C until the moisture content was 40 g $kg^{-1}$.

### *2.3 Fish experiment*

The fish experiment was conducted at the tilapia nutrition laboratory of the Norwegian University of Life Sciences, Ås. Norway (UMB). Nile tilapia was hatched from Genomar Supreme Tilapia (GST) and raised at UMB. The GST is the breeding strain from Genomar, and is originated from the

100 genetically improved farmed tilapia (GIFT) project. Before the experiment started, all fish were fed a
101 basal diet based on fishmeal and soybean meal. Two size groups of Nile tilapia were used, with an
102 average start weight of 99.6 and 224.0 g, respectively. The smallest fish were distributed in eight
103 fibre-glass tanks with 115 l water volume, while the larger ones went into eight tanks with 210 l
104 volume. Each tank was stocked with 20 fish. The tanks were covered by a transparent lid and had
105 continued light (24 h). The experiment lasted for 45 d. The tanks were supplied with recycled
106 freshwater with an average temperature of 25ºC and average dissolved oxygen level at 6.1 mol $l^{-1}$,
107 which were measured daily after the first feeding. The water flow was 8 l $min^{-1}$. Each diet was
108 assigned randomly to the fish in two small and two big tanks. Fish were fed by hand to apparent
109 satiety three times (9 am, 2 pm and 8 pm) from day 1 to 7. After that, the feeding was twice daily (9
110 am and 2 pm). Apparent feed consumption in each tank was recorded visually for each feeding.
111 Uneaten feed was collected immediately after each meal, and was only observed in the large tanks
112 after 15 days.
113
114 *2.4 Sampling*
115 Fish were weighted at the start, day 25, and at the end of the experiment. Two samples of small fish
116 and two samples of big fish, five fish in each sample, were randomly taken from the holding tank for
117 initial whole body composition. At the end of the experiment, 10 fish $tank^{-1}$ were anaesthetized with
118 MS 222 (0.2 g $l^{-1}$), and individual weight and length were measured before the abdominal cavity of
119 fish was cut open. The weight of stomach, liver, middle and distal intestines were taken from first
120 five fish. After the faecal content of the last 5 cm part of the distal intestine was collected from five
121 fish, the fish were used for analysis of whole-body chemical composition. The rest of the fish were
122 bulk-weighted and then the faecal content in the last 5 cm of the distal intestine was collected.
123

124 *2.5 Sample preparation and chemical analysis*

125 FRSM was freeze dried before homogenization in a A11 Basic Analytical Mill (IKA, Wilmington,
126 USA) and stored at 4 ºC. The faecal samples were stored at -20°C immediately after collection until
127 freeze-drying. The dry matter content of diets and freeze-dried faeces was determined at 105ºC, ash
128 by combustion at 550ºC, both until stable weight. Crude protein (Kjeldahl N×6.25) in RSM, FRSM
129 and feed was determined by using a Kjeltec auto 1035/1038 system (Tecator, Sweden). Nitrogen in
130 faeces was determined with the Dumas method (Kirsten and Hesselius, 1983) by using an EA 1108
131 element analyzer system (Uppsala, Sweden). Crude lipid was determined by an Accelerated Solvent
132 Extractor (ASE 200) from Dionex (Danvers, MA, USA). Starch was analyzed as glucose after starch
133 hydrolysis with a heat tolerant amylo-glucosidase in accordance with McCleary et al. (1994). Amino
134 acids, except tryptophan, were analysed according to EC (1998) by using a Biochrom 30 Amino
135 Analyser (Biochrom Ltd., Cambridge, UK). Formic acid and butyric acid analysis were conducted at
136 Eurofins, Moss, Norway. The samples were prepared by milling, homogenization, dilution and
137 mixing with water. The prepared sample was filtrated through a 4 micron filter prior to injection to a
138 liquid chromatography system. The separation was carried out on a cation exchange column
139 designed for separation of organic acids (Nulceogel Ion 300 OA, Macherey-nagel) at 50°C. Formic
140 acid was detected with UV (205nm) and butyric acid was detected by RI-detector. Total phosphorus
141 in the fish was analyzed by combusting the sample at 550°C until stable weight, dissolving in 1M
142 HCl, and quantification was done spectrophotometrically (Bourke and Yanagawa, 1993). Yttrium,
143 potassium, magnesium, phosphorus, and zinc in diets and faeces was determined by ICP-MS after
144 complete digestion of the homogenized and dried samples in $HNO_3$ and cooking in a micro-wave for
145 1 h. Ergosterol was determined by HPLC as described previously (Gao et al., manuscript).
146

147 *2.6 Calculation and statistics*

148 Specific growth rate (SGR) was calculated as: $G_w = \frac{\ln W_1 - \ln W_0}{T}$, where $W_1$ is the fish weight at the
149 end of the study; $W_0$ is the fish weight at the start of the experiment; and T is the interval in days.
150 Feed conversion ratio (FCR) was calculated as: $FCR = F \times G^{-1}$, where F is dietary dry matter intake
151 and G represents the weight gain. Apparent digestibility of nitrogen and amino acids were assessed
152 by the indirect method with $Y_2O_3$ as an inert marker (Austreng, et al., 2000). The calculation was
153 made as following: $100 \times (1-(D_i/F_i \times F_n/D_n))$, where D represents diet, F is faeces, and the
154 superscripts $_i$ and $_n$ are concentrations of $Y_2O_3$ and nutrient. Condition factor was calculated as: 100×
155 whole body weight (g) × body length $(cm)^{-3}$. Nutrient retention, percentage of gross or digestible
156 nutrient intake, was calculated as: $N_R = 100 \times (N_1-N_0)/N_i$, where $N_0$ and $N_1$ represent whole-body
157 nutrient content at the beginning and end of the experiment, and $N_i$ is gross or digestible nutrient
158 intake during experiment.
159
160 *2.7 Statistical analysis*
161 The results were analyzed using the GLM procedure in SAS (1999). The effects of OAB, SSF and
162 initial fish weight were analyzed by a 2x2x2 factorial analysis of variance. Significant ($P<0.05$)
163 interactions were ranked by the P-diff procedure in SAS. The results are presented as least-square
164 means and pooled standard errors of the means (S.E.M.).
165
166 **3. Results**
167 *3.1 Characteristics of fermented rapeseed meal*
168 Chemical composition and amino acid profiles of RSM and FRSM are given in Table 1. The FRSM
169 contained higher amount of crude protein, and fat, but, the starch content was lower in FRSM
170 compared with untreated RSM. The ash content was similar between the two rapeseed meals. The

171 fungal biomass was increased from 2.73 to 5.07 g (kg DM)$^{-1}$ after SSF. Moreover, SSF did not
172 influence the amino acid profile in rapeseed meal.
173
174 *3.2 Diet properties*
175 The chemical composition of the experimental diets is given in Table 2. All the diets contained
176 similar levels of crude protein and crude fat. The butyrate and formate contents in diets 2 and 4 were
177 lower than the expected level (acid moiety recovery rate: 82.3 and 80.1% for formic acid and 75.3
178 and 75.6% for butyric acid).
179
180 *3.3 Fish growth*
181 The results (Table 3 and 4) showed that adding the OAB to the feed did not significantly affect the
182 fish growth, feed intake, FCR and condition factor. The middle intestine, distal intestine and liver
183 weight percentages also were not influenced. However, the stomach weight percentage of fish fed the
184 diets containing 1.8% OAB (0.38) was significantly higher than that of fish fed the diets without
185 organic acid salts (0.34) (Table 4).
186
187 The SSF significantly affected the fish growth, feed intake and FCR, as well as the stomach and liver
188 weight in percentage of body weight (Table 3 and 4). Fish fed the diets based on RSM had
189 significantly higher mean weight at day 24 and end of the experiment, weight gain, SGR, and feed
190 intake, as compared to the fish fed the diets based on FRSM. In contrast, the FCR in the fish fed the
191 diets based on FRSM was significantly better than that in the fish fed the diet based on RSM. In
192 addition, the stomach weight in percentage of body weight was significantly lower, and the liver
193 weight in percentage of body weight was significantly higher in fish fed the diets based on RSM than
194 the fish fed the diets based on FRSM.
195

196 The start weight of fish had significant influence on a wide range of growth parameters (Table 3 and
197 4). Fish with a higher start weight had higher values of weight gain, SGR, feed intake and FCR that
198 fish starting at a lower weight. However, the SGR, stomach and middle intestine weight in the lower
199 weight fish was higher than those in the higher weight fish.
200
201 *3.4 Body composition*
202 Adding the OAB to the diets did not affect the whole body composition, but the SSF reduced the
203 body dry matter, crude fat and increased ash contents significantly (Table 3 and 4). The fish with
204 higher start weight showed higher contents of dry matter, crude fat and ash than in tilapia with lower
205 start weight. There was an interaction between the effects of OAB and fermentation for whole body
206 ash content (Table 3 and 6). Adding OAB to diet based on RSM increased the ash content from 37.2
207 to 40.2 $g\ (kg)^{-1}$. Whereas, adding OAB to the diet based on FRSM almost did not change ash content
208 (from 37.5 to 37.4 $g\ (kg)^{-1}$)(Table 6).
209
210 *3.5 Digestibility and retention*
211 Neither dietary OAB nor SSF influenced the digestibility of nitrogen, amino acids, or nitrogen
212 retention (Table 7 and 8). The start weight of fish significantly affected the digestibility of total
213 amino acids and all individual amino acids, and tended ($P = 0.052$) to affect the digestibility of
214 nitrogen.
215
216 The digestibilities of phosphorus, zinc and magnesium, as well as phosphorus retention were not
217 affected by either OAB or SSF, but the start weight of fish significantly affected the digestibility of
218 phosphorus and zinc, as well as retention of dietary phosphorus (Table 9 and 10). Moreover, an
219 interaction between SSF and start weight on magnesium digestibility was observed (Table 9 and 11).

220 SSF treatment to RSM increased magnesium digestibility from 55.8 to 70.8% for small weight fish,
221 but reduced magnesium digestibility from 73.3 to 63.4% for large weight fish (Table 11).
222
223 **4. Discussion**
224 The contents of crude protein and crude fat in the FRSM were higher than that in RSM. Our previous
225 results has proved that the recovery rate of crude protein in a SSF with *Aspergillus niger* ATCC
226 10577 was 100% (Gao et al., manuscript). Thus, the increased crude protein content in the FRSM by
227 SSF was a result of the reduction of other nutrients, as the results showed that the starch content in
228 RSM was reduced by 24% after SSF. This is in agreement with the finding from Rozan et al. (1996).
229 Besides, part of other carbohydrates may be consumed by *A. niger*, and it has been confirmed in our
230 previous results (Gao et al., manuscript). Rozan et al. (1996) concluded that hydrolysis of non-starch
231 polysaccharides (NSP) occurred during SSF, but that sugars are the preferred energy substrate for *A.*
232 *niger*. The marginal reduction of both essential and non-essential amino acids relative to total
233 nitrogen also indicated that some of the amino acids in RSM was metabolised to other nitrogen-
234 containing components during SSF. Nucleic acid synthesis (Muhammad and Oloyede, 2009) and
235 germination of conidiospores in *A. niger* (Abdelrahim and Arbab, 1985) have been suggested as
236 main reasons for loss of amino acids during fermentation with this fungi. The loss of formate and
237 butyrate was also observed in our previous study (Gao et al., 2011), and may be due to evaporation
238 during drying of the feed.
239
240 The addition of the OAB to the diets did not influence the fish growth, feed intake and feed
241 conversion ratio of Nile tilapia. The lack of improvement in feed intake and growth is in agreement
242 with our previous findings with rainbow trout (Gao et al., 2011) and with results obtained by feeding
243 comparable doses of potassium diformate to Atlantic salmon (Lückstädt, 2008b). However, the
244 results did not demonstrate the same positive effect of adding organic acids or their salts to fish feeds

245 as in previous trials with Arctic charr (Ringø, 1991), rainbow trout (deWet, 2005), Red sea bream
246 (Hossain et al., 2007; Sarker et al., 2007; Sarker et al., 2005) and tilapias (Ramli et al., 2005; Zhou et
247 al., 2009). The lack of improvement in feed conversion when the diets were supplemented with OAB
248 is contrast to previous studies with rainbow trout (Pandey and Satoh, 2008; Gao et al., 2011) and
249 hybrid tilapia (Ng et al., 2009). The reasons for the conflicting results concerning weigh gain, feed
250 intake and feed conversion between previous studies and the present experiment may be due to the
251 type of organic acid or salt used and dietary inclusion level (deWet, 2005; Ringø, 1991), diet
252 composition (Sarker et al., 2007), fish species and age (Gislason et al., 1994), fish rearing conditions
253 (Ramli et al., 2005), and feed processing (Gao et al., 2011).
254
255 The increased stomach weight percentage in the tilapia fed dietary OAB may be attributed to the
256 sodium butyrate. Butyrate has previously been shown to increase gastric mucosa thickness, stimulate
257 more cells to differentiate into enteroendocrine cells, and increase the number of parental cells per
258 gland in stomach of weaned pigs (Mazzoni et al., 2008). However, the same OAB fed to rainbow
259 trout did not increase the stomach weight (Gao et al., 2011), indicating a species difference between
260 tilapia and trout.
261
262 SSF negatively influenced growth, feed intake and feed converstion of the tilapias, in keeping with
263 our previous results (Gao et al., manuscript). The reduced feed intake may be ascribed to the
264 production of citric acid, oxalic acid, and ethanol (Abouzied and Reddy, 1986; Nakamura et al.,
265 1996), or other organic components produced during SSF. No analysis of specific toxins was done in
266 the present experiment, so factors like mycotoxin generation (Lim et al., 2001), or production of
267 potent toxins by hydrolysis of glucosinolates (review by Bell, 1984), cannot be ruled out.
268

The liver weight was reduced by the fermentation, in keeping with our previous observations (Gao et al., manuscript). This may be a result of degradation of glucosinolates during SSF, shown previously (Gao et al., manuscript), since increased dietary intake of glucosinolates generally will result in increased liver size (Duncan, 1991).

Whole fish body composition was not changed by adding the OAB to the diets, but was changed by SSF treatment. The change may be related to different final fish body weight caused by SSF treatment. This was proved by a correlation between final body weight and dry matter ($r^2$=0.71; p=0.031) or crude fat ($r^2$=0.86; p=0.0032) contents in fish with larger start weight. A similar correlation was observed in our previous investigation with rainbow trout (Gao et al., 2011).

Except for a study with broiler chickens (Chiang et al., 2010), and our previous study with Nile tilapia (Gao et al., manuscript), we are not aware of any other studies on protein digestibility and retention in monogastric animals fed FRSM. The study with broiler chickens presented a significantly better nitrogen digestibility and retention of fermented rapeseed meal compared with RSM. However, the opposite was observed in our previous study with Nile tilapia, in which the nitrogen digestibility and retention were significantly reduced by the SSF. The present study did not agree with any of those previous studies, since the present experiment showed that the nitrogen digestibility and nitrogen retention were not affected by SSF. Similarly, the amino acid digestibility was not influenced by adding the OAB to the diets, in contrast to our previous results with rainbow trout, showing that adding the same OAB to an extruded diet based on soybean meal and pea protein concentrate, reduced the amino acid digestibility (Gao et al., 2011). The design of the present experiment limits the opportunity to determine if the differences amongst these studies may be ascribed to different species (chicken, trout and tilapia), fermentation quality, dietary formulation, or feed preparation method.

294 Adding organic acid salts to the diets and SSF treatment did not influence the digestibility of
295 phosphorus, zinc and magnesium, and phosphorus retention. A previous study conducted by Usha
296 and Chandra (1998) showed that phosphorus and zinc availability was improved SSF, and our
297 previous study also revealed that magnesium digestibility was increased by the SSF (Gao et al.,
298 manuscript). In addition, using organic acids in the diets for red sea bream exhibited an improvement
299 on phosphorus utilization (Hossain et al., 2007). The reasons of the variant results amongst those
300 studies are complicated, but it seems that fermentation technology used, organic acids or salts, feed
301 preparing methods and fish species are the major reasons.
302

303 **5. Conclusion**

304 In conclusion, supplementing diets with 18 g $kg^{-1}$ of sodium formate and butyrate (ratio 2:1) did not
305 have any impact on the feed utilization, growth rate, whole fish body composition, and apparent
306 digestibility of nitrogen and minerals, nitrogen retention in Nile tilapia. Solid state fermentation
307 treatment of rapeseed meal reduced feed utilization and growth rate, changed the whole fish body
308 composition in Nile tilapia.
309

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313

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454 Table 1.

455 Chemical composition, fungal biomass and amin acid profiles of untreated, fermented rapeseed meals

| | RSM | FRSM[1] |
|---|---|---|
| *Chemical composition, g (kg DM)*$^{-1}$ | | |
| Crude protein (CP) | 385.0 | 397.5 |
| Crude fat | 43.7 | 53.3 |
| Starch | 27.8 | 21.8 |
| Ash | 72.2 | 71.2 |
| Ergosterol, *mg (kg DM)*$^{-1}$ | 15.0 | 27.9 |
| Fungal biomass[1], *g (kg DM)*$^{-1}$ | 2.73 | 5.07 |
| | | |
| Essential amino acids[2] | | |
| Arg | 5.23 | 5.00 |
| His | 2.32 | 2.37 |
| Ile | 3.52 | 3.34 |
| Leu | 5.84 | 5.85 |
| Lys | 4.66 | 4.64 |
| Met | 1.67 | 1.67 |
| Phe | 3.44 | 3.37 |
| Thr | 3.87 | 3.87 |
| Val | 4.35 | 4.33 |
| | | |
| Non-essential amino acids[2] | | |
| Cys | 1.90 | 1.86 |
| Total non-essential amino acids | 41.4 | 40.6 |
| Total amino acids | 76.3 | 75 |

456 [1] The estimate is based on an ergosterol content of 5.5 mg g$^{-1}$ fungal biomass (Charcosset and Chauvet, 2001;
457 Gessner and Chauvet, 1993)

458 [2] Presented in dehydrated form, without tryptophan. Non-essential amino acids other than cystine are not
459 presented.

460 Table 2.

461 Formulation, chemical composition and amino acid profiles of the experimental diets

| | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---|---|---|---|---|
| *Ingredients, g (kg DM)$^{-1}$* | | | | |
| Fish meal[a], g | 134.7 | 132.1 | 136.0 | 130.0 |
| Soybean meal[b], g | 193.6 | 189.9 | 195.6 | 187.0 |
| Rapeseed meal (RSM)[c], g | 511.7 | 501.9 | 0 | 0 |
| Fermented RSM, g | 0 | 0 | 506.8 | 509.7 |
| Gelatinized potato starch[d], g | 57.7 | 57.1 | 58.3 | 56.2 |
| Na-alginate[e], g | 33.4 | 33.3 | 33.7 | 32.8 |
| Soy oil[f], g | 62.6 | 48.1 | 63.3 | 47.3 |
| Na-byturate[g], g | 0 | 19.1 | 0 | 18.8 |
| Na-formate[h], g | 0 | 12.3 | 0 | 12.1 |
| Vitamin and mineral premix[i], g | 6.0 | 5.9 | 6.1 | 5.8 |
| Vitamin B9, g | 0.167 | 0.167 | 0.168 | 0.164 |
| Vitamin B12, g | 0.022 | 0.022 | 0.023 | 0.022 |
| Yttrium oxide[j], g | 0.111 | 0.111 | 0.112 | 0.109 |
| | | | | |
| *Chemical composition,* | | | | |
| Dry matter (DM), g kg$^{-1}$ | 957.4 | 949.4 | 960.8 | 963.4 |
| Crude protein (CP), *g( kg DM)*$^{-1}$ | 386.5 | 385.6 | 402.4 | 397.1 |
| Crude fat, *g( kg DM)*$^{-1}$ | 98.6 | 95.9 | 108.3 | 104.5 |
| Starch, *g( kg DM)*$^{-1}$ | 90.9 | 81.4 | 77.4 | 74.8 |
| Ash, *g( kg DM)*$^{-1}$ | 74.4 | 83 | 77.7 | 86.1 |
| Butyric acid, *g( kg DM)*$^{-1}$ | <0.1 | 4.31 | <0.1 | 3.43 |
| Formic acid, *g( kg DM)*$^{-1}$ | <0.1 | 6.68 | <0.1 | 6.41 |
| Essential amino acids[k], g (100g CP)$^{-1}$ | | | | |
| Arg | 6.08 | 5.45 | 5.38 | 4.99 |
| His | 2.49 | 2.36 | 2.32 | 2.13 |
| Ile | 3.82 | 3.64 | 3.65 | 3.35 |
| Leu | 6.46 | 6.25 | 6.25 | 5.77 |
| Lys | 5.46 | 5.28 | 5.04 | 4.51 |
| Met | 1.48 | 1.71 | 1.71 | 1.63 |
| Phe | 4.08 | 3.71 | 3.69 | 3.39 |
| Thr | 3.72 | 3.73 | 3.76 | 3.50 |
| Val | 4.3 | 4.28 | 4.28 | 3.96 |
| Total non-essential amino acids[k] | 45.2 | 42.7 | 42.6 | 39.7 |
| Cys | 1.37 | 1.40 | 1.41 | 1.32 |
| Total amino acids[k] | 83.1 | 79.2 | 78.7 | 72.9 |

462

[a] NorsECO-LT, Norsildmel, Egersund, Norway.

[b] Denosoy, extracted and toasted soybean meal, Denofa, Fredrikstad, Norway.

[c] ExPro-00E produced from "double-low" rapeseed from Sweden.

[d] Gelatinised potato starch, Lygel F 60, (moisture ≤ 90 g $kg^{-1}$; fat 1 g $kg^{-1}$; protein 1 g $kg^{-1}$; viscosity 1000 cP at 5% concentration), Lyckeby Culinar, Fjälkinge, Sweden.

[e] Protanal ® LF 20 Na-alginate, FMC BioPolymer A/S, Drammen, Norway.

[f] Egersund sildoljefabrikk, Egersund, Norway.

[g] Adimix® 30% Coated; Sodium butyrate 30 ± 2 %; Butyric acid 21.7-26.6 %; pH 10-11; Water ≤ 2.5 %; Nutri-Ad International N.V., Kasterlee, Belgium.

[h] Contents: Sodium formate ≥ 97% (App. 66% formic acid moiety); Water ≤ 0.5; Organic substances ≤ 3.0; Perstorp Specialty Chemicals AB, Perstorp, Sweden.

[i] Contents per kg: Vitamin A 2500.0 IU; Vitamin $D_3$ 2400.0 IU; Vitamin E 0.2 IU; Vitamin $K_3$ 40.0 mg; Thiamine 15.0 mg; Riboflavin 25.0 mg; d-Ca-Pantothenate 40.0 mg; Niacin 150.0 mg; Biotin 3.0 mg; Cyanocobalamine 20.0 µg; Folic acid 5.0 mg; Pyridoxine 15.0 mg; Vitamin C: 0.098 g (Stay-C 35, ascorbic acid phosphate, DSM Nutritional Products, Basel, Switzerland); Cu: 12.0 mg; Zn: 90.0 mg; Mn: 35.0 mg; I: 2.0 mg; Se: 0.2 mg; Cd ≤ 3.0 µg; Pb ≤ 28.0 µg; total Ca: 0.915 g; total K 1.38 g; total Na 0.001 g; total Cl 1.252 g; Trouw Nutrition, LA Putten, The Netherlands.

[j] Metal Rare Earth Limited, Shenzhen, China.

[k] Presented in dehydrated form, without tryptophan. Non-essential amino acids other than cystine are not presented.

Table 3.

Factorial ANOVA results (*P-values*) of feed intake, fish growth parameters, feed conversion ratio, organ weight and whole body composition for three effects of organic acid salts, fermentation and fish start weight

| | Acid salts | Fermentation | Start weight | Interaction | | |
|---|---|---|---|---|---|---|
| | (A) | (F) | (W) | A*F | A*W | F*W |
| *Feed intake* | | | | | | |
| Day 0-24 | 0.99 | <0.0001 | <0.0001 | 0.49 | 0.54 | 0.008 |
| Day 0-46 | 0.26 | <0.0001 | <0.0001 | 0.6 | 0.68 | 0.017 |
| *Mean weight* | | | | | | |
| Day 0 | 0.87 | 0.62 | <0.0001 | 0.87 | 0.19 | 0.77 |
| Day 24 | 0.47 | 0.003 | <0.0001 | 0.22 | 0.36 | 0.04 |
| Day 46 | 0.96 | 0.015 | <0.0001 | 0.72 | 0.93 | 0.026 |
| *Weight gain* | | | | | | |
| Day 0-24 | 0.36 | 0.001 | <0.0001 | 0.15 | 0.49 | 0.015 |
| Day 0-46 | 0.98 | 0.007 | 0.0002 | 0.67 | 0.74 | 0.015 |
| *SGR* | | | | | | |
| Day 0-24 | 0.28 | 0.0003 | <0.0001 | 0.25 | 0.85 | 0.085 |
| Day 0-46 | 0.78 | 0.006 | <0.0001 | 0.46 | 0.46 | 0.023 |
| *FCR* | | | | | | |
| Day 0-24 | 0.22 | 0.015 | 0.007 | 0.22 | 1.00 | 0.93 |
| Day 0-46 | 0.17 | 0.012 | 0.009 | 0.21 | 0.35 | 0.23 |
| Condition factor | 0.43 | 0.41 | 0.43 | 0.87 | 0.79 | 0.87 |
| | | | | | | |
| *Organ weight : body weight ratio* | | | | | | |
| Stomach | 0.044 | 0.027 | 0.001 | 0.52 | 0.48 | 0.6 |
| Middle intestine | 0.94 | 0.29 | 0.0002 | 0.33 | 0.53 | 0.37 |
| Distal intestine | 0.35 | 0.51 | 0.33 | 0.84 | 0.91 | 0.3 |
| Liver | 0.061 | 0.006 | 0.13 | 0.32 | 0.068 | 0.27 |
| | | | | | | |
| *Whole body chemical composition* | | | | | | |
| Dry matter | 0.67 | 0.064 | 0.024 | 1.00 | 0.84 | 0.62 |
| Crude protein | 0.61 | 0.47 | 0.20 | 0.13 | 0.79 | 0.95 |
| Crude fat | 0.65 | 0.002 | 0.003 | 0.052 | 0.36 | 0.96 |
| Ash | 0.067 | 0.038 | 0.002 | 0.031 | 0.12 | 0.64 |

Table 4.

Main effects on fish growth parameters[1], feed intake, feed conversion ratio, organ weight and whole body composition in tilapia fed diets with and without addition of organic acid salts, to diets with rapeseed meal that were or were not fermented

| | Acid salts | | Fermentation | | Start weight | | Pooled S.E.M |
|---|---|---|---|---|---|---|---|
| | 0% (Diet 1+3) | 1.8% (Diet 2+4) | Without (Diet 1+2) | With (Diet 3+4) | Small | Large | |
| *Feed intake, g fish$^{-1}$* | | | | | | | |
| Day 0-24 | 80.7 | 80.7 | $90.1^{r}$ | $71.2^{s}$ | $64.0^{y}$ | $97.3^{x}$ | 1.91 |
| Day 0-46 | 163.3 | 168.9 | $182.1^{r}$ | $150.1^{s}$ | $142.7^{y}$ | $189.5^{x}$ | 3.34 |
| *Mean weight, g* | | | | | | | |
| Day 0 | 161.7 | 161.8 | 161.6 | 161.9 | $99.6^{y}$ | $224.0^{x}$ | 0.46 |
| Day 24 | 226.0 | 224.1 | $230.1^{r}$ | $220.0^{s}$ | $152.2^{y}$ | $297.9^{x}$ | 1.47 |
| Day 46 | 277.2 | 277.5 | $283.9^{r}$ | $270.8^{s}$ | $203.4^{y}$ | $351.3^{x}$ | 3.15 |
| *Weight gain, g* | | | | | | | |
| Day 0-24 | 64.3 | 62.3 | $68.5^{r}$ | $58.1^{s}$ | $52.6^{y}$ | $73.9^{x}$ | 1.17 |
| Day 0-46 | 115.5 | 115.6 | $122.3^{r}$ | $108.9^{s}$ | $103.8^{y}$ | $127.3^{x}$ | 2.84 |
| *SGR* | | | | | | | |
| Day 0-24 | 1.5 | 1.46 | $1.57^{r}$ | $1.39^{s}$ | $1.77^{x}$ | $1.19^{y}$ | 0.02 |
| Day 0-46 | 1.28 | 1.27 | $1.32^{r}$ | $1.23^{s}$ | $1.55^{x}$ | $1.00^{y}$ | 0.02 |
| *FCR* | | | | | | | |
| Day 0-24 | 1.24 | 1.28 | $1.31^{r}$ | $1.22^{s}$ | $1.21^{y}$ | $1.31^{x}$ | 0.02 |
| Day 0-46 | 1.41 | 1.46 | $1.49^{r}$ | $1.38^{s}$ | $1.38^{y}$ | $1.49^{x}$ | 0.03 |
| *Condition factor* | 2.28 | 2.34 | 2.34 | 2.27 | 2.28 | 2.34 | 0.05 |
| *Organ weight, % of body weight* | | | | | | | |
| Stomach | $0.34^{b}$ | $0.38^{a}$ | $0.34^{s}$ | $0.38^{r}$ | $0.39^{x}$ | $0.32^{y}$ | 0.01 |
| Middle intestine | 3.54 | 3.51 | 3.33 | 3.71 | $4.58^{x}$ | $2.47^{y}$ | 0.25 |
| Distal intestine | 0.16 | 0.18 | 0.16 | 0.18 | 0.18 | 0.16 | 0.01 |
| Liver | 1.59 | 1.75 | $1.80^{r}$ | $1.54^{s}$ | 1.73 | 1.61 | 0.06 |
| *Whole body composition, g (kg)$^{-1}$* | | | | | | | |
| Dry matter | 316.2 | 314.6 | 319.2 | 311.5 | $310.4^{y}$ | $320.3^{x}$ | 1.71 |
| Crude protein | 166.1 | 165.2 | 166.3 | 165.0 | 164.8 | 166.5 | 1.02 |
| Fat | 107 | 105.9 | $111.1^{r}$ | $101.8^{s}$ | $102.0^{y}$ | $110.9^{x}$ | 1.20 |
| Ash | 38.7 | 37.4 | $37.3^{s}$ | $38.8^{r}$ | $36.7^{y}$ | $39.4^{x}$ | 0.40 |

[1]different superscripts indicate significant differences between [a,b]acid salt supplementation; [r,s]fermentation; [x,y]fish start weight.

494 Table 5.

495 Statistically significant interactions between fish start weight and fermentation

| | Small weight | | Large weight | | Pooled S.E.M |
|---|---|---|---|---|---|
| | Without fermentation (Diet 1+2) | With fermentation (Diet 3+4) | Without fermentation (Diet 1+2) | With fermentation (Diet 3+4) | |
| *Feed intake, g fish$^{-1}$* | | | | | |
| Day 0-24 | 68.9$^{c}$ | 59.2$^{d}$ | 111.3$^{a}$ | 83.3$^{b}$ | 2.45 |
| Day 0-46 | 151.8$^{c}$ | 133.7$^{d}$ | 212.3$^{a}$ | 166.6$^{b}$ | 4.50 |
| *Mean weight, g* | | | | | |
| Day 24 | 154.2$^{c}$ | 150.2$^{c}$ | 306.0$^{a}$ | 289.8$^{b}$ | 2.58 |
| Day 46 | 204.1$^{c}$ | 202.6$^{c}$ | 363.6$^{a}$ | 339.0$^{b}$ | 3.80 |
| *Weight gain, g* | | | | | |
| Day 0-24 | 54.7$^{c}$ | 50.6$^{c}$ | 82.3$^{a}$ | 65.5$^{a}$ | 2.19 |
| Day 0-46 | 104.6$^{bc}$ | 103.0$^{c}$ | 139.9$^{a}$ | 114.7$^{b}$ | 3.44 |
| *SGR* | | | | | |
| Day 0-24 | 1.83$^{a}$ | 1.71$^{b}$ | 1.31$^{c}$ | 1.07$^{d}$ | 0.03 |
| Day 0-46 | 1.56$^{a}$ | 1.54$^{a}$ | 1.08$^{b}$ | 0.92$^{c}$ | 0.02 |

496

497 Table 6.

498 Statistically significant interaction between organic acid salt and fermentation

| | 0% organic acid salt | | 1.8% organic acid salt | | Pooled S.E.M |
|---|---|---|---|---|---|
| | Without fermentation (Diet 1) | With fermentation (Diet 3) | Without fermentation (Diet 2) | With fermentation (Diet 4) | |
| Ash, $g\ (kg)^{-1}$ | 37.2 | 37.5 | 40.2 | 37.4 | 3.27 |

499

500 Table 7.

501 Factorial analysis of variance (*P-value*) of apparent digestibility of nitrogen and amino acid, and nitrogen

502 retention in tilapia fed diets with and without addition of organic acid salts, to diets with rapeseed meal that

503 were or were not fermented

| | Acid salts (A) | Fermentation (F) | Start weight (W) | Interaction | | |
|---|---|---|---|---|---|---|
| | | | | A*F | A*W | F*W |
| *Apparent digestibility, %* | | | | | | |
| Nitrogen | 0.93 | 0.86 | 0.052 | 0.51 | 0.32 | 0.12 |
| Total amino acids | 0.68 | 0.47 | 0.010 | 0.94 | 0.28 | 0.13 |
| Essential amino acids | | | | | | |
| Arg | 0.41 | 0.33 | 0.026 | 0.90 | 0.22 | 0.17 |
| His | 0.62 | 0.31 | 0.013 | 0.86 | 0.28 | 0.13 |
| Ile | 0.65 | 0.63 | 0.012 | 0.90 | 0.23 | 0.10 |
| Leu | 0.73 | 0.80 | 0.023 | 0.87 | 0.23 | 0.084 |
| Lys | 0.53 | 0.11 | 0.037 | 0.84 | 0.25 | 0.11 |
| Met | 0.31 | 0.43 | 0.014 | 0.29 | 0.34 | 0.068 |
| Phe | 0.31 | 0.45 | 0.041 | 0.92 | 0.24 | 0.075 |
| Thr | 0.78 | 0.95 | 0.012 | 0.83 | 0.34 | 0.13 |
| Val | 0.88 | 0.83 | 0.011 | 0.71 | 0.26 | 0.12 |
| Total non-essential amino acids | 0.72 | 0.45 | 0.007 | 1.00 | 0.31 | 0.15 |
| Cys | 0.95 | 0.48 | 0.007 | 0.61 | 0.42 | 0.28 |
| | | | | | | |
| *Nitrogen retention, %* | | | | | | |
| Of dietary nitrogen | 0.27 | 0.34 | 0.017 | 0.059 | 0.88 | 0.86 |
| Of digested nitrogen | 0.39 | 0.41 | 0.009 | 0.062 | 0.72 | 0.41 |

504 * Apparent digestibility of non-essential amino acids other than cystine is not presented.

505 Table 8.

506 Main effects of organic acid salts, fermentation, and start weight on apparent digestibility of nitrogen and

507 amino acids[1], and nitrogen retention in tilapia fed diets with and without addition of organic acid salts, to diets

508 with rapeseed meal that were or were not fermented

| | Acid salts | | Fermentation | | Start weight | | Pooled |
|---|---|---|---|---|---|---|---|
| | 0% (Diet 1+3) | 1.8% (Diet 2+4) | Without (Diet 1+2) | With (Diet 3+4) | Small | Large | S.E.M |
| *Apparent digestibility, %* | | | | | | | |
| Of nitrogen | 81.7 | 81.5 | 81.7 | 81.4 | 79.8 | 83.4 | 1.12 |
| Total amino acids | 84.2 | 83.6 | 84.4 | 83.4 | 81.7[b] | 86.0[a] | 0.94 |
| Essential amino acids | | | | | | | |
| Arg | 90.3 | 89.6 | 90.3 | 89.6 | 88.9[b] | 90.9[a] | 0.53 |
| His | 86.2 | 85.5 | 86.5 | 85.2 | 84.0[b] | 87.8[a] | 0.96 |
| Ile | 82.7 | 82.1 | 82.8 | 82.1 | 80.3[b] | 84.6[a] | 0.96 |
| Leu | 84.2 | 83.8 | 84.2 | 83.9 | 82.4[b] | 85.7[a] | 0.86 |
| Lys | 84.8 | 83.8 | 85.6 | 83.0 | 82.5[b] | 86.1[a] | 1.02 |
| Met | 86.2 | 87.3 | 86.4 | 87.2 | 85.3[b] | 88.3[a] | 0.70 |
| Phe | 84.1 | 82.9 | 83.9 | 83.0 | 82.1[b] | 84.8[a] | 0.81 |
| Thr | 78.6 | 78.1 | 78.3 | 78.4 | 75.7[b] | 81.1[a] | 1.20 |
| Val | 81.5 | 81.3 | 81.5 | 81.2 | 79.1[b] | 83.6[a] | 0.99 |
| Non-essential amino acids* | | | | | | | |
| Cys | 80 | 80.1 | 80.7 | 79.3 | 76.8[b] | 83.3[a] | 1.32 |
| Total non-essential amino acids | 83.9 | 83.4 | 84.2 | 83.1 | 81.2[b] | 86.1[a] | 1.01 |
| *Nitrogen retention, %* | | | | | | | |
| of dietary nitrogen | 40.3 | 38.5 | 38.7 | 40.2 | 41.5[a] | 37.3[b] | 1.03 |
| of digested nitrogen | 49.5 | 47.5 | 47.5 | 49.4 | 52.1[a] | 44.8[b] | 1.56 |

509 [1]Presented in dehydrated form, without tryptophan.

510 Table 9.

511 Factorial analysis of variance (*P-value*) of mineral digestibilities, and phosphorus retention in tilapia fed diets

512 with and without addition of organic acid salts, to diets with rapeseed meal that were or were not fermented

| | Acid salts | Fermentation | Start weight | Interaction | | |
|---|---|---|---|---|---|---|
| | (A) | (F) | (W) | A*F | A*W | F*W |
| *Apparent digestibility, %* | | | | | | |
| Phosphorus | 0.72 | 0.33 | 0.014 | 0.69 | 0.25 | 0.70 |
| Zink | 0.67 | 0.19 | 0.024 | 0.46 | 0.68 | 0.60 |
| Magnesium | 0.43 | 0.54 | 0.23 | 0.56 | 0.19 | 0.012 |
| *Phosphorus retention,%* | | | | | | |
| of dietary phosphorus | 0.26 | 0.17 | 0.059 | 0.24 | 0.38 | 0.91 |
| of digestible phosphorus | 0.24 | 0.51 | 0.86 | 0.37 | 0.84 | 0.69 |

513

514 Table 10.

515 Main effect of organic acid salts, fermentation and start weight on digestibilities of minerals, and phosphorus
516 retention in tilapia fed diets with and without the addition of organic acid salts, to diets with untreated or
517 fermented rapeseed meal

| | Acid salts | | Fermentation | | Start weight | | Pooled S.E.M |
|---|---|---|---|---|---|---|---|
| | 0% (Diet 1+3) | 1.8% (Diet 2+4) | Without (Diet 1+2) | With (Diet 3+4) | Small | Large | |
| *Apparent digestibility, %* | | | | | | | |
| Phosphorus | 56.4 | 57.9 | 55.1 | 59.2 | 51.1[b] | 63.2[a] | 2.84 |
| Zink | 24.8 | 26.6 | 28.6 | 22.8 | 20.1[b] | 31.3[a] | 2.90 |
| Magnesium | 64.2 | 67.5 | 64.5 | 67.1 | 63.3 | 68.3 | 2.80 |
| *Phosphorus retention,%* | | | | | | | |
| of dietary phosphorus | 38.9 | 34.6 | 34.1 | 39.4 | 32.9 | 40.6 | 2.52 |
| of digestible phosphorus | 68.7 | 60.4 | 62.3 | 66.8 | 65.1 | 64.0 | 4.71 |

518

519 Table 11.

520 Statistically significant interaction between fish start weight and SSF

| | Small weight | | Large weight | | Pooled |
|---|---|---|---|---|---|
| | Without fermentation (Diet 1+2) | With fermentation (Diet 3+4) | Without fermentation (Diet 1+2) | With fermentation (Diet 3+4) | S.E.M. |
| *Apparent digestibility, %* | | | | | |
| Magnesium | 55.8[b] | 70.8[a] | 73.3[a] | 63.4[ab] | 3.98 |

521

# PAPER IV

**Short communication:**

# Steam treatment does not improve nutritional value of solid state fermented rapeseed meal in diets for Nile tilapia (*Oreochromis niloticus*)

Youling Gao[1, 2], Margareth Øverland[1], Trond Storebakken[1*]

[1] Aquaculture Protein Centre, CoE, Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences (UMB), NO-1432 Ås (Norway).

[2] College of Biological and Environmental Sciences, Zhejiang Wanli University, Ningbo, Zhejiang, P.R. China.

[*]Corresponding author: Address: Department of Animal and Aquacultural Sciences, UMB, P.O. Box 5003, NO-1432 Ås (Norway) e-mail: trond.storebakken@umb.no

**Abstract**

An experiment with Nile tilapia was conducted to evaluate the effect of steam treatment on solid state fermented rapeseed meal on growth performance, whole body composition and apparent nutrient digestibilities. Two diets were prepared in accordance with a 2×2 design, the factors were with and without steam treatment after solid state fermentation of rapeseed meal, and two sizes of tilapia, small (100 g start weight) vs. large (224 g).. Each diet was fed to apparent satiation to two tanks of each fish size group for 45 d. The results showed that steam treatment increased feed conversion ratio, while growth, nutrient digestibilities and protein retention were not affected.

*Key words:* Nile tilapia; Solid state fermentation; Sodium formate and butyrate; Rapeseed meal; Growth; Apparent digestibility.

## 1. Introduction

A previous experiment (Gao et al., manuscript A) has shown that solid state fermentation (SSF) of rapeseed meal (RSM) improved the feed conversion ratio of Nile tilapia (*Oreochromis niloticus*) when used in a moist diet, while it decreased the protein retention and increased feed conversion ratio when used in an extruded diet. The study concluded that differences in nutritional value in the two experiments may partly be rationalized by differences in fermentation of the RSM, and partly by differences in hydrothermic treatment of the feed ingredients and diets.

Hydrothermic treatment may affect the nutritional value of protein-rich feed ingredients from plant seeds in several manners. Protein digestibility may be improved by inactivating heat labile antinutritional factors such as protease inhibitors and lectins (Francis et al., 2001). Mild heat-denaturing may also make storage proteins more available to digestion, an effect commonly observed by extrusion (Romarheim et al., 2005; Morken et al., 2011). Excessive heat treatment will reduce digestibility through the introduction of strong hydrogen and disulfide bindings, and chemical reactions such as the Maillard reaction between amino acids with a basic amino group and reducing sugars, oxidation of sulphur-containing amino acids, and *L→D* isomerisation (review by Storebakken et al., 2000).

The current experiment was designed in order to determine if heating solid state fermented RSM could introduce the previously observed improvement in feed conversion ratio (Gao et al., manuscript A).

## 2. Materials and methods

Preparation of seed culture for SSF of RSM with *Aspergillus niger* ATCC 10577 has been described by Gao et al. (manuscript A), and the SSF by Gao et al. (manuscript B). After 48 h of SSF**,** 12.5 kg of

fermented rapeseed meal (FRSM) was heated by steam (175°C, 8 bar) for 5 min, until the temperature was 70 ºC. The other part did not go through the steam treatment. Two diets (Table 1) were prepared with similar composition on dry matter (DM) basis, one diet with steamed FRSM and one with FRSM that had not been steamed. Briefly, all dry ingredients were mixed prior to adding water and lipid. The diets then were shaped using a pasta extruder, equipped with a cutter to produce moist pellet with a length of 3-4 mm, and dried at 65 °C until the moisture content was 40 g $kg^{-1}$.

Experimental conditions were the same as described by Gao et al. (manuscript B). Four fibre-glass tanks with 115 L water volume and four tanks with 210 l volume were used. Two size groups of Nile tilapia (*Oreochromis* niloticus) were used, with average start weights of 99.6 and 224.0 g, respectively. The smallest fish were distributed in 115 l tanks, and the larger ones in 210 l tanks (n = 20 fish $tank^{-1}$ for both groups). The fish were kept in 25 °C recycled freshwater, and fed for 45 d. Fish were fed by hand to apparent satiety three times (9 am, 2 pm and 8 pm) from day 1 to 7. After that, the feeding was twice daily (9 am and 2 pm). Apparent feed consumption in each tank was recorded for each feeding. Uneaten feed was collected immediately after each meal, and was only observed in the large tanks after 15 days.

Fish were weighted at the start, at day 25, and at the end of the experiment. Two samples of small fish and two samples of big fish, 5 fish in each sample, were sampled for initial whole body composition. At the end of experiment, 10 fish $tank^{-1}$ were euthanized with MS 222 and weighed before the abdominal cavity of fish was cut open and the faecal content of the last 5 cm of the distal intestine was collected from five fish, the fish were used for analysis of whole-body chemical composition. The rest of the fish were bulk-weighted and then the faecal content in the last 5 cm of the distal intestine was collected.

Methods and equipment for chemical analyses have been described in detail by Gao et al. (manuscript B). Briefly, the diets were analysed for dry matter, crude protein (Kjeldahl-N for feed), starch (enzymatic hydrolysis and subsequent quantification of glucose), lipid (HCl hydrolysis followed by ether extraction), ash, amino acids (EC, 1998) and yttrium oxide (ICP-MS). Freeze-dried faeces were analyzed for crude protein (Dumas method), amino acids and $Y_2O_3$. Whole tilapia bodies were analysed for dry matter, crude protein (Kjeldahl-N), lipid (ether extraction) and ash.

Specific growth rate (SGR) was calculated as: $\frac{\ln W_1 - \ln W_0}{T}$ where $W_1$ is the fish weight at the end of the study; $W_0$ is the fish weight at the start of the experiment; and T is the interval in days. Feed conversion ratio (FCR) was calculated as: $F \times G^{-1}$ where F is consumption of dry matter from feed and G represents the weight gain. Apparent digestibility of dry nitrogen and amino acids were assessed by the indirect method with $Y_2O_3$ as an inert marker, calculated as: $100 \times (1-(D_i/F_i \times F_n/D_n))$ where $D_i$ and $F_i$ are concentration of inert marker in diets and faeces, while $D_n$ and $F_n$ are nutrient concentrations in diets and faeces. Nutrient retention, percentage of gross or digestible nutrient intake, was calculated as: $100 \times (N_1-N_0)/N_i$ where $N_0$ and $N_1$ represent whole-body nutrient content at the beginning and end of the experiment and $N_i$ is gross or digestible nutrient intake.

The results were analyzed statistically by two-way analysis of variance using the GLM procedure in SAS (1999). Duncan's multiple range test was used to rank significant differences among main effects, while significant ($P<0.05$) interactions were ranked by the P-diff procedure in SAS. The results are presented as least-square means and pooled standard errors of the means (s.e.m).

## 3. Results and discussion

Short-term steam treatment is commonly used in feed processing to moisturize and heat the feed mash during conditioning and pelleting, and is known to positively affect both physical and

nutritional quality of the feed (Lundblad et al., 2011). Steam treatment did not result in notable changes in the proximate concentration of the diets (Table 1). A small loss of amino acids (0.5-3%) was, however, observed, and is also reflected in the amino acid composition of the diet. Increased concentration of amino acids in crude protein is expected if volatile nitrogen components had been flushed off by the steam heating of the FRSM. This indicates that volatile nitrogen production was not significant during SSF of RSM.

The start weight of the tilapia, and thereby fish size affected feed intake, growth rates and body composition. This has been discussed previously by Gao et al. (manuscript B), and will not be addressed here. Steam treatment did not result in significant differences in feed intake of growth rates, or in the chemical composition of the tilapias (Tables 2 and 3). Over the course of the whole experiment, the apparent feed intake, however, tended ($P=0.071$) to be higher for the tilapia fed the diet with steam treated FRSM. Because this was not reflected in improved growth, the feed conversion ratio was significantly higher for the fish fed the diet with steam treated FRSM than what was observed in the tilapia fed the diet with FRSM that had not been subject to steam treatment.

This poorer feed conversion can't be rationalized by steam processing leading to heat-induced reduction in the nutritional value of the protein (Opstvedt et al., 1984; 2003), because digestibility of crude protein, essential or total amino acids (Tables 5 and 6) did not differ significantly between dietary treatments. The small reduction in amino acid concentration caused by the steam treatment cannot be considered an important factor in explaining the differences in feed conversion, because neither total nor digestible nitrogen retentions were significantly effected by dietary treatments (Tables 5 and 6). This is further supported by the intake of all digestible essential amino acids being well above the requirement for Nile tilapia (Gao et al., manuscript A). The significant interactions observed between whole-body dry matter in the small size group and the large size group of fish,

which was lower in the small group, and the elevated whole-body ash in the large size group of tilapia fed the diet with FRSM that had not been steam treated (Tables 2 and 4), does not contribute to the explanation of the observed difference in fed conversion ratio.

Accurate feed intake assessment requires quantification of uneaten feed (Helland et al., 1996). Apparent feed intake was only assessed by visual observation in this experiment. The reasons were that the diets did not have sufficient water stability to permit collection and exposure to running water over time (Helland et al., 1996), and that the tanks were not constructed for complete recovery of uneaten feed from the water outlet. Thus, it is possible that the two diets may have caused differences in apparent eating behaviour, which may have lead to biased estimation of feed intake.

The results clearly show that 5 min steam treatment to increase the temperature of FRSM to 70$^{o}$C did not induce effects that explain the improved nutritional value of FRSM that had been pasteurised, when compared with RSM (Gao et al., manuscript A). The current result, however, do not rule out the possibility that more excessive hydrothermic treatment may have an effect on nutritional value of the FRSM, because the biomass was pasteurised for as long as 24 h at 70$^{o}$C in the previous experiment (Gao et al., manuscript A).

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181 Table 1.

182 Formulation, chemical composition and amino acid profiles of the experimental diets

| Steam | Without | With |
|---|---|---|
| Dry matter (DM), g $kg^{-1}$ | 960.8 | 965.7 |
| | | |
| *Ingredients[1], g (kg DM)$^{-1}$* | | |
| Fish meal, g | 136.0 | 135.0 |
| Soybean meal, g | 195.6 | 194.1 |
| Fermented RSM, g | 506.8 | 0 |
| Fermented RSM (steamed), g | 0 | 510.5 |
| Gelatinized potato starch, g | 58.3 | 57.9 |
| Alginate, g | 33.7 | 33.4 |
| Soy oil, g | 63.3 | 62.8 |
| Vitamin and mineral premix, g | 6.1 | 6.0 |
| Vitamin B9, g | 0.168 | 0.167 |
| Vitamin B12, g | 0.023 | 0.022 |
| Yttrium oxide, g | 0.112 | 0.112 |
| | | |
| *Chemical composition, g( kgDM)$^{-1}$* | | |
| Crude protein | 402.4 | 401.7 |
| Crude fat | 108.3 | 104.6 |
| Starch | 77.4 | 74.5 |
| Ash | 77.7 | 76.8 |
| | | |
| Amino acids[2], g (100g CP)$^{-1}$ | | |
| Total essential amino acids | 36.1 | 29.6 |
| Total amino acids | 78.7 | 73.8 |

183 [1] For detailed composition, see Gao et al., manuscript B.

184 [2] Presented in dehydrated form. Without tryptophan.

185 Table 2.

186 Two-way ANOVA (*P-value*) results of fish growth parameters, feed conversion ratio, organ weight and whole

187 body composition for two effects of fish start weight and steam treatment

| | Steam (S) | Start weight (W)[1] | S*W (Interaction) |
|---|---|---|---|
| *Weight gain* | | | |
| Day 0-24 | 0.19 | 0.0010 | 0.27 |
| Day 0-46 | 0.69 | 0.030 | 0.42 |
| *SGR* | | | |
| Day 0-24 | 0.09 | <0.0001 | 0.2 |
| Day 0-46 | 0.73 | <0.0001 | 0.33 |
| *Feed intake* | | | |
| Day 0-24 | 0.11 | 0.001 | 0.27 |
| Day 0-46 | 0.071 | 0.006 | 0.84 |
| *FCR* | | | |
| Day 0-24 | 0.23 | 0.022 | 0.67 |
| Day 0-46 | 0.023 | 0.008 | 0.39 |
| *Whole body composition* | | | |
| Dry matter | 0.36 | 0.009 | 0.033 |
| Crude protein | 0.53 | 0.087 | 0.79 |
| Fat | 0.25 | 0.079 | 0.17 |
| Ash | 0.088 | 0.005 | 0.009 |

188 [1]Differences between the two weight-classes of tilapia have been discussed by Gao et al. (manuscript B), and

189 will not be addressed here.

190 Table 3.

191 Main effects of steam treatment and start weight on fish growth parameters, feed conversion ratio, and whole

192 body composition

| | Steam | | Pooled S.E.M |
|---|---|---|---|
| | Without | With | |
| *Weight gain, g* | | | |
| Day 0-24 | 57.43 | 60.16 | 1.23 |
| Day 0-46 | 109.7 | 111 | 2.24 |
| *SGR* | | | |
| Day 0-24 | 1.39 | 1.44 | 0.02 |
| Day 0-46 | 1.24 | 1.25 | 0.01 |
| *Feed intake, g fish$^{-1}$* | | | |
| Day 0-24 | 70.3 | 77.2 | 2.36 |
| Day 0-46 | 146 | 160.2 | 4.12 |
| *FCR* | | | |
| Day 0-24 | 1.22 | 1.27 | 0.03 |
| Day 0-46 | 1.33$^{b}$ | 1.44$^{a}$ | 0.02 |
| *Whole body composition, g kg$^{-1}$* | | | |
| Dry matter | 312 | 315 | 1.9 |
| Crude protein | 167 | 165 | 1.7 |
| Crude fat | 100 | 105 | 2.8 |
| Ash | 4.02 | 3.94 | 0.02 |

193

194 Table 4.

195 Interaction between the factors of fish start weight and steam treatment on dry matter and ash content of whole

196 fish body

| | Small weight | | Large weight | | Pooled S.E.M |
|---|---|---|---|---|---|
| | Without steam | With steam | Without steam | With steam | |
| Dry matter | 301.7[b] | 313.0[a] | 322.9[a] | 317.1[a] | 2.65 |
| Ash | 3.84[b] | 3.93[b] | 4.19[a] | 3.96[b] | 0.03 |

197

198 Table 5.

199 Two-way ANOVA results (*P-value*) of apparent digestibility of nitrogen and amino acids, and nitrogen

200 retention in tilapia fed fermented rapeseed meal with and without sterilization by steaming

| | Steam (S) | Start weight (W)[1] | S*W (Interaction) |
|---|---|---|---|
| *Apparent digestibility, %* | | | |
| Nitrogen | 0.96 | 0.40 | 0.75 |
| Essential amino acids | 0.45 | 0.52 | 0.92 |
| Non-essential amino acids | 0.51 | 0.29 | 0.95 |
| Total amino acids | 0.48 | 0.37 | 0.94 |
| | | | |
| *Nitrogen retention, %* | | | |
| of feed Nitrogen | 0.14 | 0.32 | 0.73 |
| of digestible Nitrogen | 0.30 | 0.35 | 0.92 |

201 [1]Differences between the two weight-classes of tilapia have been discussed by Gao et al. (manuscript B), and

202 will not be addressed here.

203 Table 6.

204 Effects of steam and start weight on apparent digestibility of nitrogen and amino acids, and nitrogen retention

205 in tilapia fed fermented rapeseed meal with and without steam treatment

| | Steam | | Pooled S.E.M |
|---|---|---|---|
| | Without | With | |
| *Apparent digestibility, %* | | | |
| Nitrogen | 81.0 | 80.8 | 1.8 |
| Essential amino acids | 84.1 | 82.0 | 1.8 |
| Non-essential amino acids | 83.4 | 81.3 | 2.0 |
| Total amino acids | 83.7 | 81.6 | 1.9 |
| | | | |
| *Nitrogen retention, %* | | | |
| of feed Nitrogen | 42.6 | 38.5 | 1.6 |
| of digestible Nitrogen | 52.6 | 47.8 | 3.0 |

206