Investigation of potential bio-active properties and effects on production performance of aquafeed ingredients derived from fish processing waste by way of enzymatic autolysis

by

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Declaration

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Date: .....

This dissertation includes 4 original papers published in peer-reviewed journals and/or books and 2 unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contribution of co-authors.

Opsomming

Hierdie studie vorm deel van die voortdurende en wêreldwye soeke na nuwe akwakultuur voerbestanddele wat nie net vismeel en –olie kan vervang as bron van noodsaaklike voedingstowwe nie, maar wat ook gespesialiseerde funksionele eienskappe openbaar wanneer dit in akwavoere ingesluit word. As gevolg van beperkings in die voorsiening van vismeel en –olie afkomstig vanaf wilde visserye, word die voortdurend groeiende akwavoer bedryf genoodsaak om nuwe grondstowwe te benut vir die vervaardiging van hoë kwaliteit voere.

Die doelwit van die studie was om moontlike gespesialiseerde funksionele eienskappe (met spesifieke klem op potensiële immuun-stimulasie) van voerbestanddele te ondersoek wat herwin is vanaf reënboogforel proseseringsafval, na ensiematiese hidrolise d.m.v. endogene ensieme teenwoordig in die afval (outolise). Verdere doelwitte was om (i) effekte op produksie prestasie van hierdie bestanddele te kwantifiseer in twee akwakultuur spesies relevant tot die Suid-Afrikaanse bedryf (naamlik die Mosambiekse kurper *Oreochromis mossambicus* en die Suid-Afrikaanse perlemoen *Haliotis midae*), (ii) om produksie prestasie te vergelyk met kommersieel beskikbare voerbestanddele voorberei d.m.v. ensiemtegnologie, en (iii) om die moontlike effekte van die voerbestanddele te skei van die van die sure gebruik om die bestanddele te preserveer tydens die outolitiese proses.

Daar is bevind dat olie herwin na outolise van reënboogforel ingewande ‘n goeie voerbestanddeel is wat gunstige effekte op die nie-spesifieke, sellulêre immuniteit van beide Mosambiekse kurper en Suid-Afrikaanse perlemoen gehad het. In die Suid-Afrikaanse perlemoen het verbeterde immuunfunksie gepaard gegaan met ‘n verswakking in produksie prestasie. Die gehidroliseerde proteïen komponent van ge-outoliseerde prosesseringsafval het ook beduidende verbetering in nie spesifieke immuniteit en oorlewing van Mosambiekse kurper tot gevolg gehad - onafhanklik van die preserverende suur - maar dieselfde is nie in die Suid-Afrikaanse perlemoen waargeneem nie. Produksie prestasie was afhanklik van die insluitingsvlakke van gehidroliseerde proteïen in beide spesies en dit is bevind dat oormatige insluiting produksie nadelig beïnvloed. Prestasie van proteïen afkomstig van outolise en die van ‘n kommersiële produksieproses het beduidend verskil, moontlik as gevolg van verskillende grondstowwe en prosesseringstegnieke. Daar is verder vir die eerste keer getoon dat mieresuur ‘n beduidende verbetering in waterstabiliteit in sekere perlemoenvoere teweeg kan bring.

Die studie kom tot die slotsom dat die eenvoudige outolise proses funksionele akwavoerbestanddele kan produseer wat kan bydra tot ‘n verbetering in produksie prestasie in die Mosambiekse kurper en Suid-Afrikaanse perlemoen. Die resultaat kan bydra tot verbeterde volhoubaarheid van die akwavoer bedryf, deur vismeel en –olie afkomstig van wilde visserye, te vervang.

Abstract

This study is part of the continuing global research effort dedicated to finding alternative aquafeed ingredients, which not only replace fish meal and fish oil as sources of essential nutrients in aquafeeds, but also provide specialist functional properties when included in feeds. Due to constraints in supply of fish meal and fish oil originating from wild capture fisheries, the continually growing aquafeed industry requires new sources of raw materials for the production of high quality feeds.

The aim of the study was to investigate the specialist functional properties of feed ingredients (with emphasis on immune-stimulation potential) derived from fish processing waste after enzymatic hydrolysis by endogenous proteolytic enzymes (autolysis). Further aims were to (i) quantify effects of these feed ingredients on production performance of two species relevant to the South African aquaculture industry, namely Mozambique tilapia *Oreochromis mossambicus* and South African abalone *Haliotis midae*, (ii) compare performance to commercially available enzymatically produced feed ingredients, and (iii) separate the functional effects of these fish processing waste derived feed ingredients from the acid used to preserve them against bacterial spoilage during the autolysis process.

Oil recovered after autolysis of rainbow trout viscera proved to be an attractive feed ingredient due to favourable effects on the non-specific cellular immune function of both Mozambique tilapia and South African abalone. However, in South African abalone, increased immune function due to inclusion of fish oil was accompanied by a significant decrease in production performance. The inclusion of hydrolysed proteins, obtained by autolysis of fish waste, in aquaculture feeds also improved non-specific immunity and survival of Mozambique tilapia significantly – independently of the preserving acid – although the same was not observed for South African abalone. Production performance was dependent on dietary hydrolysed protein inclusion levels in both species; excessive inclusion resulted in decreased production performance. The performance of dietary hydrolysed protein from autolysis and those from commercial production processes were significantly different, possibly as a result of different raw material origins and production processes. It is further shown that formic acid can contribute to improved water stability in abalone feeds, a novel mode of action not previously described.

The study concludes that the simple autolysis process for processing of fish waste can provide aquafeed ingredients with immune stimulatory potential, which can contribute to improved production performance in the Mozambique tilapia and the South African abalone. The result can contribute to improved sustainability of the aquafeed industry, through substitution of fish meal and fish oil derived from capture fisheries with processed fish waste components.

Declaration of contributions to the work

Declaration by the candidate

With regard to the chapters as detailed below, the nature and scope of my contribution were as follows:

Chapter	Pages	Nature of contribution	Extent of contribution (%)
5	37 – 62	Detailed description provided on page 38	70 %
6	63 – 87	Detailed description provided on page 64	80 %
7	89 – 107	Detailed description provided on page 89	80 %
8	109 – 130	Detailed description provided on page 110	80 %
9	131 – 150	Detailed description provided on page 132	80 %
10	151 - 166	Detailed description provided on page 151	80 %

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Declaration with signature in possession of candidate and supervisor Date: ……..

Declaration by co-authors:

The undersigned hereby confirm that:

1. the declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to the chapters as specified above,

2. no other authors contributed to these chapters besides those specified above, and

3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in this dissertation.

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	Feed Technology Group, Stellenbosch University	
	Department of Microbiology, Stellenbosch University	
	Department of Microbiology, Stellenbosch University	

Preface

This dissertation is the partial account of a journey that started off in the same yellow wood where Robert Frost found himself many years ago. During a cold and wet winter, alone on a London train platform between Christmas and New Year and being one of the very few people heading to the office, the oppressing morning closed in around me and I shared the same sentiment as Charlie Marlow: "...resenting the sight of people hurrying through the streets to filch a little money from each other, to devour their infamous cookery, to gulp their unwholesome beer, to dream their insignificant and silly dreams. They trespassed upon my thoughts." (Joseph Conrad, *Heart of Darkness*). I was actually staring down the two proverbial paths: one heading toward the security and predictability of a position at a reputable company in a world city; the other heading into the general direction of PhD studies and academia, but nobody could be certain. Being as stubborn and naive as I am, I will one day be able to say of these two roads that "I took the one less travelled by".

I mentioned that this dissertation is only part of the full story. Upon setting out on this PhD journey, I aimed to change the world. Eventually I settled for obtaining a PhD, and hoped that these two are somehow related. This document details the scientific investigations and findings of the study, but not the personal journey that was interwoven with the scientific one. Unfortunately, the personal journey is impossible to convey within the confines of words, written or spoken; again I quote Joseph Conrad from *Heart of Darkness*: "No, it is impossible; it is impossible to convey the life-sensation of any given epoch of one's existence – that which makes its truth; its meaning – its subtle and penetrating essence. It is impossible. We live, as we dream – alone..."

What was true in my case, is that the intensity of the battles fought as part of the personal journey far outweighs that of any of the scientific ones; yet these were the battles which contributed most to the success of the overall project. In the fighting of these personal battles, the isolation that often besets PhD candidates was a constant companion. And caught up in this world where only I seemed to live, countless times I found myself having to fight myself first before I could continue the fight with the task at hand, not unlike Bilbo where "He fought the real battle in the tunnel alone, before he ever saw the vast danger that lay in wait." (J.R.R. Tolkien, *The Hobbit*) I have been more fortunate than most in that I was surrounded throughout with the love of Christ and a group of people dedicated to seeing me succeed in this journey; for that I am immeasurably thankful. And this journey, this epoch is now at its end. After all "So comes snow after fire, and even dragons have their endings." J.R.R. Tolkien, *The Hobbit.*

Neill Goosen (11 May 2014)

Acknowledgements

One thing that is almost universally true of any PhD study is that it is not accomplished without considerable help from various sources. However, how does one properly acknowledge all the people who contributed to a body of work that essentially spanned more than 4 years? However impossible it is, I have to try.

Firstly, my immeasurable gratitude goes to my Lord and saviour Jesus Christ, for inspiration and the ability to commence and complete this work.

Then to my study leaders: thank you for academic guidance throughout the study, and for freedom to work independently whilst not allowing me to do nothing. However, I am much more grateful for understanding, support and level-headed advice during times of considerable personal turmoil. It has been a privilege studying under you.

To my family: thank you for your dedication in supporting me during this venture. However long it took, I believe we have reached the end. May I be given the opportunity to be a blessing to you, as you are to me. Many thanks also to my church, Shofar Christian Church Stellenbosch, for support and motivation when these seemed to be in short supply.

To friends, colleagues and colleagues turned friends, I cannot thank each one individually in this document (as it will double its length), but I can list your names here: Tiaan, Warren, Faf, Heinrich, Gustav, Francois, Walter, Iakovos, Stefan, Stephan and another Stefan, Wiehan, Amy, Desmaré, Le-Daan, Schalk, Bernard, Tanya, Carol, Pottie, Pieter and Pieter, Thinus, Charl, Jurie, Gerhard, Jason, Andrew, Willem, Kosie. I know I have probably left out people. Please accept my apologies.

A special word of thanks to Pastor Hercules Opperman: you guided me through tough times and constantly modelled to me how a true servant of God conducts himself.

The following people and organisations are acknowledged for assistance with various aspects of the trials: Syster and Sanna van Wyk, Lisa Uys, the certificate students at Aquaculture, Mr Matthias Wessels, Dr László Ardó, Dr Galina Jeney and Dr Vincent Fournier, Anneke de Bruyn, Prof Karin Jacobs.

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To colleagues at the Department of Process Engineering, Stellenbosch University: thank you for the encouragement and the freedom to complete the write-up of this study. And to the coffee-drinking squad (mostly Percy, Sunel, Tosca and on special occasions Christie), thank you for endless discussions about students, studying and anything else worthy or unworthy of discussion.

Dedication

This work is dedicated to my family.

My father Jurgens, my mother Neranzé, sister Dominique and brother Carl: through all the tumultuous and uncertain times God has given us the grace to make the best of things. That doesn't make you bunch of Goosens an easy lot to live with, but I still love you.

And to the family of fish breath Jonkershoek Clawless Cape Otters, who were so kind as to devour every single fish supposed to be my entire first feeding trial: "May the hairs on your toes never fall out".

List of abbreviations

ANOVA	analysis of variance
CFU	colony forming units
DHA	docosahexaenoic acid
DOH	degree of hydrolysis
DWG	daily weight gain
EPA	eicosapentaenoic acid
F	test statistic
FAME	fatty acid methyl esters
FCR	feed conversion ratio
FL	fold length
FM	fish meal
FPH	fish protein hydrolysate(s)
FW	fold width
GC	gas chromatography
GIT	gastro intestinal tract
H	height
IG	serum immunoglobulin
IP	inner perimeter
Jones CF	Jones condition factor
L	length
LSD	Least significant difference
L:W	Length to width ratio of intestinal folds
mg	milligram
ml	millilitre
MUFA	monounsaturated fatty acids
OP	outer perimeter
P	probability value
PBS	phosphate buffered saline
PEG	poly-ethylene glycol
PSD	pooled standard deviation
PUFA	polyunsaturated fatty acids
RFI	relative feed intake
SD	standard deviation
SE	standard error of the mean

SEM	standard error of the mean
SFA	saturated fatty acids
SGR	specific growth rate
t	time
TCA	trichloroacetic acid
TP	serum total protein
W	weight
Wid	width
µg	microgram

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List of Publications

The following academic publications have resulted from the work reported in this dissertation:

Peer reviewed journal publications:

Goosen, N.J., De Wet, L.F., Görgens, J.F., Jacobs, K., De Bruyn, A., 2014. Fish silage oil from rainbow trout processing waste as alternative to conventional fish oil in formulated diets for Mozambique tilapia *Oreochromis mossambicus*. Animal Feed Science and Technology. 188, 74 - 84.

Goosen, N.J., De Wet, L.F., Görgens, J.F.,2014. The effects of protein hydrolysates on the immunity and growth of the abalone *Haliotis midae*. Aquaculture 428-429C, pp. 243-248.

Goosen, N.J., De Wet, L.F., Görgens, J.F.,2014. Rainbow trout silage oil as immunity enhancing feed ingredient in formulated diets for South African abalone *Haliotis midae*. Aquaculture 430, pp 28 - 33

Goosen, N.J., De Wet, L.F., Görgens, J.F., 2014. Rainbow trout silage as immune stimulant and feed ingredient in diets for Mozambique tilapia (*Oreochromis mossambicus*). Aquaculture Research, doi:10.1111/are.12497

Conference proceedings:

Goosen, N.J., De Wet, L.F., Görgens, J.F., 2012. Improving the sustainability of Tilapia aquaculture through the utilisation of fish oil recovered from fish silage as aquafeed ingredient, Poster presentation XV ISFNF - 15th International Symposium on Fish Nutrition and Feeding, Molde, Norway

Goosen, N.J., De Wet, L.F., Görgens, J.F., 2012. Improving the sustainability of Tilapia aquaculture by using fish silage as feed ingredient in formulated diets. Poster presentation, XV ISFNF - 15th International Symposium on Fish Nutrition and Feeding, Molde, Norway

Goosen, N.J., De Wet, L.F., Görgens, J.F., 2013. Hydrolysed proteins in feeds for Mozambique tilapia. Bi-annual conference of the Aquaculture Association of Southern Africa, Stellenbosch, South Africa.

Goosen, N.J., De Wet, L.F., Görgens, J.F., 2014, Oil recovered from fish silage in formulated diets for South African abalone Haliotis midae, XVI ISFNF - 16th International Symposium on Fish Nutrition and Feeding, Cairns, Australia

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Chapter 1: Introduction

"In the beginning was the Word, and the Word was with God, and the Word was God." John 1:1

"There are no safe paths in this part of the world. Remember you are over the Edge of the Wild now, and in for all sorts of fun wherever you go." J.R.R. Tolkien, The Hobbit

The research embarked upon in this dissertation is set against the backdrop of stagnating or declining wild fisheries and a continually growing aquaculture industry, thus requiring increasing volumes of quality feed ingredients. The need to optimally utilise current resources and find alternatives to current practice has become apparent to the aquaculture industry, specifically the provision of feeds to this industry in a sustainable manner. As a result, much recent and current research is devoted to the search for alternative feed ingredients or alternative feedstocks for the production of high quality ingredients for the aquafeed industry, and to the detailing of the growth performance of these feed ingredients when applied to a large variety of aquatic species.

Another research area in the aquafeed industry attracting much attention is the field of functional feed ingredients, where feed ingredients not only serve solely as sources of nutrients, but also as modulators of specialist physiological processes in the animals (e.g., the immune system or digestive enzyme secretion), or enhance the feed in some way (e.g., increasing the feed palatability). Due to differences in digestive physiology, feeding habits and tolerance to specific feed ingredients between aquaculture species, the potential effects on production performance and physiological processes by new and alternative feed ingredients remain to be tested.

The research documented in the following chapters combines both the improved utilisation of fish wastes as sources of aquaculture feeds, and the measurement of functional properties of feeds derived from fish waste processing in selected aquaculture species. Fish waste as a potential feedstock for the production of aquafeed ingredients was identified from the local fish processing industry; after processing the experimental ingredients were evaluated for functionality and effects on growth performance in two aquatic species important to the South African aquaculture industry.

The chapters that follow include a literature survey, the novel contributions made by the study, detailed reports on various aspects investigated and finally a concluding chapter. The chapters were constructed in such a way to highlight the parallels between the research done on the Mozambique tilapia and that on the South African abalone. Due to the differences between the species in terms of culturing techniques and typical grow-out times, nutritional requirements and biology, there are some differences in the feed formulation and

preparation, and in the analytical methods employed to quantify the effects of the feed ingredients. For these reasons a common 'Materials and Methods' section was not feasible and the specific methods employed for each part of the study are detailed in the particular chapter.

The study is reported in the form of six individual scientific articles as chapters, four of which have been accepted for publication in peer reviewed journals at the time of writing the dissertation. Four individual feeding trials were done: three on Mozambique tilapia (Chapters 5 – 7), and one on South African abalone (Chapters 8 – 10). As a result of the different requirements from different scientific journals, there are some slight differences between the chapters in terms of structure, order of sections, title page details, manner how units are reported etc. Lists of all Tables and Figures are provided as reference before the Table of Contents of the dissertation. In Appendix A, the certificate providing ethical clearance for trials on Mozambique tilapia is provided: at the time when trials were performed no such clearance was required for South African abalone. Permission to reproduce work published in or accepted by peer reviewed journals is available in Appendix B.

It is the belief of the author that the research reported contributes significant advances in the field of alternative or novel feed ingredient use in aquafeeds. Further, due to the utilisation of processing by-products as feed ingredients, a contribution to sustainable aquafeed production has been made since fish processing waste represents a sustainable source of raw materials, both from fishing and aquaculture. The advances made during the project have the potential to contribute to the continued and sustainable expansion of the aquaculture industry in South Africa.

Chapter 2: Aims and design of the study

"But seek first the kingdom of God and His righteousness, and all these things shall be added to you." Matthew 6:33

"Try to become not a man of success, but try rather to become a man of value." Albert Einstein

Aims

In order to address the shortcomings in literature (see Chapter 3) regarding the possible bio-activities and effects on production performance of the protein and lipid components in fish silage when included in aquaculture feeds, and the possible effects of the acid used during fish silage preparation, the study aimed to:

I. **Ascertain the potential bio-activities and effects on production efficiency of both the hydrolysed protein and the lipid components of fish silage when included in aquaculture feeds.**

   The potential bio-activities of both the protein and lipid components of fish silage are poorly described when included as feed ingredients for aquaculture species. It was envisaged that the planned investigations would contribute fundamental knowledge regarding the application of hydrolysed proteins from fish silage, and silage oil, in aquaculture species, and elucidate whether these components of fish silage affect production performance or exert valuable biological activity when utilised as feed ingredients. The investigations of Chapters 5 and 6 address this aim in Mozambique tilapia, and Chapters 8 and 9 address it in the case of the South African abalone.

II. **Determine the bio-active properties and impact on production efficiency of the hydrolysed protein and lipid components of fish silage, when included in feeds for two aquaculture species that are important to the South African aquaculture industry namely the Mozambique tilapia (*Oreochromis mossambicus*) and the South African abalone (*Haliotis midae*).**

   Information regarding the application of hydrolysed proteins and lipids as potentially bio-active feed ingredients in diets for tilapia and abalone are lacking in general; however this is even more so in the Mozambique tilapia and the South African abalone. The fundamental knowledge produced will be directly applicable in the local aquafeed industry, which will contribute toward the ongoing efforts in aquafeed

improvement. Chapters 5 and 6 focus on Mozambique tilapia, while Chapters 8 and 9 focuses on the South African abalone.

III. **Compare the performance of the hydrolysed protein component in the fish silage against commercial fish protein hydrolysates produced from different raw materials.**

The effects of dietary hydrolysed fish proteins on physiological responses and production performance in a particular aquaculture species are dependent on the final product characteristics, which in turn differ according to the raw material origin and subsequent processing. From the perspective of an aquafeed manufacturer, there are very few studies that adequately compare dietary fish protein hydrolysates produced from different raw materials; again this is even more pronounced in the case of the two species used in the current study. This study specifically compared the hydrolysed protein component of fish silage to commercial fish protein hydrolysates in Mozambique tilapia (Chapter 7) and South African abalone (Chapter 9), in order to quantify the differences in growth and physiological responses resulting from the different dietary ingredients.

IV. **Establish the role of the acid used during silage preparation, and thus included in the FPH products, in any observed effects on production performance or physiological responses observed during inclusion of these FPHs in aquaculture feeds.**

Dietary organic acids are known to influence aquaculture production efficiency through different mechanisms; however, the effects of organic acids used during fish silage preparation are normally discounted when fish silage is incorporated in aquafeeds. The effects of organic acids in aquafeeds need to be determined in order to distinguish between the effects of the hydrolysed proteins in fish silage, and those of the acids used during silage preparation. Chapter 6 reports on the role of formic acid in diets for Mozambique tilapia, while Chapter 10 addresses this aspect in the South African abalone.

Design of the study

The aims of the study were achieved by way of feeding trials. The experimental species were chosen based on the fact that information regarding the potential bio-active effects of FPH and fish oil for both these species is lacking. Further, both these species are important to the South African aquaculture industry: the culture of the marine South African abalone is well established and generates significant revenue annually, whilst the Mozambique tilapia is a promising freshwater candidate species.

It was decided to employ a chemical ensiling method due to the simplicity of the process and the minimal additional infrastructure requirement compared to fermentative ensiling (e.g. the growth and maintenance of a bacterial inoculum). The ensiling acid was chosen as formic acid, as it is generally employed for the preparation of chemical fish silage, and it is a suitable candidate acid to distinguish between potential effects of hydrolysed proteins in fish silage and the acid used for preparation. Formic acid been shown to potentially impact production performance in the South African abalone (Goosen, et al., 2011), and growth and disease resistance in the Nile tilapia (Hassaan, et al., 2014; Ramli, et al., 2005). All batches of silage employed in feeding trials were prepared using a common ensiling method, as described in Appendix C.

In total, 4 feeding trials were conducted: 3 with Mozambique tilapia and 1 with South African abalone. The results of each tilapia trial are reported in a separate paper (Chapters 5 – 7). The abalone trial was conducted on a commercial abalone farm (Haga-Haga, Eastern Cape, South Africa) between August 2011 and January 2012. Due to limited on-farm capacity and resources, all investigations had to be performed simultaneously in a single trial; however, the results are reported in 3 separate papers (Chapters 8 – 10). In order to achieve this, appropriate control diets containing formic acid and trout silage oil were employed in the abalone trial. For the sake of clarity, the treatments, ingredients and treatment abbreviations used in the abalone trial, and the papers in which the results are reported, are summarised in Table 2-1. The experimental design of each part of the abalone study, and the justification for the ingredient choices and inclusion levels are reported in each separate paper.

The statistical design of different feeding trials varied, as there were physical and logistical limitations as to the number of replicates that could be included in trials, and adjustments were made to later trials based on results of previous trials. For example, the first trials completed on tilapia (reported in Chapters 5 and 6) employed 5 replicate tanks per treatment and 8 fish per tank; the number of treatments was limited by the availability of trial animals. In the final tilapia trial (reported in Chapter 7), the experimental design was

adjusted to 6 tanks per treatment and 12 fish per tank in in an attempt to increase the statistical power to detect significant differences between treatments. In the case of the abalone feeding trials (Chapters 8, 9 and 10), the number of treatments and replicates employed in the trial was determined by the space available for trials in the on-farm experimental system, and the availability of experimental animals.

Chapters that report experimental results contain abbreviated statistical analysis. For a more comprehensive description of the results of statistical analysis, refer to the supplementary statistical data in Appendix D.

Table 2-1 Summary of experimental layout and treatment abbreviations for the South African abalone feeding trial

Dietary ingredients	Nomenclature: Chapter 8	Nomenclature: Chapter 9	Nomenclature: Chapter 10
Ref	NC	-	A0
Ref + FA	-	-	A1
Ref + TO	NC + SO	C	A2
Ref + TO + FA	-	-	A3
Ref + TV	-	-	A4
Ref + TV + FA	-	-	A5
Ref + TO + TSL	-	SL	-
Ref + TO + TSH	-	SH	-
Ref + TO + TSH (phase)	-	SH, Phase	-
Ref + TO + HPL	-	HPL	A6
Ref + HPL TO + FA	-	-	A7
Ref + TO + HPH	-	HPH	-
Ref + TO + HPH (phase)	-	HPH, Phase	-

Abbreviations: Ref – Reference diet; FA – formic acid; TO – Trout silage oil; TV – Trout viscera; TSL – Trout silage, low inclusion level; TSH – Trout silage, high level inclusion level; (phase) – Phase-feeding; HPL – Commercial hydrolysate, low inclusion level; HPH – Commercial hydrolysate, high inclusion level.

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Chapter 3: Literature survey

"Moreover it is required in stewards that one be found faithful" 1 Corinthians 4:2

Background

Historically, the aquafeed industry has relied largely on capture fisheries to provide wild caught fish as source of proteins and lipids, as fish meal and fish oil respectively, for the manufacture of aquafeeds (Gatlin, et al., 2007; Glencross, et al., 2007; Olsen and Hasan, 2012). However, most wild fish stocks are either fully exploited or overexploited (FAO, 2010; 2012) and increased demand for aquafeed ingredients from a continually expanding aquaculture sector cannot be met by wild fisheries alone (Naylor, et al., 2009; Tacon, et al., 2011). Alternative sources of feed ingredients must therefore be utilized to ensure continued supply to fuel the aquafeed industry's expansion.

Fish processing waste from capture fisheries or aquaculture is an important alternative source of potential aquafeed ingredients. Even though significant advances have been made to substitute fish meal (FM) and fish oil in aquafeeds with non-fish ingredients, fish based products remain sought after in the industry due to their excellent nutrient profiles, e.g. balanced amino acid composition and high ω-3 polyunsaturated fatty acid (PUFA) content (Gatlin, et al., 2007; Naylor, et al., 2009; Tacon, et al., 2011). Fish meal derived from fish processing discards and by-products already makes a significant contribution to global fish meal production (FAO, 2012; Tacon, et al., 2011) and production from this source is projected to increase as the aquaculture industry expands (Tacon and Metian, 2008). However some barriers exist to the utilisation of processing wastes for fish meal and fish oil production, including economic consideration in cases where relatively small amounts of by-products are produced, final product characteristics and concerns about oil-soluble dioxin and polychlorinated biphenyl build-up (Naylor, et al., 2009). As a result, fish processing by-products are still discarded in some instances (Blanco, et al., 2007; Bower and Hietala, 2008; FAO, 2012).

Enzymatic protein hydrolysis has been advocated as an alternative method for the targeted recovery of value-added products from fish processing waste (Dumay, et al., 2004; Ghaly, et al., 2013; Guerard, et al., 2002; Harnedy and FitzGerald, 2012; Kechaou, et al., 2013; Kim and Mendis, 2006; Kristinsson and Rasco, 2000a; Ngo, et al., 2012). Enzymatic proteolysis of processing waste results in a protein component that is mostly hydrolysed to free amino acids and short chain peptides; some of the resulting fish protein hydrolysates (FPH) have been shown to have a variety of functional and bio-active properties (Kim and Mendis, 2006; Kristinsson and Rasco, 2000a; Ngo, et al., 2012) that are not characteristic of

the native proteins in the raw material (Balti, et al., 2010; Harnedy and FitzGerald, 2012; Kristinsson and Rasco, 2000a; b). Bio-active hydrolysates are defined as those that have potential physiological effects in addition to acting as a source of amino acids and nitrogen; typical potential bio-activities in human nutrition include anti-hypertensive, antioxidant, and appetite suppressing properties among others (Harnedy and FitzGerald, 2012). Important functional properties of protein hydrolysates include solubility, water holding capacity, emulsifying and foaming properties, fat absorption ability and sensory qualities (Kristinsson and Rasco, 2000a). These improved and/or novel properties of FPH increase the economic value of the end product, as these properties can serve to increase production efficiency when included in aquaculture feeds.

Additionally, the enzymatic hydrolysis facilitates the liberation and recovery of oil from the material (Dumay, et al., 2004; Gbogouri, et al., 2006; Gildberg, 1993; Linder, et al., 2005; Raa and Gildberg, 1982; Rai, et al., 2010; Vidotti, et al., 2011) at lower temperatures (up to 50 ºC) than the temperatures of up to 95 ºC applied in the conventional fish oil manufacturing processes. The lower temperatures prevent the temperature dependent oxidation of PUFA (Aidos, et al., 2002; Dumay, et al., 2004; EFSA, 2010; Gbogouri, et al., 2006). The components in fish oils that are of most value, specifically the ω-3 PUFA, are therefore preserved, thereby increasing the economic value of the oils (Dumay, et al., 2004).

Considerable research effort has been devoted to enzymatic proteolysis of fish material, as a means to extract valuable protein components. Recent progress has been in the area of utilizing specifically selected enzymes and controlled hydrolysis conditions for FPH production, and the application of FPH in aquaculture. It is acknowledged that chemical methods for protein extraction and/or hydrolysis from low value fish material are also available; however these methods generally result in proteins with poor functionality and nutritional properties (Kristinsson and Rasco, 2000a). As such, these methods fall outside the scope of this investigation.

Enzymatic hydrolysis of fish material

A number of different enzymatic methods are available for production of fish protein hydrolysates, depending among other factors on the type of fish material to be hydrolysed, the intended end use of the hydrolysates, the required final product properties, and the capital investment required. For the purpose of this text, the different types of hydrolysates will be referred to on the basis of the production method.

An important distinction between different hydrolysates lies in the origin of the enzymes employed for the hydrolysis. Autolysates are produced through initially preserving the raw material against microbial spoilage, and then allowing the endogenous proteolytic enzymes to hydrolyse the proteins (Kristinsson and Rasco, 2000a; Raa and Gildberg, 1982). The alternative production method is to add a known enzyme or mixture of proteolytic enzymes to the fish raw material, and to control the hydrolysis time and processing conditions (Kristinsson and Rasco, 2000a). Various important aspects of each production method are discussed below.

Production of autolysates

During autolysis, one of the key requirements is to achieve preservation from microbial spoilage of the highly perishable fish by-products and processing waste (Bower and Hietala, 2008; Espe and Lied, 1999). There are predominantly two methods employed to achieve preservation: through the addition of high concentrations of salt (sodium chloride), or through the addition of an acid, either directly or through the production of lactic acid through microbial fermentation of a carbohydrate source (Faid, et al., 1994; Raa and Gildberg, 1982; Vidotti, et al., 2002). The autolysed product resulting from salt-preserved fish material is generally known as fish-sauce and is utilised as a food condiment (Gildberg, 2004; Kristinsson and Rasco, 2000a); acid or bacterially preserved fish material that has subsequently undergone autolysis is known as fish silage and is employed as an animal feed ingredient (Kristinsson and Rasco, 2000a; Raa and Gildberg, 1982; Ruthu, et al., 2012).

Fish sauce

Due to its application as human food product, fish sauce falls outside the scope of the current investigation, although there are some commonalities with the autolysates that are the primary focus of the present work. Fish sauce is an autolysed product that is normally produced from whole small fish or fish waste (Gildberg, 1993) in the presence of high concentrations (25% or more) of sodium chloride (Orejana and Liston, 1981) at a pH that is close to neutral (Kristinsson and Rasco, 2000a). It results in a liquid product that is used as a condiment on rice and vegetable dishes (Gildberg, 2004; Kristinsson and Rasco,

2000a). The endogenous enzymes achieve autolysis over a long period of time of typically a few months, and high levels of free amino acids and small peptides are produced (Kristinsson and Rasco, 2000a; Orejana and Liston, 1981); fish sauce is consequently a good source of essential amino acids (Gildberg, 2004). The production time is extended due to the inhibition of endogenous proteolytic enzyme activity by the high salt concentration in the mixture (Gildberg, 1993; Kristinsson and Rasco, 2000a; Orejana and Liston, 1981).

Fish silage

Fish silage is a non-homogenous liquid product made from low-value fish or processing waste (Espe, et al., 1992; Hasan, 2003; Tatterson, 1982; Vidotti, et al., 2002; Vidotti, et al., 2003). The addition of acid to the starting raw material causes a lowering in the pH, which serves two purposes: the lowered pH has an anti-microbial effect in the material, thus avoiding microbial spoilage, but simultaneously also activating the endogenous proteolytic enzymes and thereby accelerating the protein hydrolysis (Espe, et al., 1992; Raa and Gildberg, 1982; Rai, et al., 2010; Stone, et al., 1989; Tatterson, 1982; Vidotti, et al., 2002). When centrifuged, fish silage separates into a floating oil phase, an aqueous phase containing soluble proteins, peptides, amino acids, and the acid employed during silage preparation, and a solid sediment phase containing hydrolysis-resistant, insoluble proteins and any bones that were present in the original raw material. The aqueous phase is separated from the oil and sediment emulsion phases, consisting of light and heavy emulsified lipid-proteins, respectively (Kristinsson and Rasco, 2000a; Raa and Gildberg, 1982).

When acids are added directly to fish waste for silage production, the silage is classified as chemical silage; when carbohydrates and microorganisms are used to produce acids through fermentation, the silage is classified as fermented silage. Added acids can further be grouped into strong mineral acids (e.g. hydrochloric, sulphuric and phosphoric acid) or weak organic acids such as formic, acetic and propionic acid. The mechanism through which added acids achieve preservation from microbial spoilage differ: the strong mineral acids do so purely through a lowering of the silage pH (Raa and Gildberg, 1982), while some organic acids are known to have antimicrobial properties linked to their ability to diffuse across microbial cell membranes when in undissociated form (Lückstädt, 2008). Organic acids therefore achieve preservation from microbial spoilage at higher pH values than in the case of mineral acids (Bower and Hietala, 2008; Lückstädt, 2008; Partanen and Mroz, 1999; Raa and Gildberg, 1982). When using mineral acids, the pH of the silage needs to be lowered to less than pH 2 to achieve microbial stability; however, in the case of organic acids, the silage is stable at pH values of 3.5 – 4.0 (Lückstädt, 2008; Raa and Gildberg, 1982). In feed applications, the high acidity of mineral acid silages needs to be neutralized

with a base before silages can be fed to animals, leading to undesirably high levels of inorganic salt formation in these silages (Gildberg and Raa, 1977; Raa and Gildberg, 1982)

The production of fermented fish silage relies on the metabolic products of micro-organisms to prevent the growth of spoilage bacteria in the ensiled material. In the most cases where fermented silage is produced, lactic acid bacteria are employed to ferment an added carbohydrate source to produce antimicrobial organic acids and bacteriocins (Bower and Hietala, 2008; Ruthu, et al., 2012), although mixed lactic acid and alcoholic fermentation has also been employed (Faid, et al., 1994; Faid, et al., 1997). As with chemical silage, the resulting product is a liquid which consists of lipids, solubilized proteins, peptides, amino acids and the water soluble components e.g. organic acids produced by the micro-organisms employed during the ensiling process (Dong, et al., 1993). The pH at which fermented silage is microbially stable is higher than chemical silages produced using mineral acids and pH values ranging between 4.0 – 4.5 have been deemed sufficient to produce silage that prevented spoilage and growth of pathogenic organisms (Ahamed and Mahendrakar, 1996; Ahmed and Mahendrakar, 1996; Dong, et al., 1993).

The lipids present in fish silage can play an important role in determining the nutritional quality thereof. The PUFA content of fish lipids is relatively high; PUFA is also the component vulnerable to oxidation (Vidotti, et al., 2011; Zhong, et al., 2007). Lipid oxidation could decrease the nutritional and sensory value of the oil through destruction of PUFA or the production of toxic oxidation products and off-flavours (Aidos, et al., 2002; Choe and Min, 2006; Hoyle and Merrit, 1994; Yano, et al., 2008). The amount and PUFA content of the lipids in fish silage is mainly dependent on that of the starting material; it has been shown that the final proximate composition of a silage largely reflects that of the raw material it was produced from, irrespective of the ensiling method employed (Espe and Lied, 1999; Espe, et al., 1992; Mach and Nortvedt, 2009; Ndaw, et al., 2008; Vidotti, et al., 2002). For these reasons, it is therefore important to take into account the lipid contained in fish silage when evaluating silage quality (De Arruda, et al., 2007), and not solely focus on the protein component.

Controlled hydrolysis with added enzymes

When producing hydrolysates by addition of selected, exogenous enzymes, the hydrolysis parameters such as temperature, pH, enzyme to substrate ratio, etc. are more tightly controlled than is the case when producing autolysates. FPH can be produced from low value fish, by-products and waste materials using exogenous enzymes (Aspmo, et al., 2005b; Liaset, et al., 2000; Šližyte, et al., 2005). Similar to fish silage, hydrolysates produced with added enzymes result in a non-homogenous product containing soluble and insoluble proteins, lipid-protein emulsions, lipids and residual bones, if these were present in the starting material (Kristinsson and Rasco, 2000a; Liaset, et al., 2003). Depending on the starting raw material and the FPH characteristics, the final products can be employed in animal feeds, human foods or nutraceuticals (Ghaly, et al., 2013; Harnedy and FitzGerald, 2012; Kim and Mendis, 2006; Kristinsson and Rasco, 2000a; Ngo, et al., 2012).

Due to a high degree of control over the active enzymes and hydrolysis conditions when added enzymes are employed, the final characteristics of the hydrolysis products can be better predicted and controlled than is the case for autolysis (Hathwar, et al., 2011; Liaset, et al., 2003). Functional and bio-active properties of FPH are dependent on the average molecular weight of the final product (Bourseau, et al., 2009; Deeslie and Cheryan, 1991; Saidi, et al., 2013), which can be manipulated through the hydrolysis process conditions and choice of proteolytic enzyme addition (Kristinsson and Rasco, 2000a; b).

As in the case of autolysates, the lipids present in the fish raw material need to be taken into account during hydrolysis with added enzymes. The type of hydrolytic enzyme employed will affect the distribution of lipids in the different fractions (aqueous, emulsion, oil and sludge phases) (Šližyte, et al., 2004; Šližyte, et al., 2014), and therefore raw materials with relatively high levels of lipids might influence the choice of proteolytic enzyme (Šližyte, et al., 2005). Various processing conditions are known to affect lipid quality in fish hydrolysates (Zhong, et al., 2007); some pre-treatment methods used to prepare raw material for protein hydrolysis e.g. heat treatment to inactivate endogenous enzymes or water addition prior to hydrolysis might lead to increased oxidation or an increase in the amount of free fatty acids, both of which might affect the nutritional quality of the lipids contained in the FPH fraction (Chantachum, et al., 2000; Ramakrishnan, et al., 2013; Šližyte, et al., 2005; Šližyte, et al., 2004; Wu and Bechtel, 2008). For these reasons it might be advantageous to remove lipids from fish protein hydrolysates, which can be achieved through centrifugation technology (Kim and Mendis, 2006; Kristinsson and Rasco, 2000a; Šližyte, et al., 2004).

Comparison of fish protein hydrolysates

Although the end products of autolysis and enzymatic hydrolysis using added enzymes are similar in the case of FPH (i.e., peptides and free amino acids), there are important differences in the final product characteristics and production methods used to obtain these products. These differences will play an important role in determining the production method employed to produce FPH from a particular raw material, and in determining the most suitable end-use of the FPH.

The economics of producing fish silage and FPH with autolysis or with added enzymes differ substantially. Production of fish silage relies on enzymes that occur naturally in the raw material (Espe, et al., 1992; Kristinsson and Rasco, 2000a; Raa and Gildberg, 1982); this method will therefore not incur enzyme costs. The technology employed to produce silage is further relatively simple and the production cost is nearly independent of the scale of production (Bower and Hietala, 2008; De Arruda, et al., 2007; Wassef, 2005). Silage production is therefore a suitable production route in cases where relatively low amounts of fish processing waste does not justify high capital outlay of alternative processing plants, or where processing waste is only available seasonally or produced in remote areas (Bower and Hietala, 2008; Dong, et al., 1993). Due to the relatively higher degree of process control employed when FPH are produced using added enzymes, the production technology is more sophisticated than in the case of autolysates (Shahidi, et al., 1995). This leads to relatively higher operating costs and higher initial capital investment, and the additional cost of proteolytic enzymes, which contributes a significant portion of the operating costs (Aspmo, et al., 2005b; Benhabiles, et al., 2012; Deeslie and Cheryan, 1988; Kristinsson and Rasco, 2000a; Prieto, et al., 2008; Prieto, et al., 2007; Shahidi, et al., 1995)

The different production methods employed to prepare fish silage and controlled hydrolysis FPH are mainly responsible for the differences in final hydrolysate properties. One of the main differences is the extent of the proteolysis of the final product: the proteins in fish silage are subjected to extensive and unchecked hydrolysis, resulting in relatively high amounts of free amino acids and short chain peptides that have a low average molecular weight (Gildberg, 1993; 2004; Kristinsson and Rasco, 2000a). The endogenous enzyme mixture responsible for protein hydrolysis is further complex and varies both between fish species, and within species according to seasons, genders, life stages and body tissues (Aspmo, et al., 2005b; Dong, et al., 1993; Herpandi, et al., 2011; Kristinsson and Rasco, 2000a; Mukundan, et al., 1986; Raghunath and McCurdy, 1990). This variation in the enzyme composition responsible for hydrolysis, and the high degree of protein hydrolysis (DOH), makes it difficult to produce fish silage with good functional properties (Kristinsson and Rasco, 2000a; Shahidi, et al., 1995). An advantage of FPH production via controlled hydrolysis is that functional properties can be manipulated through the choice of enzyme and

hydrolysis conditions (Balti, et al., 2010; Benjakul and Morrissey, 1997; D'Alvise, et al., 2000; Gildberg, 1993; Guerard, et al., 2002; Shahidi, et al., 1995); downstream processing of the FPH (e.g. through the application of filtration membranes) can further result in products that have molecular weight distribution within desired ranges (Jeon, et al., 1999; Kim and Mendis, 2006).

Due to the generally poor functional properties of fish silage it is viewed as being only suitable as protein source in animal feeds, and is mostly employed in this capacity (De Arruda, et al., 2007; Kristinsson and Rasco, 2000a); thus regarded as a low value product (Aspmo, et al., 2005a; Kim and Mendis, 2006). FPH produced from controlled hydrolysis, however, are regarded as products with higher economic value resulting from their improved functional properties or specialist bio-activities (Ghaly, et al., 2013; Harnedy and FitzGerald, 2012; Ngo, et al., 2012; Pires, et al., 2013); these products are produced for specific higher value end uses and target markets, and can therefore justify higher production costs than is the case for fish silage manufacture.

Application of enzymatically derived proteins and oils in aquafeeds

FPH have found wide applications as ingredients in formulated aquafeeds, due to favourable nutritional properties of FPH, e.g. balanced amino acid profile, essential nutrient content and lack of anti-nutritional factors (Bui, et al., 2014; Chalamaiah, et al., 2012; Leal, et al., 2010; Lian, et al., 2005), coupled with the apparent requirement that carnivorous fish have some level of protein from marine origin in their diets to ensure good performance (Aksnes, et al., 2006b). A large number of studies have investigated the potential of both fish silage and FPH produced via controlled hydrolysis to be employed as feed ingredients in aquafeeds for herbivorous, omnivorous and carnivorous species. A large proportion of these studies specifically investigated the potential of FPH products to substitute fish meal (FM) in aquafeeds (Table 3-1). Another body of literature specifically deals with the potential bio-active properties of FPH products and their physiological and growth effects when incorporated into aquafeeds (Table 3-2). Some important aspects of oils recovered during FPH production and their application are also discussed.

FPH as aquafeed ingredient

A wide range of aquaculture organisms have been studied to determine the suitability of silage as feed ingredient. The list includes a natural herbivore, various omnivores and carnivores, and both fresh and saltwater species. The species can further be divided into finfish, crustaceans and molluscs (refer to Table 3-1).

FPH products have been found to be a good source of protein in aquafeeds for a wide variety of aquaculture species. Further, the total substitution of FM in aquafeeds has been achieved using FPH products alone or in combination with other non-fish proteins in diets for the herbivorous abalone *Haliotis fulgens* (Guzmán and Viana, 1998) and in a number of studies on omnivorous species e.g. tilapia, whiteleg shrimp and pacu (Gallardo, et al., 2012; Macedo-Viegas, et al., 2004; Wassef, 2005). However only a single study reported the total replacement of FM in a carnivorous aquaculture species without growth impairment; the FM replacement was using a mixture of FPH, feather meal and soybean meal (Hardy, et al., 1984). Protein and amino acid digestibility of PFH products have generally been found to be relatively high (> 70%) for both fermented and chemical fish silage products in omnivorous species. It has also been found, however, that in certain cases high dietary levels of both chemical and fermented fish silage can negatively impact on animal growth performance (Guzel, et al., 2011; Hardy, et al., 1984; Macedo-Viegas, et al., 2004; Nwanna, 2003; Soltan, et al., 2008; Wassef, 2005).

Table 3-1 Investigation of nutritional properties and fish meal (FM) replacement potential of FPH in aquafeeds.

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Herbivorous species					
Haliotis fulgens	Juvenile	Chemical silage	Abalone viscera	100% substitution of FM achieved with a mixture of silage + soybean meal	(Guzmán and Viana, 1998)
Haliotis fulgens	Juvenile	Chemical silage	Mackerel without head and bones and abalone viscera	Silage diets resulted in higher growth than kelp; silage was concluded to be suitable dietary protein source	(Viana, et al., 1996)
Haliotis fulgens	Juvenile	Chemical silage	Mackerel without head and bones	Heated and unheated silage diets resulted in lower growth than higher protein commercial diet	(Viana, et al., 1999)
Omnivorous species					
Clarias gariepinus	Juvenile	Fermented silage	Shrimp head waste	Protein digestibility 76.2% and higher for silage alone, or in combination with various other protein sources	(Fagbenro and Bello-Olusoji, 1997)
Clarias gariepinus	Juvenile	Fermented silage	Non-edible fish by-products	Up to 50% substitution of FM before growth is negatively impacted	(Soltan, et al., 2008)
Clarias gariepinus	Juvenile	Fermented silage	Shrimp waste	Up to 30% FM substitution before negative growth effects become apparent	(Nwanna, 2003)
Clarias gariepinus	Juvenile	Fermented silage	Minced tilapia	75% FM replaced with 1:1 silage – soybean mixture	(Fagbenro, et al., 1994)

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Cyprinus carpio	Juvenile	Chemical silage	Grouper processing waste	Improved growth rates; acid silage concluded to be good feed ingredient	(Ramasubburayan, et al., 2013)
Cyprinus carpio	Larvae	Controlled hydrolysis FPH	Commercial product, raw material origin not stated	DOH important in larval nutrition	(Carvalho, et al., 1997)
Cyprinus carpio	Juvenile	Chemical silage	Minced whole whiting	Silage resulted in depressed growth and increased mortality	(Wood, et al., 1985)
Labeo rohita	Juvenile	Fermented silage	Viscera of mixed carp species	50% FM substituted, with simultaneous improvement in growth	(Mondal, et al., 2007)
Litopenaeus vannamei	Juvenile	Chemical silage	Mixed marine processing waste	Silage alone replaced 100% FM with equivalent growth; silage + soy improved growth at 100% FM replacement	(Gallardo, et al., 2012)
Litopenaeus vannamei	Juvenile	Fermented silage	Mixed tuna by-products	Improved ingredient digestibility and animal growth	(Hernández, et al., 2011)
Macrobrachium rosenbergii	Juvenile	Chemical and fermented silage	Mixed type raw minced fish	100% substitution of FM by both silages, without growth impairment	(Ali and Sahu, 2002)
Oreochromis aureus	Juvenile	Chemical silage	Whole minced Indian oil sardines	High silage protein digestibility (95.1%); not different from high quality FM	(Goddard and Al-Yahyai, 2001)
Oreochromis aureus	Fry	Chemical fish silage	Not stated	100% substitution of FM without negative consequences on growth rate	(Wassef, 2005)
Oreochromis aureus	Juvenile	Chemical fish silage	Not stated	100% substitution of FM without negative consequences on growth rate	(Wassef, 2005)

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Oreochromis niloticus	Fry	Chemical fish silage	Not stated	100% substitution of FM without negative consequences on growth rate	(Wassef, 2005)
Oreochromis niloticus	Juvenile	Chemical fish silage	Not stated	100% substitution of FM without negative consequences on growth rate	(Wassef, 2005)
Oreochromis niloticus	Fry	Fermented fish silage	Not stated	Up to 50% substitution of FM before growth is negatively impacted	(Wassef, 2005)
Oreochromis niloticus	Juvenile	Fermented fish silage	Not stated	Up to 50% substitution of FM before growth is negatively impacted	(Wassef, 2005)
Oreochromis niloticus	Juvenile	Fermented silage	Minced tilapia	Protein digestibility was relatively high (>83.5%) when for silage combined with various other protein sources	(Fagbenro and Jauncey, 1998)
Oreochromis niloticus	Juvenile	Temperature controlled autolysis	Shrimp heads	Up to 20% FM successfully substituted without adversely affecting growth	(Leal, et al., 2010)
Oreochromis niloticus	Juvenile	Chemical, fermented silage; acid silage with added enzymes	Mixed tilapia filleting residue and whole minced tilapia	All silages showed high protein digestibility (89.1% - 93.7%)	(Borghesi, et al., 2008)
Oreochromis niloticus	Juvenile	Fermented silage	Minced tilapia	75% FM replaced with 1:1 silage – soybean mixture	(Fagbenro, et al., 1994)
Oreochromis niloticus	Juvenile	Fermented silage with or without extracts or formalin	Whole minced tilapia	Untreated silage resulted in highest protein digestibility (90.4%)	(Fagbenro and Jauncey, 1994)
Oreochromis niloticus	Juvenile	Fermented silage; raw, cooked or supplemented with salt	Whole minced tilapia	All silages showed high protein digestibility (90.4% - 96.2%)	(Fagbenro and Jauncey, 1993)

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Oreochromis niloticus	Juvenile	Chemical silage	Minced trash fish	Improvement in growth at 100% FM substitution; decreased body fat deposition	(El-Hakim, et al., 2007)
Oreochromis niloticus	Juvenile	Fermented silage	Mixed fish by-products	Up to 50% FM substitution without negatively affecting growth	(Soltan and El-Laithy, 2008)
Oreochromis niloticus	Fry	Fermented silage	Shrimp head waste	Up to 20% FM substitution without negatively affecting growth	(Plascencia-Jatomea, et al., 2002)
Oreochromis niloticus	Juvenile	Fermented silage	Minced small tilapia	Up to 50% FM substitution without negatively affecting growth or feed conversion	(Wassef, et al., 2001)
Piaractus mesopotamicus	Larvae	Chemical fish silage	Tilapia filleting by-products (including viscera)	100% substitution of FM without affecting growth or survival	(Macedo-Viegas, et al., 2004)
Piaractus mesopotamicus	Larvae	Controlled hydrolysis FPH	Viscera free tilapia filleting by-products	100% substitution of FM without affecting growth or survival	(Macedo-Viegas, et al., 2004)
Piaractus mesopotamicus	Larvae	Controlled hydrolysis FPH	Tilapia viscera	Decreased growth upon 100% FM substitution; no effect on survival	(Macedo-Viegas, et al., 2004)
Piaractus mesopotamicus	Juvenile	Fermented and chemical fish silages	Fresh and saltwater fish and tilapia filleting residue	All silages displayed relatively high protein digestibility (72.5% - 80%)	(Vidotti, et al., 2002)

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Carnivorous species					
Anguilla anguilla	Juvenile	Not stated; presume chemical silage	Sardine and blue whiting	20% FM replacement resulted in higher animal weight	(Goncalves, et al., 1989)
Gadus morhua	Juvenile	Controlled hydrolysis FPH	Viscera free salmon by-products	Successfully substituted 33% of FM in high plant protein diets	(Aksnes, et al., 2006b)
Heteropneustes fossilis	Juvenile	Fermented silage	Viscera of mixed carp species	Successfully substituted 50% FM with simultaneous increase in fish growth	(Mondal, et al., 2008)
Oncorhynchus mykiss	Juvenile	Not stated	Unknown	50% FM replacement without affecting growth; 100% replacement decreased growth	(Guzel, et al., 2011)
Oncorhynchus mykiss	Juvenile	Chemical silage	Whole minced Pacific whiting	All silage treatments resulted in decreased final animal weights	(Hardy, et al., 1984)
Oncorhynchus mykiss	Juvenile	Temperature controlled autolysis	Whole minced Pacific whiting	100% FM replaced with FPH + soybean/feather meal mixture, without affecting growth	(Hardy, et al., 1984)
Oncorhynchus mykiss	Juvenile	Chemical silage	Pacific whiting processing waste	100% FM substitution decreased final animal weight; silage protein utilised less efficiently than from FM	(Stone, et al., 1989)
Rachycentron canadum	Juvenile	Chemical silage	Fish and crab	Silage diets showed decreased growth compared to diets using identical raw material, but without the ensiling process	(Mach, et al., 2010)
Salmo salar	Fry	Controlled hydrolysis FPH	Commercial product, raw material origin not stated	8% FM substitution and improved growth rates	(Berge and Storebakken, 1996)

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Salmo salar	Post-smolt	Chemical silage	Whole herring or offal	Successfully substituted up to 15% of FM before negatively impacting growth	(Espe, et al., 1999)
Salmo salar	Post-smolt	Controlled hydrolysis FPH	Whole herring	Successfully substituted up to 30% FM without negative impacts on growth	(Hevrøy, et al., 2005)
Salmo salar	Juvenile	Chemical silage	Spiny dogfish head and viscera	Dogfish silage was concluded to be a sufficient feed ingredient for salmon aquaculture	(Heras, et al., 1994)
Salmo salar	Juvenile	Chemical silage	Saithe offal	Protein utilisation not affected by silage DOH; higher DOH resulted in lower carcass fat deposition	(Espe, et al., 1992)
Salmo salar	Juvenile	Chemical silage	Fresh whole sprat	Silage proved to be good feed ingredient for salmon	(Jackson, et al., 1984a)
Scophthalmus maximus	Juvenile	Controlled hydrolysis FPH	Commercial product, raw material origin not stated	Successfully substituted 35% FM without negative impacts on growth	(Oliva-Teles, et al., 1999)
Sparus aurata	Fry	Chemical fish silage	Whole marine pelagic fish	Successfully substituted 100% FM in short term growth trial	(Wassef, 2005)

Abbreviations: DOH – Degree of hydrolysis of protein; FM – Fish meal

Fermented and chemical silages constitute the majority of FPH products investigated as protein source in aquaculture diets. This might be related to the relatively poor functional properties of fish silage from autolysis, difficulty in predicting final product characteristics and the perception that silage is a lower value product when compared to controlled hydrolysis FPH products.

There is little distinction made in aquaculture trials between whether growth and other effects are exerted by the hydrolysed protein component of silage or by the acid employed to prepare the silage. It is known that various organic acids employed for silage production have growth enhancing and antimicrobial effects in aquaculture species (De Wet, 2005; Goosen, et al., 2011; Hassaan, et al., 2014; Lückstädt, 2008; Ng, et al., 2009; Zhou, et al., 2009) and can enhance ingredient digestibility (Khajepour and Hosseini, 2012; Sarker, et al., 2012). It is important to distinguish whether observed effects are as a result of the organic acids, or the hydrolysis products.

Bio-activities exhibited by FPH when included in aquafeeds

Dietary FPH products have exhibited an array of desirable bio-active properties that altered physiological and growth responses in aquaculture trials (Table 3-2). These included stimulation of the non-specific immunity and increased survival with or without bacterial challenge (Bui, et al., 2014; Kotzamanis, et al., 2007; Liang, et al., 2006; Nguyen, et al., 2012; Tang, et al., 2008), improved intestinal development, altered gastrointestinal enzyme activity and improved digestive function (Cahu, 1999; Còrdova-Murueta and García-Carreño, 2002; Kotzamanis, et al., 2007; Santos, et al., 2013; Srichanun, et al., 2014; Taheri, et al., 2010; Tonheim, et al., 2005) and increased feed intake and growth (Chotikachinda, et al., 2013; Kousoulaki, et al., 2013; Refstie, et al., 2004; Viana, et al., 1994). The various bio-activities exerted by FPH products are attributed to the low molecular weight peptides and free amino acids that occur as part of the FPH. This has been shown in turbot, Japanese flounder and red seabream where the observed growth stimulation, improved non-specific immunity, higher insulin-like growth factor I levels and higher antioxidative capacity where all attributed to low molecular weight peptides found in the FPH (Bui, et al., 2014; Zheng, et al., 2012; 2013). Conversely, the removal of low molecular weight components from FPH has been shown to negatively impact growth and feed utilisation in rainbow trout (Aksnes, et al., 2006a).

Table 3-2 Some bio-activities of dietary FPH investigated in different species during different life-stages.

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Herbivorous species					
Haliotis fulgens	Juvenile	Chemical silages (various)	Marine fish and abalone	Abalone silage improved feed attraction	(Viana, et al., 1994)
Omnivorous species					
Oreochromis niloticus	Juvenile	Controlled hydrolysis FPH	Shrimp waste	Altered digestive enzyme activity	(Santos, et al., 2013)
Penaeus vannamei	Juvenile	Controlled hydrolysis FPH	Commercial products from krill and mixed marine fish	Enhanced digestive enzyme activity and growth	(Còrdova-Murueta and García-Carreño, 2002)
Penaeus vannamei	Juvenile	Controlled hydrolysis FPH	Tuna heads	Improved survival and growth	(Nguyen, et al., 2012)
Carnivorous species					
Anarhichas minor	Newly hatched / juvenile	Not stated	Shrimp waste	None detected	(Savoie, et al., 2006)
Dicentrarchus labrax	Larvae	Silage and controlled hydrolysis FPH	Silage: sardine by-products; controlled hydrolysis FPH raw material not stated	Controlled hydrolysis FPH had improved survival, growth and intestinal development	(Kotzamanis, et al., 2007)
Dicentrarchus labrax	Larvae	Controlled hydrolysis FPH	Commercial product, raw material origin not stated	Enhanced digestive tract development and survival	(Cahu, 1999)

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Gadus morhua	Juvenile	Controlled hydrolysis FPH	Salmon frames and heads (no viscera)	Altered distribution of intestinal peptide transporter	(Bakke, et al., 2010)
Hippoglossus hippoglossus	Larvae	Controlled hydrolysis FPH	Salmon serum protein	Improved protein adsorption efficiency and rate	(Tonheim, et al., 2005)
Lateolabrax japonicus	Juvenile	Silage produced using added enzymes	Pollock waste	Improved non-specific immunity	(Liang, et al., 2006)
Lates calcarifer	Larvae	Controlled hydrolysis FPH	Fish muscle and squid mantle	Improved digestive capacity and growth	(Srichanun, et al., 2014)
Lates calcarifer	Juvenile	Controlled hydrolysis FPH	Tuna viscera	Improved feed intake	(Chotikachinda, et al., 2013)
Oncorhynchus kisutch	Juvenile	Controlled hydrolysis FPH	Mixed filleting by-products	No effect on non-specific immunity	(Murray, et al., 2003)
Oncorhynchus mykiss	Juvenile	Controlled hydrolysis FPH	Commercial product from viscera free salmon frames and heads	Low molecular weight FPH components required to sustain high growth rates	(Aksnes, et al., 2006a)
Oncorhynchus mykiss	Alevin	Controlled hydrolysis FPH	Gold striped sardine	Increased growth, digestive enzyme activity and survival after bacterial challenge	(Taheri, et al., 2010)
Oncorhynchus mykiss	Juvenile	Chemical silage	Shrimp by-products	Improved astaxanthin digestion and accumulation in flesh	(Torrisen, et al., 1981/1982)
Pagrus major	Juvenile	Controlled hydrolysis FPH	Mixed sources	Increased non-specific immunity and growth	(Bui, et al., 2014)
Paralichthys olivaceus	Juvenile	Controlled hydrolysis FPH	Pollock by-products	Increased plasma levels of insulin-like growth factor	(Zheng, et al., 2012)

Studied species	Life stage	FPH type	FPH raw material	Main finding	Citation
Pseudosciaena crocea	Adult	Controlled hydrolysis FPH	Pollock	Significantly enhanced non-specific immunity	(Tang, et al., 2008)
Salmo salar	Post-smolt	Controlled hydrolysis FPH	Pollock by-products	Stimulated feed intake and improved growth	(Refstie, et al., 2004)
Salmo salar	Fry	Controlled hydrolysis FPH	Cod muscle	None detected	(Gildberg, et al., 1995)
Salmo salar	Post-smolt	Controlled hydrolysis FPH	Blue whiting	FPH lead to reduced viscera mass, but could not improve growth in low FM diets	(Espe, et al., 2012)
Salmo salar	Post-smolt	Controlled hydrolysis FPH	Krill	Stimulated feed intake and growth in low FM diets	(Kousoulaki, et al., 2013)
Scophthalmus maximus	Juvenile	Controlled hydrolysis FPH	Viscera free frames and heads of pollock	No effect on non-specific immunity	(Zheng, et al., 2013)

There is a distinct lack of information regarding the possible bio-activities of the hydrolysed protein component of fish silage in aquaculture diets (Table 3-2). The majority of studies that investigated bio-activities of FPH in aquafeeds employed products produced through a controlled hydrolysis process. This is despite the fact that hydrolysis products similar to those of controlled hydrolysis are formed during silage production (Kristinsson and Rasco, 2000a), and fish silages generally have lower production costs than those of controlled hydrolysis FPH. A limited number of investigations detected bio-activity of fish silage when included in aquaculture diets: fish silage functioned as feed attractants in abalone (Viana, et al., 1994), and improved non-specific immunity and pigment adsorption in finfish (Liang, et al., 2006; Torrisen, et al., 1981/1982).

A further shortcoming in the field of FPH research is that there is little comparison of the bio-activities and nutritional aspects of FPH originating from different raw materials (Bui, et al., 2014). Most studies evaluate a single FPH product originating from a single raw material. However, characteristics of specific FPH products will differ according to the original raw material and the production processes employed (Kristinsson and Rasco, 2000a; b; Murray, et al., 2003), and bio-activities and physiological responses might also differ accordingly.

Fish oils from enzymatic hydrolysis in aquaculture

There is little information available regarding the application of fish oils recovered from enzymatic hydrolysis in aquaculture. It is known that enzymatic hydrolysis facilitates the recovery of fish oil from the resulting hydrolysate (Dumay, et al., 2004; Raa and Gildberg, 1982; Rai, et al., 2010), but oil is normally seen as a component that could decrease nutritional value of FPH due to lipid oxidation (Jackson, et al., 1984b; Raa and Gildberg, 1982). Fish oil from both controlled hydrolysis and fish silage can be of sufficient quality to be used as aquafeed ingredients, with high essential fatty acid and PUFA content (Dumay, et al., 2004; Heras, et al., 1994; Rai, et al., 2010; Turchini, et al., 2003; Vidotti, et al., 2011). Dietary PUFA are known to play various important biological roles in fish nutrition and health, and are routinely supplemented to aquaculture diets to ensure good growth and health (Kiron, 2012; Trichet, 2010).

Fish oil recovered from enzymatic hydrolysates is viewed as being similar to fish oil recovered from conventional processes, even though production conditions for the two processes differ. This assumption might be particularly inappropriate in the case where fish silage is produced, due to the possible carry-over of organic acids into the lipids (Rai, et al., 2010). Dietary organic acids are known to affect production performance in aquaculture species (Goosen, et al., 2011; Lückstädt, 2008) and the presence of these substances in fish

silage and oil recovered from fish silage could therefore have additional effects when such oils are incorporated into aquafeeds.

Conclusions

The following is concluded from the literature surveyed:

I. Enzymatic protein hydrolysis is a suitable method for the production of value added products from low-value fish processing waste and by-products. Due to their nutritional characteristics, FPH products are good ingredients for use in aquaculture feeds and can substitute a proportion of FM in formulated diets, depending on the specific properties of the FPH and the cultured species.

II. Some of the products of protein hydrolysis exhibit bio-activities when included in formulated aquaculture feeds, which enhance aquaculture production through improvements in production parameters or physiologic responses in cultured animals. Due to the improvement in aquaculture production, the economic value of these FPH products is increased.

III. There is a distinct shortage of information regarding the possible bio-activities of autolysed FPH, even though the products of autolysis are peptides and amino acids (as is the case with controlled hydrolysis FPH).

IV. In fish silage research, little or no distinction has been made between whether growth and other effects are related to the protein hydrolysis products when included in aquafeeds, or as a result of the acid employed during fish silage preparation. As organic acids are known to affect animal growth performance, it is important to ascertain whether the organic acids contribute to the effects of fish silage included in aquafeeds.

V. Information regarding the use of fish oil recovered from protein hydrolysis processes in aquaculture is scarce, even though these oils could differ substantially from conventional fish oils as a result of the differences in production methods.

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Chapter 4: Novel contributions of the study

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed."
Albert Einstein

The work reported in this dissertation makes the following novel contributions:

I. Dietary oil recovered from rainbow trout silage is demonstrated to significantly enhance the non-specific cellular immunity of both Mozambique tilapia (Chapter 5) and the South African abalone (Chapter 8). It is the first report which conclusively shows the immune stimulatory potential of dietary lipids in any abalone species, and to the best of the author's knowledge, the first such report specifically in the Mozambique tilapia.

II. Dietary silage oil supplementation resulted in a favourable fillet fatty acid profile with high PUFA content in Mozambique tilapia (Chapter 5), while foot muscle fatty acid profile was unaffected in the South African abalone. The fatty acid profile of cultured South African abalone is reported (Chapter 8).

III. In Mozambique tilapia, two more effects of silage oil are shown: the silage oil exerted antimicrobial properties in the gastrointestinal tract and in the feed; however the silage oil supplementation resulted in significantly decreased length of intestinal folds in the experimental animals, although this did not affect production performance (Chapter 5).

IV. It is shown that hydrolysed fish proteins can significantly enhance the non-specific cellular immunity in both the Mozambique tilapia (Chapter 6) and the South African abalone (Chapter 9). In Mozambique tilapia, the hydrolysed protein component of fish silage caused the stimulation of cellular immunity; however, this could not be shown in abalone. Cellular non-specific immunity was enhanced in the South African

abalone by dietary commercial hydrolysed fish proteins, which is the first such report in any abalone species.

V. In both experimental species, a maximum dietary inclusion level of fish silage is demonstrated, above which the production performance of the animals starts to deteriorate. In Mozambique tilapia the decrease in growth might be attributed to negative metabolic effects of high amount dietary free amino acids and short chain peptides (Chapter 6), and in the South African abalone the negative impacts on production are correlated with the decreasing feed water stability as fish silage inclusion increases (Chapter 9).

VI. In both experimental species it is clearly demonstrated that raw material origin and inclusion level of fish protein hydrolysates are important parameters to take into account to ensure optimal production performance. In both species, significant differences were found between treatments incorporating dietary hydrolysed fish proteins at different inclusion levels (Chapters 7 and 9).

VII. A new mode of action of formic acid has been described in abalone diets, namely improved feed water stability (Chapter 10). However, this was only observed in diets that incorporated raw unprocessed trout viscera.

VIII. It is shown that formic acid is not responsible for effects on non-specific immunity or production performance in the Mozambique tilapia (Chapter 6).

IX. The study showed that both the lipid and hydrolysed protein components of chemical fish silage can be employed as bio-active feed ingredients in the two species selected for experimental work.

X. Through the contributions listed above, significant advances have been made toward the application of hydrolysed fish proteins and oil recovered from chemical fish silage in formulated diets for two aquaculture species that are relevant to the South African aquaculture industry.

Chapter 5: Fish silage oil in diets for Mozambique tilapia

"'Fish,' he said, 'I love you and respect you very much. But I will kill you dead before this day ends.'" Ernest Hemingway, The Old Man and the Sea

This chapter is also published in the form of a journal article:

Goosen, N.J., De Wet, L.F., Görgens, J.F., Jacobs, K., De Bruyn, A., 2014. Fish silage oil from rainbow trout processing waste as alternative to conventional fish oil in formulated diets for Mozambique tilapia *Oreochromis mossambicus*. Anim. Feed Sci. Technol. 188, 74 - 84.

Abstract

This chapter aimed to determine the bio-active properties and effects on production performance of silage oil recovered from rainbow trout processing waste in Mozambique tilapia (according to aims I and II of the study). Bio-activity was quantified through monitoring haematology, cellular and non-cellular non-specific immunity, and gross intestinal morphology. Additionally, the effects on gastrointestinal and feed microflora were monitored, and the final fillet fatty acid profile of experimental animals were determined. A commercial fish oil of pelagic origin was used as a control. It was found that dietary rainbow trout silage oil supplementation significantly enhanced cellular non-specific immunity by and animal survival, decreased microbial numbers both in the feed and gastrointestinal tract, and caused significantly shorter intestinal fold length in the mid-intestine of experimental animals. It was further found that the fatty acid profile of experimental animals contained high levels of PUFA, which reflected the high PUFA level of the rainbow trout silage oil. It is concluded that rainbow trout silage oil is a good feed ingredient which displays positive bio-active properties, and it can fully substitute conventional fish oil in diets for Mozambique tilapia without negatively impacting production performance.

The work described in this chapter makes the following novel contributions:

1) It clearly demonstrates the enhancement of the non-specific cellular immunity of dietary rainbow trout silage oil in Mozambique tilapia, with consequent increases in animal survival.
2) Antimicrobial action of the silage oil is demonstrated both in the feed and gastrointestinal tract of animals.
3) The effect of silage oil on the morphological structure of the gastrointestinal tract is documented.

4) It is shown that dietary rainbow trout silage oil results in a favourable fillet fatty acid profile in Mozambique tilapia, with high levels of PUFA. The fatty acid profile of the rainbow trout silage oil is also described.

Summary of authors' contributions

The respective contritubions of the authors to the article published from this chapter are:

Goosen, N.J.: Responsible for majority of trial planning, ingredient preparation, experimental design (excluding microbiological studies), feed formulation, preparation of experimental feed ingredients and experimental feeds; performed daily feeding trial duties; responsible for sampling of animals for production performance, determination of immunology, haematology, intestinal morphology and fillet fatty acid composition; performed analytical procedures to determine immunity, haematology and intestinal morphology; performed statistical analysis of data; responsible for writing draft manuscript and incorporating comments of co-authors to prepare for journal submission. Estimated % contribution to the work: 70%

De Wet, L.F.: Assisted with feed formulation, interpretation of final data and reviewed the completed manuscript. Took part in general discussion surrounding the project. Estimated % contribution to the work: 10 %

Görgens, J.F.: Assisted with interpretation of final data and reviewed the completed manuscript. Took part in general discussion surrounding the project. Estimated % contribution to the work: 5 %

Jacobs, K.: Contributed to the experimental design of microbiological studies, interpretation of final data and reviewed the completed manuscript. Estimated % contribution to the work: 5 %

De Bruyn, A.: Responsible for performing all microbiological work, including sampling of animals and determining viable bacteria in the intestine and experimental feeds. Estimated % contribution to the work: 10 %

Fish silage oil from rainbow trout processing waste as alternative to conventional fish oil in formulated diets for Mozambique tilapia *Oreochromis mossambicus*

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Key Words:

antimicrobial activity; aquaculture; fish silage; polyunsaturated fatty acids; sustainability; waste utilization

Abbreviations:

CFU – colony forming units; DHA – docosahexaenoic acid; EPA – eicosapentaenoic acid; FCR – feed conversion ratio; FL – fold length; FW – fold width; GC – gas chromatography; GIT – gastro intestinal tract; IP – inner perimeter; Jones CF – Jones condition factor; MUFA – monounsaturated fatty acids; OP – outer perimeter; PEG – poly-ethylene glycol; PSD – pooled standard deviation; PUFA – polyunsaturated fatty acids; SD – standard deviation; SFA – saturated fatty acids; SGR – specific growth rate

Abstract

Fish oil is a critical and globally constrained aquaculture feed ingredient. Oil recovered from fish silage can contribute to fish oil production and is a potential low-cost alternative for conventional fish oil in aquaculture. The purpose of the study was to evaluate silage oil recovered from rainbow trout processing waste as an alternative for commercial pelagic fish oil in diets for Mozambique tilapia, and to determine the effects on fillet fatty acid profile, production parameters, intestinal microflora and gut histology. A feeding trial with the experimental treatment incorporating silage oil, and a control incorporating commercial pelagic marine fish oil was conducted. Silage oil successfully substituted the commercial oil with no negative effects on production parameters, and improved cellular non-specific immunity by 33%. The silage oil proved to be a good source of polyunsaturated fatty acids (36.9 g/100g total fatty acids), exhibited antimicrobial properties in the feed and gastro-intestinal tract, and caused a significant shortening of intestinal folds (34.4%) in the mid-intestine of experimental fish. It is concluded that rainbow trout silage oil is a cost-effective alternative dietary oil for tilapia diets, with advantages over some conventional fish oils.

Introduction

Fish oil is a critical feed ingredient in the aquaculture industry, with approximately 74% of the global supply utilized in aquafeeds (FAO, 2011). The expanding aquaculture industry is mainly dependent on capture fisheries to provide fish oil for feed manufacture; however, the scope for increased supply of fish oil from capture fisheries is limited, as output from global fisheries has stabilised and is not anticipated to increase in future (FAO, 2012; Naylor, et al., 2009; Tacon and Metian, 2008). There are further concerns about the sustainability of using wild caught fish for fish oil production (WWF, 2012). This situation calls for the optimal utilization of existing sources of fish oil or developing aquafeeds with lower fish oil inclusion levels, in order to ensure continued supply of feed ingredients for the expanding aquaculture industry . Substitution of dietary fish oil with alternative oils in aquafeeds is, however, not readily accomplished, due to the lack of long-chain n-3 polyunsaturated fatty acids in alternative oils (Naylor, et al., 2009; Turchini, et al., 2009). Recovery of fish oil from fish processing waste can make a contribution to annual fish oil production, as is the case with fish meal where by-product recovery contributes substantially to annual fish meal output (FAO, 2012).

Recovery of fish oil from fish silage could have advantages in oil quality compared to conventional fish oil production, due to differences in recovery methods. Preparation of fish silage is an enzymatic method of protein hydrolysis that is accomplished at mild temperatures, which may prevent lipid oxidation that occurs during high temperature (up to 95 °C) conventional fish oil recovery processes (Dumay, et al., 2004; EFSA, 2010). The protein hydrolysis facilitates the recovery of fish oil, as the aqueous layer containing protein hydrolysate is readily separated from the floating oil, which is then easily recovered (Dumay, et al., 2004; Raa and Gildberg, 1982). The ensiling process is well suited to recovery of fish oil from relatively small amounts of fish waste, since the process is fairly simple with low initial capital expenditure, and nearly independent of economies of scale (De Arruda, et al., 2007; Raa and Gildberg, 1982).

Silage oil derived from fish processing waste can find application as feed ingredient in the aquaculture industry due to its lower production costs that result from inexpensive feedstock and processing methods (De Arruda, et al., 2007). Aquaculture is dependent on cost effective lipid sources for the provision of energy and essential nutrients like polyunsaturated fatty acids in aquafeeds, and silage oil can potentially serve as low cost replacement of conventional fish oil. The aquafeed industry currently

competes with the higher-value human nutrition sector for fish oil (FAO, 2012), where it is an important source of functional n-3 polyunsaturated fatty acids (Mourente and Bell, 2006). As a product derived from fish processing waste, silage oil has the further advantage that it does not lead to increased wild fish harvesting and will thereby contribute to improving the sustainability of aquafeeds.

Current literature on the utilisation of silage oil as aquafeed ingredient is limited. The aim of the study was, therefore, to evaluate silage oil recovered from rainbow trout processing waste as an alternative for commercially available pelagic fish oil in diets for Mozambique tilapia *Oreochromis mossambicus*.

Materials and Methods

Experimental design, animals and system

A feeding trial was used to evaluate the efficacy of rainbow trout silage oil as dietary ingredient for Mozambique tilapia *Oreochromis mossambicus*. Parameters investigated included production performance, fatty acid composition of fillets, haematology, non-specific immunity, intestinal microbiology and intestinal morphology. The trial employed two diets: a control (C) diet supplemented with 100 g/kg commercial fish oil of mixed pelagic origin from the South African coastline (Energy Oil, Wadeville, South Africa), and an experimental diet (SO) containing 100 g/kg silage oil (production method described below). Each treatment was replicated 5 times in 5 randomly selected tanks, with each tank initially containing 8 randomly allocated, sex reversed Mozambique tilapia (40 fish initially in each treatment) with mean weight ± standard deviation (SD) of 42.5 ± 5.08 g.

The experimental system is located at the Welgevallen Experimental Farm, Stellenbosch University and consists of an indoor, heated recirculation system, with 80 litre tanks supplied with continuous aeration. Water temperature was between 26 °C and 29 °C throughout. The trial was run for a period of 52 days and fish were sampled for evaluation of non-specific immunity, haematology and growth parameters at two sampling times (Sampling 1 and Sampling 2, respectively 23 and 52 days after trial initiation). Intestinal morphology and fillet fatty acid profile were only determined at the conclusion of the trial (at Sampling 2), while intestinal microbial communities were evaluated at trial initiation and at each sampling.

Silage oil recovery and diet preparation

Fresh rainbow trout viscera were ensiled with 25 g/kg formic acid (85%) and protein hydrolysis proceeded at ambient temperature (15 °C - 25 °C). Viscera were obtained from Three Streams Smokehouse, Franschhoek. Silage oil was recovered after 3 days, stabilised through the addition of antioxidant (1 ml/litre of butylated hydroxytoluene/ butylated hydroxyanisole mixture, Oxipet L, Bitek) and stored in plastic containers at 4 °C for 7 days until diet preparation.

Diets were prepared by mixing commercial feed pellets with oil in a ratio of 9:1 (w/w) and stored in airtight containers. The basal diet for all treatments consisted of a fish meal based diet containing 480 g/kg crude protein, 60 g/kg crude fat, 70 g/kg ash and 25 g/kg crude fibre at a moisture content of 100 g/kg. See Table 5-1 for final composition of experimental feeds. Proximate feed analyses were done according to standard procedures: Nitrogen was determined according to method 992.15 (AOAC, 1992) and crude protein was determined as Nx6.25. Crude fat was determined according to Lee et al. (1996); feed moisture (AOAC, 2002a) and ash (AOAC, 2002b) were done according to AOAC.

Experimental and analytical procedures

Fish were fed at a rate of 30 g feed/kg body weight per day in 3 equal rations during weekdays and 2 equal rations over weekends. At every feeding, mortalities were removed from containers and recorded. All handling of live animals happened subsequent to being anaesthetized using AQUI-S (Lower Hutt, New Zealand). Fish were weighed to 0.01 g and measured to the closest mm (total length) at the initiation of the trial and at Sampling 1 and Sampling 2, using a laboratory scale and ruler. At the conclusion of the trial, fish height was also recorded in order to compute the Jones Condition Factor (Jones CF).

Samples of the silage and commercial fish oils were analysed in triplicate for determination of fatty acid profiles. At the trial conclusion two randomly selected male fish from different tanks were slaughtered per treatment for analysis of fillet fatty acid profiles. Fillets were stored in airtight plastic bags at -80 °C until analyzed. Lipids were extracted from each tilapia fillet according to Folch et al. (1957) and the fatty acid profile of each fillet was characterized separately using gas chromatography (GC).

GC samples were prepared through transmethylation with a 19:1 methanol:sulphuric acid mixture. An internal standard of heptadecanoic acid (C17:0, 98%, Sigma Cat no. H3500) and a standard fatty acid methyl ester mixture (Supelco 37

Component fatty acid methyl ester mix Cat no. 47885-U, Bellefonte, USA) were used. The gas chromatograph is a Thermo Finnigan Focus GC (Thermo Electron S.p.A., Milan, Italy), fitted with BPX70 column, length 60 m and inner diameter 0.25 mm (SGE International, Victoria, Australia); carrier gas was hydrogen at 20 ml/min with a split of 20:120; initial and final temperatures were 60 °C and 120 °C respectively, with a rate of increase of 1.7 °C/min. Injector and detector temperatures were 220 °C and 260 °C, respectively.

Production performance parameters were determined from the measurements of weight, length and height of animals and the amount of feed consumed. Specific growth rate (SGR) was calculated as SGR = $100*[\ln(W_{i+1}/W_i)/(t_{i+1} - t_i)]$, with *W* and *t* in units of grams and days, respectively, at sampling time *i*. The feed conversion ratio (FCR) was determined as the gain in wet fish weight per unit dry feed given, and Jones CF was calculated as Jones CF = $1000*W/L^2H$, where *W* is weight (g), *L* is length (cm) and *H* is height (cm) respectively (Jones, et al., 1999).

After blood sampling at the conclusion of the trial, one fish per tank was specifically slaughtered for histological purposes and the viscera removed. The intestinal tract was unwound on a smooth wet surface to prevent mechanical damage, and three portions of 2 - 3 cm each were sampled: the proximal intestine (2 - 3 cm from the stomach), the mid-intestine (halfway between the stomach and anus) and the distal intestine (2 - 3 cm from the anus). Samples were preserved in 10% (w/v) formalin solution, processed according to standard histology protocols and stained with hematoxylin and eosin. Stained samples were evaluated under a light microscope and analyzed using NIS-Elements version 3.00 software.

Viable cell counts were performed to determine the number of viable cells in the gastrointestinal tract (GIT). At the initiation of the trial, a baseline microbial count was established by slaughtering 3 fish from the same container as the experimental animals and colony forming units (CFU) from the GIT was determined. At Sampling 1 and Sampling 2, three random fish per treatment were used to determine the CFU count from the GIT (the animals sampled for microbiology were the same ones sampled for determination of haematology and non-specific immunity).

To determine the CFU, entire GITs were aseptically removed and placed in 10 ml sterile saline solution (9 g/l NaCl) containing acid-washed glass beads (Sigma, South Africa) and stored at 4 °C until used. Within 24 hours after sampling, each GIT was homogenized and dilutions were plated in triplicate onto Tryptone Soya Agar. Plates were incubated at 26 °C for 48 hours after which colony forming units were counted for each plate.

At the end of the trial, samples of feed were analysed for total viable bacteria using plate counts. For each feed, 1 g of ground pellets was suspended in 9 ml sterile saline solution and 1 ml of the suspension was plated out in triplicate onto nutrient agar (meat extract 1 g/l, yeast extract 2 g/l, peptone 5 g/l, sodium chloride 8 g/l and agar 15 g/l). Plates were incubated at 26 °C for 48 hours to determine total colony forming units.

At each sampling, one fish per replicate (five per treatment) was sampled for determination of haematology and non-specific immunity. Sampled animals were not replaced in tanks after Sampling 1. Fish were anaesthetized prior to blood collection, which was done through caudal puncture using 27 gauge heparinzed syringes (Stellenbosch University Ethics Approval Certificate number: SU_ACUM_00018). Blood samples were divided: half was put into Eppendorf tubes with sodium heparinate as anti-coagulant, and the remaining blood was allowed to clot overnight at 4 °C in untreated Eppendorf tubes. Blood serum was collected by centrifuging the clotted blood samples at 500 × *g* for 5 minutes. Serum was stored at -20 °C until analysis. Haematocrit values, manual cell counts and estimation of phagocytic activity of leukocytes were completed using heparinized blood.

Manual cell counts of erythrocytes and leukocytes were performed using a light microscope and haemocytometer. Haematocrit values were determined directly after sampling by centrifuging samples in glass capillary tubes for 5 minutes in a microhaematocrit centrifuge and expressed as erythrocyte volume as a proportion of total blood volume.

The method of Cai et al. (2004) was used for determination of phagocytic activity of leucocytes, with some modifications: volumes of heparinised blood and suspended baker's yeast used during the procedure were 250 µl in order to ensure equal volumes. Furthermore, to ensure frequent mixing and prevent partial blood clotting, the time intervals for mixing of blood/yeast mixture during incubation were decreased to 5 minutes. Baker's yeast suspension was prepared from commercial baker's yeast (NCP, Modderfontein, South Africa) by autoclaving and re-suspending in phosphate buffered saline. Phagocytic activity was determined as follows: 250 µl of blood and baker's yeast were put in an Eppendorf tube and incubated for 30 minutes at room temperature, and mixed every 5 minutes through careful inversion. Blood smears were prepared after incubation, then fixed with methanol and stained with 4% Giemsa-solution. Approximately 100 leukocytes per slide were counted under a light microscope. Phagocytic activity was determined as the proportion of total leucocytes counted that ingested yeast cells, and expressed as an index value relative to the mean phagocytic activity of the control.

The method Sankaran and Gurnani (1972) was used to determine lysozyme activity. Standard lysozyme (Sigma) solution or blood serum (50 µl) was added in duplicate to 250 µl of a suspension of 0.075% (w/v) *Micrococcus lysodeikticus* (Sigma) in phosphate buffer (0.05 M at pH 6.2), in a 96-well microplate. After 5 minutes incubation, the absorbance at 530 nm was measured, and again after 20 minutes. The decrease in absorbance was plotted against the known lysozyme concentration to prepare a standard curve; serum lysozyme concentrations were calculated from the standard curve.

Serum total protein was measured spectrophotometrically using the linearized Bradford assay (Zor and Selinger, 1996) with bovine serum albumin as standard. Immunoglobulin was determined according to Ardó et al. (2008): equal volumes of serum and 12% (w/v) poly-ethylene glycol (PEG, average molecular weight of 10,000 Dalton, Sigma) solution were added to Eppendorf tubes (giving a final concentration of 6% (w/v) PEG) and incubated at room temperature for 2 hours. Tubes were then centrifuged at 14000 rpm for 5 minutes and the total protein concentration of the supernatant determined using the linearized Bradford method. Immunoglobulin concentration was calculated as the difference between protein concentration of the serum and supernatant after PEG precipitation (also taking into account the dilution by the PEG solution).

Statistical analysis

All means were compared using one-way analysis of variance (ANOVA) using Statistica version 10 software (StatSoft, Inc.). The following linear statistical model was employed: $Y_i = b_0 + b_1X_i + \varepsilon_{i,}$ where Y_i is the dependent variable, X_i is the effect of silage oil and ε_i is the random error. The values of b_0 and b_1 were determined according to the least squares method. Differences between means were viewed to be significant for $p < 0.05$.

Results

Fatty acid profiles of silage oil and tilapia fillets

The fatty acid composition of the silage oil and pelagic fish oil control are given in Table 5-2. The silage oil has lower saturated fatty acid (SFA) content than the control oil (25.4 g/100g fatty acids vs. 34.6 g/100g) and higher mono-unsaturated fatty acid (MUFA) levels (37.6 g/100g vs. 27 g/100g), while polyunsaturated fatty acid (PUFA) content did not differ significantly (36.9 g/100g in silage oil and 38.3 g/100g in control oil). Within the PUFA component of the silage oil the most prevalent fatty acids were C18:2n6*cis*, C22:5n3 and C22:6n3 with approximately equal amounts (11.7 g/100g, 11.5 g/100g and 11.4 g/100g respectively) of each, yielding a final (n-6):(n-3) ratio of 0.54. In the marine fish oil, the largest contribution to the PUFA component was made by C22:5n3 and C22:6n3 (23.8 g/100g and 11.3 g/100g of total fatty acids respectively) and a (n-6):(n-3) ratio of 0.05 was found.

Production performance parameters

Despite sex-reversal treatment, breeding and territorial behaviour was exhibited by experimental animals after Sampling 1. In each tank, a male fish became dominant after Sampling 1 and established an area that it defended from both male and female intrusion, and frequent fighting occurred between dominant and non-dominant males. The dominant fish in each tank could be identified by their black body colouration. The territorial behaviour was consistent with that described by Shubha and Reddy (2011). Decreased feed intake was observed among female fish. As a corrective measure, the gender of each fish was recorded (determined visually through inspection of urogenital papillae and secondary sexual features) at Sampling 2 in order to evaluate production performance separately for each gender where possible. Results of production parameters are summarised in Table 5-3 and cumulative mortalities are shown in Figure 5-1. At Sampling 1, every tank in the control contained 8 fish, except one tank which contained 7; every tank in the silage oil treatment contained 8 fish. At the trial conclusion (Sampling 2), the 5 tanks in the control contained 6, 4, 2, 2 and 4 fish respectively, while the silage oil treatment contained 6, 5, 4, 3 and 7 fish in the different tanks.

There were no statistically significant differences between treatments in SGR or FCR; neither were there any differences in mean Weight, Length or Height for male or female fish between treatments. Jones CF was significantly higher (9.0%) in male fish

for treatment SO, while no differences were found in female fish. Mortalities increased sharply after day 30 for treatment SO and day 38 for the control diet. At the conclusion of the trial, cumulative mortalities were 0.23 and 0.37 (measured as a proportion of initial number of fish in each treatment) for treatment SO and the control, respectively. Within each treatment, male fish were heavier at the end of the trial than female fish.

Gut histology

The results of the various measurements of the intestinal histology are shown in Table 5-4. Silage oil resulted in mean intestinal fold length (FL) decreasing significantly by 34.4% in the mid intestine compared to the control. All other morphological measurements and calculated intestinal parameters in treatment SO that are dependent on FL (inner perimeter length, fold length : fold width, inner perimeter : outer perimeter, fold length : outer perimeter) also differed significantly from the control in the mid-intestine. No significant differences were detected in the proximate or distal regions of the intestine.

Microbiology of intestine and feed

Results for microbial counts for the two feeds at the end of the trial, and CFU counts of the GIT at trial initiation and at each sampling are shown in Figure 5-2(A) and (B), respectively. Feed CFU counts (mean ± SD) were significantly lower in the silage oil treatment (6.67 ± 11.6 cells/ml) than in the control (60.0 ± 26.5 cells/ml). In the silage oil treatment, bacterial growth was only observed on the plates with the lowest dilution (10^{-1}), and only on one of the three replicate plates. For the silage oil treatment, the total CFU counts in the GIT were significantly lower than the control at Sampling 1 (63.8 ± 3.66 cells/ml for silage oil treatment and 145.1 ± 22.0 cells/ml for the control) and Sampling 2 (10.3 ± 5.14 cells/ml and 88.3 ± 20.0 cells/ml, respectively). By the end of the trial the CFU in the GIT for the silage oil treatment decreased significantly from 100.44 ± 28.85 cells/ml to 10.3 ± 5.14 cells/ml, but no detectable change occurred in the control.

Haematology and non-specific immunity

Non-specific immunity was characterised through evaluation of cellular response mechanisms (phagocytic activity of leucocytes) and non-cellular mechanisms (lysozyme, immunoglobulin and total protein concentration of serum). Data for non-specific immunity for the different treatments are presented in Table 5-5. Cellular

immune response in the silage oil treatment was enhanced by 33% at Sampling 1. Phagocytic activity of leucocytes could not be determined successfully at sampling 2, as the heparin treated blood coagulated upon contact with the yeast suspension and reliable blood smears could not be made. There were no significant differences between treatments for any of the other non-specific immune parameters.

Concentrations of erythrocytes and leucocytes, and hematocrit values are presented in Table 5-5. There were no significant differences between treatments at either sampling date. The leucocyte counts decreased from Sampling 1 to Sampling 2, by 34.0% and 44.8% for the silage oil treatment and the control, respectively.

Discussion

Fatty acid profiles

The fatty acid profile of silage oil compared favourably to the commercial oil. The silage oil was a good source of the important polyunsaturated fatty acids, which constituted 36.9 g per 100 g total fatty acids, and did not differ significantly from the control oil. The silage oil was further also a good source of the n-3 fatty acid docosahexaenoic acid (DHA or C22:6n3), which constituted 11.4 g/100g of total fatty acids. Fish oils are valued for their high PUFA content, especially the long chain n-3 fatty acids (Kolanowski and Laufenberg, 2006). Among the n-3 fatty acids in fish oil, eicosapentaenoic acid (EPA or C20:5n3) and DHA are of particular interest in human nutrition due to their role as essential nutrients for normal development and health (Connor, 2000). These fatty acids are mainly ingested by humans via fish and fish oil products (Kolanowski and Laufenberg, 2006). Enrichment in PUFA of aquaculture products can be achieved by supplementing aquafeeds for certain species with oils rich in PUFA, which are then deposited in the organism's tissues and eventually ingested by humans.

Supplementation of diets for Mozambique tilapia with high PUFA fish oils resulted in favourable fatty acid profiles high in PUFA. Of the total fatty acids of fillets, PUFA comprised 41.1 g/100g fatty acids in the silage oil treatment and 35.7 g/100g in the control. These values are high compared to wild caught *O. mossambicus*, where PUFA levels varied between 8.0 g/100g and 18.4 g/100g (Dhanapal, et al., 2012; Rahman, et al., 1995; Ravichandran, et al., 2011). Furthermore, n-3 fatty acid levels were higher than n-6 fatty acids with a n-6:n-3 fatty acid ratio of 0.27 in the control and 0.28 in treatment SO. The modern Western diet is thought to be partially deficient in n-

3 fatty acids and an increased consumption of n-3 fatty acids relative to n-6 fatty acids is desirable (Candela, et al., 2011; Simopoulos, 2008). The most common PUFA present in fillets of fish from both treatments was the important n-3 fatty acid DHA (with 23.9 g/100g and 18.8 g/100g of total fatty acids in treatment SO and the control, respectively).

The different dietary oils resulted in differences in the fillet fatty acid composition of the experimental animals (Table 5-2). Total fillet SFA levels for the silage oil diet were significantly lower than the marine fish oil diet, which reflects the lower SFA level of silage oil when compared to the marine fish oil. No differences in total MUFA or PUFA levels occurred. Within the SFA portion of fillets, there were significant differences between the minor fatty acids C14:0 (0.4 g/100g vs. 1.6 g/100g of total fatty acids) and C15:0 (0.21 g/100g vs. 0.40 g/100g), where the silage oil resulted in significantly lower levels in both cases. One MUFA (C16:1) was significantly lower in treatment SO compared to the control (1.7 g/100g vs 3.6 g/100g of total fatty acids), but there were no significant differences among fatty acids in the PUFA component of fillets.

Production performance

The results of the trial showed that the rainbow trout silage oil was able to fully substitute marine pelagic fish oil in tilapia diets without negatively impacting production performance. Positive effects of silage oil inclusion were lower mortalities for treatment SO than for the control, and higher average Jones CF in male fish. Condition factors are mostly used in fisheries management sciences and are characterized by the assumption that fish with a higher condition factor (heavier fish of a specific length) are healthier animals and therefore in a better general condition (Jones, et al., 1999). Male fish in treatment SO at the end of the trial could therefore be said to have been in better general condition than those in the control group. It is unclear whether this was due to an effect of dietary silage oil addition, or a consequence of the lower mortality observed in treatment SO. Growth disparities between male and female fish within each treatment was probably a result of the breeding and territorial behaviour that developed after Sampling 1, coupled with the decreased feed intake of females observed during this phase of the experiment.

The mortalities experienced during the trial could have contributed to the breeding and territorial behaviour. It has been shown that lower stocking densities lead to increased spawning activity in *O. mossambicus* (Shubha and Reddy, 2011). Mortalities experienced in the tanks effectively decreased fish stocking density and could thereby have led to increased breeding behaviour.

Gut histology

The substitution of commercial fish oil with silage oil affected the intestinal histology of the experimental fish; however, these effects were limited to the mid-intestine. The shorter intestinal folds were not detrimental to animal performance in the current trial, as there were no negative impacts on animal production efficiency (SGR or FCR) or health status (mortalities or non-specific immunity). There is some ambiguity around whether the shortening of intestinal folds will be positive or detrimental to animal performance. Shorter intestinal folds will result in a smaller nutrient absorption area for the uptake of nutrients and might affect absorption efficiency (Gargiulo, et al., 1998). Specifically in tilapia, significantly increased fold length was seen in the proximal and distal portions of the intestine and was thought to be related to a non-significant improvement in FCR (Pirarat, et al., 2011), while in pigs the opposite was seen when dietary formic acid addition led to a slight decrease in the length of villi within the proximal jejunum, but a concurrent significant improvement in feed conversion was recorded (Manzanilla, et al., 2004); however, the specific relation between villi height and feed conversion was unclear. In the current trial, a non-significant improvement in FCR for the silage oil diet coincided with a significantly decreased length of intestinal folds in the mid-intestine; but again the relation between fold length and FCR (if any), is unclear.

The cause of shortened intestinal folds in the mid intestine in treatment SO was not established, but might be a result of some formic acid in the silage oil. Formic acid caused shortened villi in the jejunum in pigs (Manzanilla, et al., 2004). Formic acid is used in the preparation of fish silage and from distribution coefficient data, a small proportion of the formic acid used in silage preparation will also dissolve in the lipid (less polar) phase (Sangster, 1989). Although the level of formic acid in the lipid phase was not determined, it might be responsible for the effects seen in the tilapia mid intestine.

Microbiology of intestine and feed

The silage oil exhibited antimicrobial properties, both in the feed and in the GIT of tilapia. This antimicrobial property of the silage oil might be advantageous to producers, both in the feed and in the GIT. Lower bacterial numbers in aquafeeds will improve feed hygiene, while lower bacterial numbers in the GIT will decrease competition for nutrients between the host animal and the microflora of the GIT. In terrestrial animal husbandry, it is commonly believed that production efficiency can be

improved through a reduction in the number of intestinal microbes (Hardy, 2002), as more nutrients are available to the host animal.

The specific cause of the decreased microbial numbers in the feed and GIT of fish in the current trial is not known, but again might be related to the presence of formic acid in the silage oil. Formic acid is known to have antimicrobial properties and a salt of formic acid (potassium diformate) has been shown to alter the composition of the intestinal microflora in tilapia (Zhou, et al., 2009) while also decreasing bacterial numbers in the faeces of tilapia (Ng, et al., 2009).

Haematology and non-specific immunity

Proper immune function is key to ensure disease resistance in animals (Kiron, 2012). The improvement of cellular immune response by the silage oil may have contributed to the decreased mortality observed at the end of the trial in treatment SO (0.23 vs. 0.37 in the control). The reason for improved phagocytic activity of leukocytes in fish fed the silage oil diet is not obvious, but might be related to an improvement in dietary fatty acid balance. The fatty acid balance of leucocyte membranes are important and might affect phagocytic capacity (Montero, et al., 2003). Other investigators found that cellular immunity of gilthead seabream was negatively affected when fish oil was partially substituted by rapeseed and soybean oil, while no effects were seen on plasma lysozyme concentration (Montero, et al., 2003). The effects on cellular immunity were linked to possible imbalances created in the leucocyte membrane fatty acid composition by the different dietary oils. In the current trial, although both oils had high total PUFA content, there were many fatty acids of which the levels in the two oils were significantly different. It is therefore possible that dietary silage oil resulted in a more balanced fatty acid intake, which in turn resulted in improved cellular non-specific immunity through ensuring a balanced combination of fatty acids in leukocyte membranes.

Dietary silage oil inclusion did not affect any of the measured blood parameters. However, leucocyte numbers decreased between Sampling 1 and 2. This decrease in leukocyte numbers also coincided with increased mortalities and could indicate increased stress levels in the experimental fish. Changes in white blood cells can be an indicator of stress in fish (Barton and Iwama, 1991), which in turn can lead to increased mortalities.

Conclusion

It is concluded that silage oil recovered from rainbow trout waste is a cost effective feed ingredient that has advantages over some conventional fish oils in tilapia feeds. Dietary silage oil effectively substituted the control oil without any negative effects on production performance, while improving cellular non-specific immunity and simultaneously decreasing total mortalities. Silage oil also exhibited significant antimicrobial effects in the feed and GIT of experimental fish. These results confirm that utilisation of the ensiling process for silage oil recovery will increase overall production of fish oil, which is a critical ingredient for continued aquaculture expansion.

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Tables

Table 5-1 Formulation and final composition of the control (C) and silage oil supplemented (SO) diets.

	Diet	
Feed ingredient (g/kg)	**C**	**SO**
Basal diet	900	900
Control oil	100	0
Silage oil	0	100
Feed composition (g/kg)		
Moisture	82	81
Ash	101	102
Crude fat	144	144
Crude protein	450	452
Crude fibre	23	23
Energy content (MJ/kg)	19.2	19.2

Table 5-2 Fatty acid profiles of oils used in feeds, and of fillets of experimental fish. Data are presented as g/100g total fatty acids. Pooled standard deviation and P-values are applicable to fatty acid profiles of fillets only. C – control, SO – silage oil treatment

	Oils (g/100g)		Fillets (g/100g)			
Fatty acid	**C**	**SO**	**C**	**SO**	**PSD[a]**	**P-value**
C12:0	0.01	0.02	ND[b]	ND	-	-
C14:0	8.03	1.19	1.60	0.40	0.18	0.02
C15:0	0.13	0.08	0.38	0.21	0.03	0.02
C16:0	25.1	22.0	25.5	21.9	1.05	0.08
C18:0	0.97	1.04	12.7	14.3	1.34	0.35
C20:0	0.17	0.18	0.66	0.40	0.34	0.52
C21:0	0.01	0.10	0.18	0.22	0.09	0.69
C22:0	0.14	0.36	1.23	1.26	0.28	0.93
C24:0	0.05	0.39	ND	ND	-	-
C14:1	0.01	0.02	ND	ND	-	-
C15:1	0.03	0.02	ND	ND	-	-
C16:1	14.1	10.9	3.62	1.67	0.38	0.04
C18:1n9*cis*	12.6	26.2	15.7	17.8	5.32	0.73
C18:1n9*trans*	0.04	0.06	0.26	0.23	0.08	0.74
C20:1	0.05	0.08	0.24	0.25	0.10	0.94
C22:1n9	0.03	0.09	0.45	0.26	0.23	0.49
C24:1	0.18	0.34	ND	ND	-	-
C18:2n6*cis*	0.56	11.7	4.96	5.68	1.04	0.56
C18:2n6*trans*	0.03	0.02	ND	ND	-	-
C18:3n6	0.20	0.36	0.45	0.45	0.19	0.98
C18:3n3	1.02	0.59	2.34	1.50	0.27	0.09
C20:2	0.05	0.20	0.65	1.01	0.31	0.37
C20:3n6	0.33	0.09	1.40	1.52	0.24	0.67
C20:3n3	0.03	0.04	0.21	0.17	0.06	0.56
C20:4n6	0.79	0.61	1.08	0.72	0.14	0.12
C20:5n3	0.15	0.17	ND	ND	-	-
C22:2	0.05	0.17	ND	ND	-	-
C22:5n3	23.8	11.5	7.57	6.03	0.55	0.11
C22:6n3	11.3	11.4	18.8	23.9	4.74	0.39
SFA[c]	34.6	25.4	42.3	38.7	0.83	0.05
MUFA[d]	27.0	37.6	20.0	20.0	5.06	0.99
PUFA[e]	38.3	36.9	37.5	41.1	4.22	0.48
PUFA:SFA	1.11	1.45	0.89	1.06	0.09	0.18
(n-6):(n-3)	0.05	0.54	0.27	0.28	0.08	0.87

[a] Pooled standard deviation; [b] Not detected; [c] Saturated fatty acids; [d] Monounsaturated fatty acids; [e] Polyunsaturated fatty acids

Table 5-3 Production performance data for the trial. Values are given as means with pooled standard deviation. C – control diet; SO – silage oil supplemented diet

	Treatment			
	C	**SO**	**PSD[a]**	**P-value**
FCR and SGR for all fish, calculated over the entire trial				
FCR[b]	2.4	1.8	0.95	0.96
SGR[c]	1.0	1.1	0.37	0.82
Measurements of male fish at Sampling 2				
Jones CF[d]	52.5	57.2	3.53	0.009
Weight (g)	89.0	92.1	12.1	0.54
Length (mm)	179	178	9.66	0.90
Height (mm)	52.8	50.7	2.96	0.13
Measurements of female fish at Sampling 2				
Jones CF	58.1	56.3	3.11	0.30
Weight (g)	56.8	59.5	7.04	0.42
Length (mm)	150	154	5.18	0.18
Height (mm)	43.2	44.2	2.31	0.44

[a] Pooled standard deviation

[b] Feed conversion ratio

[c] Specific growth rate

[d] Jones condition factor

Table 5-4 Data for intestinal morphology determined in the different regions of the intestine. Values are given as means with pooled standard deviation. All lengths are in units of µm and ratios are dimensionless. C – control diet; SO – silage oil supplemented diet

	Treatment			
	C	**SO**	**PSD[a]**	**P-value**
Proximal intestine				
FL[b]	301	323.3	42.9	0.44
FW[c]	114	125.1	21.4	0.44
IP[d]	25270	28593	4100	0.24
OP[e]	6013	7256	1003	0.09
FL : FW	2.68	2.65	0.56	0.93
IP: OP	4.26	3.98	0.71	0.55
FL : OP	0.05	0.05	0.01	0.40
Mid intestine				
FL	231	152	31.9	0.004
FW	109	97.4	16.2	0.28
IP	13947	8735	3494	0.046
OP	3948	3661	587	0.46
FL : FW	2.13	1.54	0.25	0.005
IP: OP	3.53	2.35	0.70	0.029
FL : OP	0.06	0.04	0.01	0.015
Distal intestine				
FL	159	183	46.2	0.43
FW	87.3	96.3	14.0	0.34
IP	7543	7658	2772	0.94
OP	2846	3162	395	0.24
FL : FW	1.87	1.88	0.52	0.96
IP: OP	2.60	2.41	0.73	0.69
FL : OP	0.06	0.06	0.02	0.89

[a] Pooled standard deviation; [b] Fold length; [c] Fold width; [d] Inner Perimeter; [e] Outer perimeter

Table 5-5 Haematology and non-specific immunity parameters measured during the trial. Values are reported as means with pooled standard deviation. C – control diet; SO – silage oil supplemented diet.

	Treatment			
	C	**SO**	**PSD[a]**	**P-value**
Sampling 1 (23 days)				
Erythrocytes (10^6/mm^3)	2.91	3.01	0.46	0.76
Leukocytes (10^5/mm^3)	1.45	1.47	0.27	0.92
Hematocrit (ml/ml)	0.37	0.38	0.04	0.68
Phagocytic activity	1.00	1.33	0.12	0.002
Lysozyme (µg/ml)	10.1	5.79	7.62	0.53
Immunoglobulin (mg/ml)	2.60	2.60	0.90	0.97
Total protein (mg/ml)	18.1	20.4	2.19	0.22
Sampling 2 (52 days)				
Erythrocytes (10^6/mm^3)	2.63	3.16	0.87	0.46
Leukocytes (10^5/mm^3)	0.80	0.97	0.18	0.29
Hematocrit (ml/ml)	0.33	0.354	0.03	0.47
Phagocytic activity	-	-		
Lysozyme (µg/ml)	6.93	7.18	4.11	0.95
Immunoglobulin (mg/ml)	2.47	1.46	1.70	0.51
Total protein (mg/ml)	19.0	17.1	2.16	0.26

[a] Pooled standard deviation

Figures

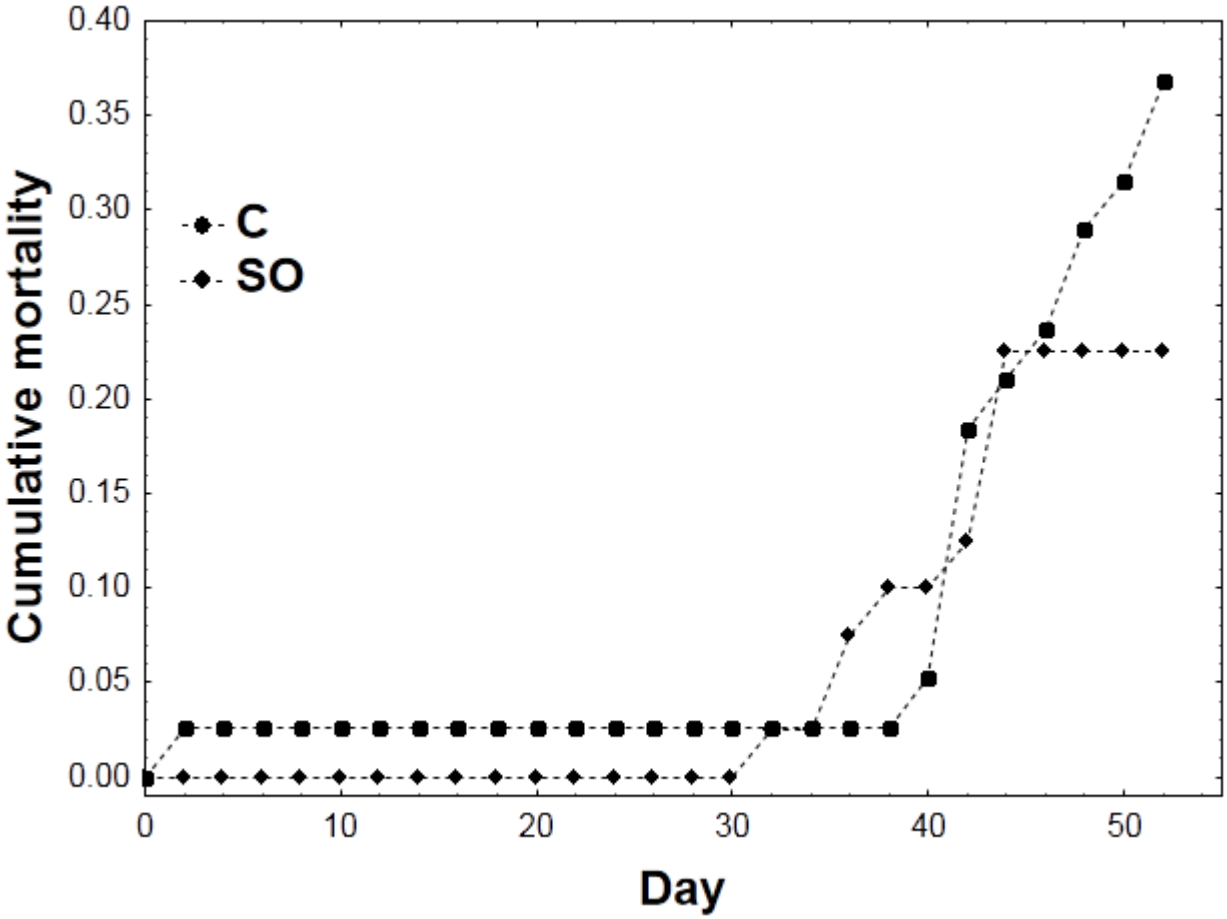

Figure 5-1 Cumulative mortalities recorded during the trial, as proportion of initial number of animals in each treatment. C – control diet; SO – silage oil diet

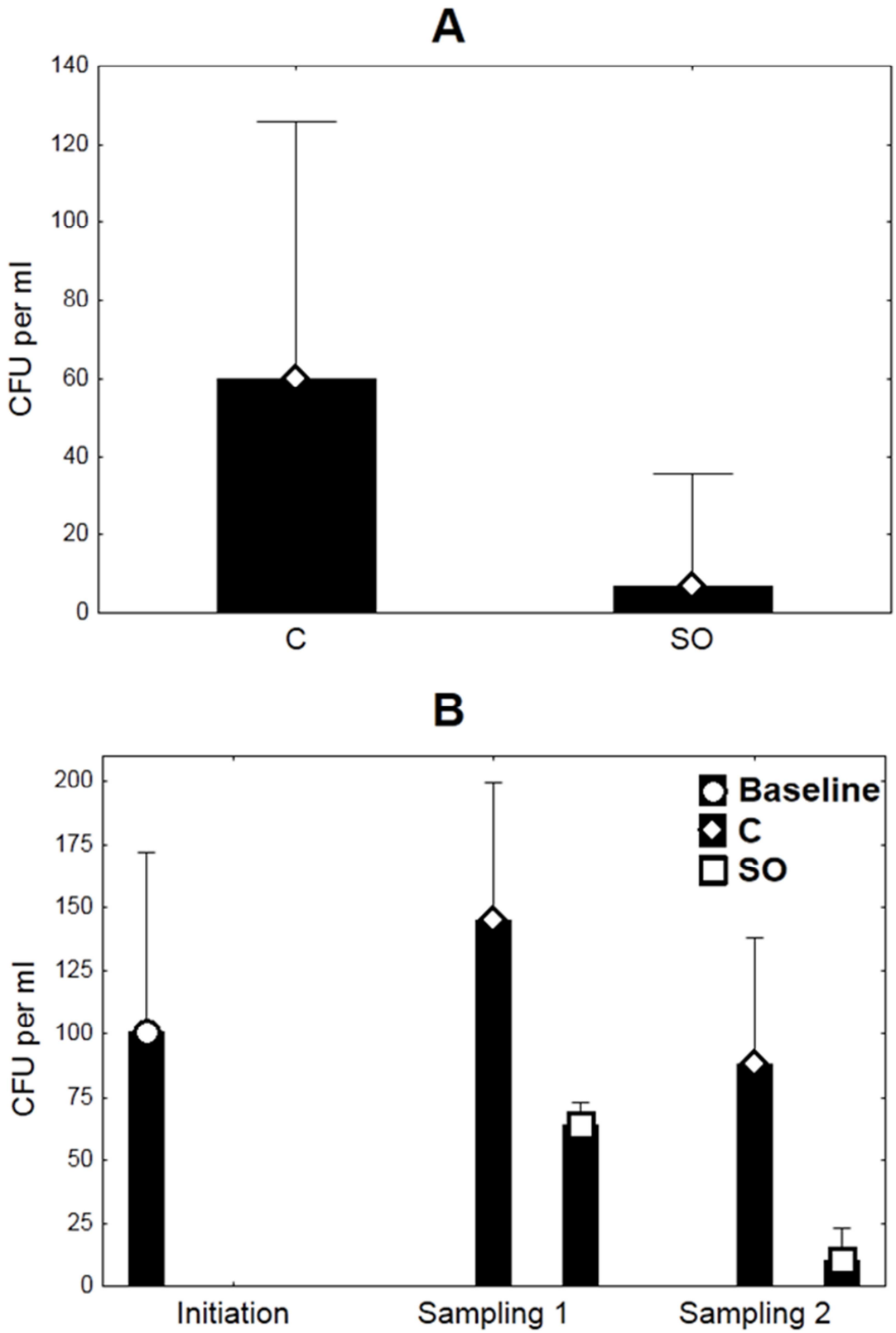

Figure 5-2 Colony forming units (CFU) for feeds incorporating control (C) and silage (SO) oils (A) and CFU of the gastro intestinal tract at trial initiation and at both samplings, for each treatment (B). Data are shown as mean values ± 95% confidence intervals.

Chapter 6: Fish silage and formic acid in diets for Mozambique tilapia

"I may not be as strong as I think, but I know many tricks and I have resolution."
Ernest Hemingway, The Old Man and the Sea

"For of Him and through Him and to Him are all things, to whom be glory forever. Amen." Romans 11:36

This chapter is also published in the form of a journal article:

Goosen, N.J., De Wet, L.F., Görgens, J.F., 2014. Rainbow trout silage as immune stimulant and feed ingredient in diets for Mozambique tilapia (*Oreochromis mossambicus*). Aquaculture Research. doi:10.1111/are.12497, 1 - 12.

Abstract

This chapter investigated bio-active properties and effects on production performance of chemical fish silage, at two dietary inclusion levels in diets for Mozambique tilapia (according to aims I and II of the study) . Further, the possible effects of formic acid used during silage preparation were investigated in order to distinguish between effects of silage and that of the acid (according to aim IV of the study). Bio-activity was quantified through monitoring haematology, cellular and non-cellular non-specific immunity, and gross intestinal morphology. It was found that dietary fish silage supplementation can significantly enhanced cellular non-specific immunity and animal survival; however, effects are dependent on the dietary silage inclusion level. Excessive inclusion levels resulted in decreased production performance and increased mortality. It was further shown that formic acid was not responsible for the improved immunity; therefore the improved cellular immune function is attributable to the hydrolysed protein component of the fish silage. It is concluded that chemical fish silage from rainbow trout processing waste is a good feed ingredient in formulated diets for Mozambique tilapia, and can contribute to improved fish health through stimulation of cellular non-specific immunity. Further, it can contribute to dietary protein supply and essential amino acid requirements.

The work described in this chapter makes the following novel contributions:

1) Dietary fish silage is shown to significantly enhance non-specific cellular immunity and survival of Mozambique tilapia, without negative impacts on production performance or intestinal morphology.

2) The effects on non-specific immunity are attributable to the hydrolysed protein component of the fish silage, and not to the formic acid used in silage preparation.
3) It is demonstrated that dietary silage inclusion level is critical to ensure good production performance in Mozambique tilapia, as excessive inclusion will negatively impact on production performance and animal survival.

Summary of authors' contributions

Goosen, N.J.: Responsible for majority of trial planning, experimental design, feed formulation, feed manufacturing; daily trial operation; sampling of animals for production performance, immunology, haematology and intestinal morphology; analysis of immunity, haematology and intestinal morphology; statistical analysis of data; writing draft manuscript and incorporating comments of co-authors to prepare for journal submission. Estimated % contribution to the work: 80 %

De Wet, L.F.: Assisted with feed formulation, interpretation of final data and reviewed the completed manuscript. Took part in general discussion surrounding the project. Estimated % contribution to the work: 10 %

Görgens, J.F.: Assisted with interpretation of final data and reviewed the completed manuscript. Took part in general discussion surrounding the project. Estimated % contribution to the work: 10 %

Rainbow trout silage as immune stimulant and feed ingredient in diets for Mozambique tilapia (*Oreochromis mossambicus*)

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Running title:

Fish silage as immune stimulant in tilapia

Key words:

Fish processing waste; Hydrolysed protein; Protein replacement; Sustainability; Formic acid; Non-specific immunity

Conflict of interest:

The authors have no conflict of interest to declare.

Abbreviations:

SD: standard deviation. SEM: standard error of the mean. DOH: Degree of hydrolysis. TCA: Trichloroacetic acid. PEG: Polyethylene glycol. FPH: Fish protein hydrolysate. L:W: Length to width ratio of intestinal folds. IP: Inner perimeter of intestine.

Abstract

Peptides and free amino acids resulting from protein hydrolysis could act as stimulants of fish non-specific immunity. The study aimed to determine the potential of rainbow trout viscera silage as immune stimulant and feed ingredient for Mozambique tilapia, and to establish whether formic acid used during silage preparation contributed to any effects. Four diets were evaluated: a reference diet (R), one containing 6.5 g kg^{-1} formic acid (FA) and two silage diets containing 160 g kg^{-1} (SL, low inclusion) and 285 g kg^{-1} (SH, high inclusion) silage. Low silage inclusion improved phagocytic activity of leukocytes compared to the reference, while high inclusion showed no improvement. No other non-specific immunity parameters or haematology were affected by any treatments. High silage inclusion significantly decreased growth and led to higher mortality, while formic acid had no effect on growth. It is concluded that rainbow trout viscera silage can stimulate the cellular non-specific immunity of *Oreochromis mossambicus*, and that protein hydrolysis products (and not formic acid) is responsible for the stimulation. The silage can also serve as source of dietary protein and essential amino acids in tilapia diets. However, both fish growth performance and improvement in cellular immunity are dependent on silage inclusion level.

Introduction

One of the challenges facing expansion in tilapia culture is that of improving the disease resistance of animals (Watanabe, et al., 2002). Although tilapia species generally exhibit good disease resistance, the intensification of production systems necessitates increased attention to disease management (Abdel-Tawwab, et al., 2008; Watanabe, et al., 2002), especially in the light of the development of bacterial resistance to certain therapeutic agents (Sudhakaran, et al., 2006). The various tilapia species are very important in aquaculture globally, with production of Nile tilapia *Oreochromis niloticus* alone reaching an output of approximately 2.3 million tonnes in 2008 (Bostock, et al., 2010; FAO, 2010), and further expansion in tilapia culture is expected. To ensure the continued and sustainable growth of this sector, it is important to develop feed ingredients that not only supply nutrients, but also promote animal health and disease resistance (Kiron, 2012).

An effective method of increasing disease resistance in tilapia is to ensure a good immune response, which can be achieved through dietary intervention. Diverse substances have been reported to act as stimulants of non-specific immunity in tilapia, including traditional Chinese herbs, vitamin C, inulin, ß-glucan, live Spirulina and baker's yeast in Nile tilapia, (Abdel-Tawwab and Ahmad, 2009; Abdel-Tawwab, et al., 2008; Ardó, et al., 2008;

Ibrahem, et al., 2010; Sahan and Duman, 2010; Yin, et al., 2006), and extracts of an Indian medicinal herb in Mozambique tilapia (Sudhakaran, et al., 2006). Disease resistance subsequent to a bacterial challenge trial was increased in hybrid tilapia through inclusion of dietary potassium diformate (Ramli, et al., 2005), but effects on immune parameters were not reported.

Immune stimulating effects by dietary fish silage in species other than tilapia have been reported. Fish silage is prepared to prevent microbial spoilage through acidification of fish material (mostly low-value waste from fisheries or processing operations), and allowing endogenous digestive enzymes to hydrolyze the protein fraction. The main components of nutritional significance in the liquidised product are hydrolysed proteins, peptides and free amino acids, and fish oil (Raa and Gildberg, 1982). Disease resistance of European sea bass larvae was improved by inclusion of sardine silage in the diets, however it was unclear whether this was due to immune stimulation of the larvae, or as a result of change in the composition of the larval microbial community. As protein hydrolysates are routinely used in bacterial growth media, it could play a role in altering the composition of microflora associated with the larvae (Kotzamanis, et al., 2007). Another study reported immune stimulation of Japanese sea bass by a hybrid-type fish protein hydrolysate/silage product (produced through the addition of both formic acid as preservative, and an acid protease as proteolytic enzyme) in the diet (Liang, et al., 2006). No similar reports on the possible immune stimulating effects of fish silage in diets for tilapia are available.

Fish silage has been shown to be a good feed ingredient sustaining satisfactory growth rates when incorporated in formulated feeds for Nile tilapia (Fagbenro, et al., 1994; Fagbenro and Jauncey, 1998) and various other finfish species, including Atlantic salmon (Espe, et al., 1992; Jackson, et al., 1984), African catfish (Fagbenro, et al., 1994; Soltan, et al., 2008), and pacu (Macedo-Viegas, et al., 2004; Vidotti, et al., 2002). Formic acid is routinely used in the manufacture of fish silage, and formic acid salts have been shown to increase disease resistance in tilapia (Ramli, et al., 2005) and to alter the intestinal microbial community (Zhou, et al., 2009), which in turn can also affect disease resistance. It is therefore necessary to determine whether the formic acid used in silage preparation could act as immune stimulant in tilapia diets.

The study aimed to determine the immune stimulation potential of dietary fish silage in Mozambique tilapia *Oreochromis mossambicus* and to distinguish between the effects of the hydrolysis products found in fish silage and those of the formic acid used during silage production. This was done by *i*) evaluating the effects of fish silage produced from locally sourced processing waste on the non-specific immunity, intestinal morphology and growth performance parameters during a growth trial *ii*) at both a low and high silage inclusion level

and *iii*) comparing the results to a formic acid containing treatment to establish if any effects occur due to formic acid addition during silage preparation.

Materials and Methods

Preparation of fish silage

Fresh rainbow trout viscera were sourced from a local processing operation (Three Streams Smokehouse, Franschhoek) and ensiled immediately after collection through the addition of 25 g kg^{-1} formic acid (85%). The silage was stirred manually daily for the first week to ensure adequate mixing and hydrolysis was allowed to take place at ambient temperature (ranging between 15 °C – 25 °C). The silage contained high levels of oil resulting from the high oil content of the trout viscera (525 g kg^{-1} crude fat). The floating oil was manually decanted from the silage after 3 days. In order to remove the remaining oil and avoid possible mixing problems encountered in mackerel silage containing 276 g kg^{-1} fat (Espe and Lied, 1999), the silage was de-oiled using a centrifugal dairy separator (Elecrem Model 1) 37 days post ensiling. Forty nine day old silage was included in experimental diets.

Preparation of experimental diets

Four experimental diets (described below) were formulated to be on iso-nutrient and iso-energetic basis, extruded at 70 °C, dried at 55 °C and stored in airtight containers. The basal diet for all treatments consisted of AquaNutro Finfish Starter (NutroScience, Malmesbury, South Africa). Diet formulations and proximate composition are given in Table 6-1.

Experimental design, system and animals

The feeding trial consisted of four diets: a reference diet (R), one diet supplemented with 6.5 g kg^{-1} formic acid (FA) and two diets supplemented with silage at a low (SL) and high (SH) inclusion levels. Formic acid inclusion in diet FA was chosen to be equal to the final formic acid content of diet SH. The final formic acid content in diet SL was calculated to be approximately 50% of that in diet FA. The silage inclusion levels were chosen such that the protein contribution (after drying) would constitute 25 g kg^{-1} and 50 g kg^{-1} of the total feed, for the low and high inclusion levels respectively. Treatment SL included 160 g kg^{-1} wet silage, while treatment SH included 285 g kg^{-1}; these inclusion levels ensured that the total nitrogen contribution (including protein, hydrolysed protein, peptides and free amino

acids) from the silage would be less than which caused growth depression in tilapia in other investigations; Plascencia-Jatomea and co-workers (2002) found that dried fermented shrimp silage at inclusion above 200 g kg^{-1} caused growth depression, while Wassef (2005) showed that wet inclusion of 250 g kg^{-1} fish silage did not affect growth in *O. niloticus* fingerlings. The degree of hydrolysis (DOH) of silage is as an approximate measure of the amount of the protein in the silage that exists in the form of short chain peptides and free amino acids.

The experimental system consisted of continuously aerated 80 litre tanks in an in-door, heated recirculation system at Welgevallen Experimental Farm, University of Stellenbosch. Each treatment was replicated 5 times, with each diet fed to 5 randomly allocated tanks. Fish were sampled for evaluation of non-specific immunity, haematology, and growth parameters at day 23 (Sampling 1) and at day 52 (Sampling 2). One fish per tank was randomly selected for determination of immunity and haematology, while all fish in each tank were measured and weighed to determine growth performance parameters. At Sampling 2, all animals sampled for determination of immune parameters were also sacrificed for evaluation of intestinal morphology (refer so section 3.4.5). At the trial initiation, each tank contained 8 randomly allocated, mixed sex Mozambique tilapia (*O. mossambicus*) with initial mean weight ± standard deviation (SD) of 42.01 ± 5.41g.

Experimental procedures

Animal care and measurement

Fish were fed 3 times daily during the week and twice daily over weekends, at 3% of body weight per day. Mortalities were recorded and removed at every feeding period. Live animals were handled only after being anaesthetized using AQUI-S (Lower Hutt, New Zealand). Fish were weighed and measured (total length) at the initiation of the trial and at Sampling 1 (after 23 days) and Sampling 2 (after 52 days), using a laboratory scale and ruler. At the conclusion of the trial, fish height was also recorded in order to compute the Jones Condition Factor (Jones CF) according to Jones et al. (1999).

Determination of growth performance parameters

Growth performance parameters were determined from the measurements of weight, length and height of animals and the amount of feed consumed. Specific growth rate (SGR) was calculated as $SGR = 100*[\ln(W_{i+1}/W_i)/(t_{i+1} - t_i)]$, with units of grams and days for *W* and *t* respectively, at sampling time *i*; feed conversion ratio (FCR) was determined as fish wet weight gain per unit dry feed given, and Jones CF was calculated as Jones CF = 1000*

W/L^2H, where W is weight in grams, and L and H are length and height respectively, in units of cm (Jones, et al., 1999).

Analysis of silage and feeds

Proximate analyses of the diets were performed according to standard AOAC methods. Nitrogen was determined according to method 992.15 (AOAC, 1992) and crude protein was determined as Nx6.25; moisture (AOAC, 2002a) and ash (AOAC, 2002b) were also determined according to standard AOAC procedures . Crude fat was determined according to Lee et al. (1996).

The degree of hydrolysis (DOH) of the silage was determined through the trichloroacetic acid (TCA) precipitation method (Hoyle and Merrit, 1994). Briefly, equal volumes of de-oiled silage and 20% (w/v) TCA were mixed to yield a final TCA concentration of 10%, and incubated at room temperature for 1 hour. Samples were centrifuged at 14000 rpm for 5 minutes and the supernatant collected and analyzed for nitrogen as described above. DOH was calculated as the ratio of non-precipitated nitrogen to total sample nitrogen, and expressed as a percentage.

In order to determine the amino acid composition, the fish silage was acid hydrolysed, subjected to an AccQ Tag Ultra Derivitization Kit (Waters Corporation, Milford, USA) and elucidated using a Waters API Quattro Micro mass spectrometer. The amino acid composition of the fish silage is shown in Table 6-2.

Blood measurements

Animal sampling and blood collection

Prior to weighing and measuring fish, one fish per tank (5 per treatment) was sampled for determination of non-specific immune status. Blood was collected under anaesthesia, through caudal puncture with 27 gauge heparinzed syringes (Stellenbosch University Ethics Approval Certificate number: SU_ACUM_00018), and split into two parts: half was put into eppendorf tubes containing two drops of sodium heparinate as anti-coagulant, with the remainder allowed to clot overnight at 4 °C in an untreated eppendorf tube. Serum was collected post-clotting and after centrifuging tubes at 500×*g* for 5 minutes, and stored until analysis at -20 °C. Heparin treated blood was used for determination of hematocrit values, cell counts and estimation of phagocytic activity of leukocytes. Sampled animals were removed from the experimental system in order to prevent repeated sampling of the same animal, and were not replaced.

Haematology

Erythrocytes and leukocytes were counted manually under a light microscope using a haemocytometer. Haematocrit values were determined directly after sampling by using heparin treated blood in capillary glass tubes and centrifuging for 5 minutes in a microhaematocrit centrifuge.

Non-specific immunity

Phagocytic activity was determined according to the method of Cai et al. (2004) with some modifications: To ensure equal volumes of fluids during all procedures, aliquots of heparinised blood and suspended baker's yeast were fixed at 250µl each; the time between mixing of the blood/yeast mixture during incubation was shortened to 5 minutes to ensure frequent mixing to prevent the partial clotting of the sample. The baker's yeast suspension was prepared by autoclaving commercial baker's yeast (NCP, Modderfontein, South Africa) and re-suspending in phosphate buffered saline. To perform phagocytic activity protocol, aliquots of heparinised blood and baker's yeast were added to an eppendorf tube, incubated for 30 minutes at room temperature and mixed through careful inversion every 5 minutes. After incubation, blood smears were prepared, fixed with methanol and stained with 4% Giemsa-solution. Stained slides were evaluated under a light microscope; approximately 100 leukocytes per slide were counted. Phagocytic activity was expressed as an index value, relative to the overall average of the reference diet.

Lysozyme activity was determined according to the method described by Sankaran and Gurnani (1972). In a 96-well microplate, standard lysozyme solutions or serum (50µl) was added in duplicate to 250µl of a suspension of 0.075% (w/v) *Micrococcus lysodeikticus* in phosphate buffer (0.05 M at pH 6.2). The absorbance at 530nm was measured after 5 minutes and again after 20 minutes. A standard curve was prepared by plotting the decrease in absorbance against the known lysozyme concentration; serum lysozyme concentrations of samples were determined from the standard curve. *Micrococcus lysodeikticus* and lysozyme standard were purchased from Sigma.

Total protein content of serum was determined spectrophotometrically according to the linearized Bradford assay (Zor and Selinger, 1996) using bovine serum albumin as standard. Total immunoglobulin was determined according to Ardó, et al. (2008) by precipitating with poly-ethylene glycol (PEG, average molecular weight of 10,000 Dalton, Sigma). Equal volumes of serum and 12% (w/v) PEG were added to eppendorf tubes (giving a final concentration of 6% (w/v) PEG), mixed and incubated at room temperature for 2 hours. At the conclusion of the incubation period, tubes were centrifuged at 14000 rpm for 5 minutes and the protein content of the supernatant was determined according to the

linearized Bradford method. The immunoglobulin content was calculated as the difference between total serum protein and the protein content of the supernatant after PEG precipitation (taking into account the dilution by the PEG solution).

Determination of intestinal morphology

At the end of the trial, one fish per tank was taken to evaluate the morphological structure of the intestine to monitor whether silage inclusion had any effects on the intestine that might influence nutrient absorption. The intestinal morphology was evaluated in the proximal, mid and distal intestine, according to the method described previously by Goosen and co-workers (2014).

Statistical analysis

All data were evaluated with one-way ANOVA, followed by Duncan's multiple range test if significant differences were detected. Differences were viewed to be significant for $P < 0.05$. All analyses were done with Statistica version 10 software (StatSoft, Inc.).

Results

Silage formation and characterisation

Trout viscera liquefied within 2-3 days; a layer of clear, floating oil was formed and recovered readily through decantation. The degree of hydrolysis (DOH) was determined 49 days post ensiling as 89.5%.

All essential amino acids except tryptophan were detected in the silage. Tryptophan was not determined as it is known to be acid labile and will therefore be destroyed in the acidic medium of the silage.

Growth performance

Animals reached sexual maturity during the course of the trial, with breeding and territorial behaviour increasing unexpectedly after Sampling 1. Feed intake of female fish harbouring eggs or fry decreased markedly or ceased totally. As a result of the decreased feed intake from female fish, the gender of each fish was recorded at the final sampling procedure of the trial (determined visually) and growth parameters were evaluated separately for each gender. Results of the growth parameters and gender specific data and the final ratio of male : female fish at the end of the trial are summarised in Table 6-3.

There were no statistically significant differences in FCR or SGR over the duration of the trial. In male fish, Jones CF also showed no differences between treatments, while statistically significant differences were observed for final male weight, length and height. Male fish in treatment SH were 18.5%, 13.3% and 13.7% lighter than R, SL and FA respectively; final length of males in treatment SH were 5.3% and 5.1% lower than those of and R and FA, while treatment SL did not differ from any other treatments. Mean male height was 8.0% lower for treatment SH compared to R, while there were no differences between any other treatments. For female fish, no significant differences were observed for Jones CF or final mean weight between treatments, while significant differences were seen in length and height. The final mean length of females in treatment SL were 3.5%, 3.2% and 3.6% lower than R, FA and SH respectively. Mean female height of treatment SH was 5.4% and 7.0% higher than treatments FA and SL respectively, but did not differ from R, with SL also being 5.7% lower than R (see Table 6-3). The sex ratios (male : female) at the conclusion of the trial were 1.0:1 for FA, 0.83:1 for SL, 1.09:1 for SH and 0.89:1 for R.

Cumulative mortalities during the trial are shown in Figure 6-1. Mortalities recorded in treatment SH (31%) were considerably higher than any of the other treatments, where values ranged from 5.1% - 11.6%. The lowest mortality was seen in treatment SL. The majority of the mortalities (91.3%) occurred during the second part of the trial (between the first and final blood sampling at days 23 and 52 respectively). For treatment SH, mortalities started increasing immediately post-sampling and continued rising from 2.4% pre-sampling to reach 31.0% at the conclusion of the trial.

Haematology and non-specific immunity

Results of manual cell counts and hematocrit values at each sampling are given in Table 6-4. No differences were detected between treatments for any of the haematological parameters, at either sampling. Leukocyte counts decreased from Sampling 1 to Sampling 2: counts were 37.6% lower in R, 52.3% lower in FA, 42.6% in SL and 49.7% in SH.

Data for the different immunity parameters at both samplings are shown in Table 6-5. At Sampling 1, phagocytic activity for treatment SL was 18% and 21.6% higher than R and FA, while SH did not differ from any treatment. Phagocytic activity could not be measured successfully at Sampling 2, as the heparin treated blood coagulated upon contact with the yeast suspension and reliable blood smears could not be made. There were no differences between treatments for serum lysozyme concentration, immunoglobulin or total protein concentration.

Intestinal morphology

Results of the intestinal characterisation are shown in Table 6-6. There were no statistically significant differences between treatments for any of the intestinal morphological features, in any of the intestinal regions studied.

Discussion

The current trial showed that dietary fish silage acted as stimulant of the cellular non-specific immunity of the experimental fish. Fish fed diet SL had significantly increased phagocytic activity compared to the reference diet at day 23 of the trial, and showed the lowest cumulative mortalities at the trial conclusion. This result is significant as it adds *O. mossambicus* to the list of finfish species that have shown immune stimulation as a result of dietary silage or fish protein hydrolysate (FPH) products. Dietary FPH supplementation improved non-specific immune parameters in Japanese sea bass (Liang, et al., 2006) and large yellow croaker (Tang, et al., 2008) and increased survival in European sea bass larvae (Cahu, 1999); in the same species, an experimental fish silage evaluated simultaneously with a commercial FPH product significantly decreased mortality subsequent to bacterial challenge (Kotzamanis, et al., 2007), but unfortunately this study used no control diet without silage or protein hydrolysate. In other studies, however, FPH failed to elicit any non-specific immune stimulation or increased disease resistance in coho salmon, (Murray, et al., 2003), Atlantic salmon (Gildberg, et al., 1995) or in spotted wolffish (Savoie, et al., 2006). Failure of FPH to elicit immune stimulation might be related to the molecular weight or dietary inclusion level of these products (Murray, et al., 2003).

The rainbow trout viscera silage is a good feed ingredient that can provide essential amino acids and proteins in tilapia diets. The rainbow trout silage contained all essential amino acids (except tryptophan, which was not determined). Further, no growth depression was seen at the lower inclusion level of fish silage, which means the silage can be used to replace a portion of the fish meal in tilapia diets. There were also no effects on intestinal morphology at either silage inclusion level, indicating that fish silage had no negative impacts on the intestinal morphological structure of the experimental animals. The finding agrees with a number of other studies that investigated fermented fish and shrimp silage as sources of protein in diets for tilapia (Fagbenro and Jauncey, 1993; Fagbenro, et al., 1994; Plascencia-Jatomea, et al., 2002; Wassef, 2005) and generally found that it is a good feed ingredient that can sustain satisfactory animal growth rates and can partially replace fish meal. In one study it was even found that growth performance was improved under total fish

meal replacement (El-Hakim, et al., 2007). Within the context of the projected increase in global aquaculture (including tilapia culture) and subsequent increased demand for aquafeed ingredients (Olsen and Hasan, 2012), this result contributes toward the sustainable growth of this sector.

Improved cellular immunity observed in the current trial can be attributed to the effect of protein hydrolysis products, as formic acid showed no effects on the measured immune parameters or on growth performance. Formic acid and its salts increased growth rate in tilapia (Lückstädt, et al., 2012; Ramli, et al., 2005), and enhanced animal disease resistance against *Vibrio anguillarum* (Ramli, et al., 2005). The mechanisms by which formic acid and/or its salts have had these effects are not wholly understood, but according to the current investigation, it does not function as immune stimulant in tilapia. Most of the mechanisms associated with increased disease resistance and growth promotion of formic acid are assumed to be linked to its antimicrobial properties. Dietary potassium diformate succeeded in altering the composition of the gut microflora of tilapia (Zhou et al., 2009); however the implications of the altered gut microflora for animal growth or health could not be established.

It was shown that there is an upper limit to inclusion of fish silage in diets for *O. mossambicus*. Treatment SH containing 285 g kg^{-1} fish silage showed growth depression in male fish compared to the reference diet, while treatment SL containing 160 g kg^{-1} fish silage showed similar growth compared to the reference diet. The lower inclusion level was therefore below the inhibitory level where dietary silage inclusion negatively impacts growth performance of *O. mossambicus*. The inclusion level of hydrolysed protein products in aquafeeds is a critical factor when utilising these ingredients, as excessive inclusion may adversely affect feed performance. Growth depression as a result of high inclusion of fish and shrimp silage has been reported in rainbow trout (Hardy, et al., 1984; Rungruangsak and Utne, 1981; Stone, et al., 1989), African catfish (Nwanna, 2003; Soltan, et al., 2008), carp (Wood, et al., 1985) and Nile tilapia (Plascencia-Jatomea, et al., 2002). Decreased growth was also reported in carp fingerlings fed high amounts of hydrolysed casein and the existence of a maximum dietary inclusion level of hydrolysed casein was hypothesised (Carvalho, et al., 2004).

The decreased growth performance of treatment SH may be attributed to relatively high amounts of free amino acids and short chain peptides introduced into the diet by the highly hydrolysed fish silage. Decreased growth caused by diets incorporating high levels of fish silage have been attributed to decreased palatability resulting from high diet acidity (Rungruangsak and Utne, 1981), and to negative metabolic effects of high levels of free amino acids and peptides that are characteristic of highly hydrolysed silages (Espe, et al., 1992). There is evidence to support the second hypothesis in a study which fed increasing

levels of a relatively younger fish silage (implying lesser degrees of hydrolysis) to Nile tilapia and African catfish; no negative effects on growth was seen at any of the inclusion levels (Fagbenro, et al., 1994). In the current study, the possibility of decreased palatability as a result of formic acid was eliminated by use of the acid containing treatment, which showed no difference in growth or feed conversion compared to the reference.

The increased mortalities observed during the second half of the trial might have resulted as a consequence of increased stress levels in the experimental animals. Mortalities increased in all treatments after Sampling 1 (refer to Figure 6-1). Increased stress is known to be partially responsible for higher mortalities in aquaculture (Barton and Iwama, 1991) due to a possible decrease in disease resistance or suppression of the immune function (Ashley, 2007; Pickering and Pottinger, 1989). Signs of immune suppression were seen in the decrease in white blood cells from Sampling 1 to Sampling 2, which was independent of dietary treatment. Changes in white blood cell concentration is thought to be an indicator of stress in fish (Barton and Iwama, 1991; Wedermeyer, et al., 1984) and a decrease of white blood cells over time indicated a negative effect on fish health (Omoregie, 1998). A possible source of stress in the current trial might have been the territorial and breeding behaviour observed after Sampling 1. The weighing and measuring of the fish during Sampling 1 may have acted as a short-term stressor to the fish, but the contribution thereof to the long-term stress of the animals is unknown.

The use of mixed sex populations of *O. mossambicus* for experimental purposes should be avoided if possible, as it might cause difficulties during experiments and in the analysis and interpretation of the data. Previous studies have been able to detect statistically significant differences in growth using mixed sex tilapia populations (El-Sayed, 1998; Ridha and Azad, 2011), but the mixed sex population used in the current study masked growth differences. The lack of statistically significant differences in SGR between treatments of the current study might lead to wrongfully concluding that there were no differences in growth, even though male fish in treatment SH weighed less than those of other treatments. Further, the presence of sexually mature female fish in the experimental groups led to growth disparities between the sexes. Breeding females' feed intake and subsequently SGR decreased, as shown by the lower mean weight of females compared to males, in all treatments. Between treatments, differences in growth rates of female fish could not be shown; even though there were statistically significant differences in female length and height no differences occurred in final female weight. These data and the uncertainty caused by female breeding behaviour precludes reaching any clear conclusion regarding the growth rates of female fish, and growth is thus only evaluated based on male fish data. The decreased feed intake of females is one of the main factors for the high FCR values (for dry, pelleted diets) obtained for all treatments during the trial, as feed rations

were determined based on total weight of all fish per container; uneaten feed rationed for females led to feed wastage and thereby increased average FCR values.

In conclusion, it has been shown that dietary rainbow trout viscera silage can act as stimulant of the cellular non-specific immunity of Mozambique tilapia. The stimulation was the result of the enzymatic hydrolysis products, and not due to the formic acid used during silage preparation. The silage was also a good feed ingredient that contained essential amino acids, and it successfully replaced a portion of the fish meal in the tilapia diets; however, excessive silage inclusion resulted in impaired growth performance. The study contributes to the sustainable expansion of aquaculture through the production of functional aquafeed ingredients from waste products.

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Tables

Table 6-1 Formulation and composition of experimental diets.

Feed ingredient (g kg^{-1})	**FA**	**SL**	**SH**	**R**
Basal diet[a]	851.5	801	715	857
Trout oil	20	-	-	20
Fish meal[b]	122	39	-	123
Trout silage[c]	-	160	285	-
Formic acid	6.5	-	-	-
Total (g)	1000	1000	1000	1000
Feed composition (g kg^{-1})				
Moisture	41	47	43	61
Ash	122	111	102	119
Crude fat	70	57	67	69
Crude protein	523	527	518	514
Crude fibre	26	25	25	26
Carbohydrates[d]	218	233	245	211
Energy content (MJ kg^{-1})	18.9	18.8	19.2	18.5

Treatments: FA - formic acid diet; SL – low silage diet; SH – high silage diet; R - reference diet.

a – The basal diet consisted of a proprietary blend of soybean meal (460 g kg^{-1} crude protein); white maize (pre-gelatinized); poultry meal (640 g kg^{-1} crude protein); fish meal (640 g kg^{-1} crude protein); yellow maize prime gluten (600 g kg^{-1} crude protein); fish oil; vitamin-mineral premix (DSM, Switzerland) and monocalcium phosphate. The final proximate composition of the basal diet was: Moisture 100 g kg^{-1}; Ash 70 g kg^{-1}; Crude fat 60 g kg^{-1}; Crude protein 450 g kg^{-1}; Crude fibre 25 g kg^{-1}

b – Proximate analysis of fish meal: Moisture 89 g kg^{-1}; Ash 242 g kg^{-1}; Crude fat 64 g kg^{-1}; Crude protein 577 g kg^{-1}

c – Proximate analysis of trout silage: Moisture 794 g kg^{-1}; Ash 10 g kg^{-1}; Crude fat 63 g kg^{-1}; Crude protein 107 g kg^{-1}

d – Determined by difference

Table 6-2 Amino acid profile of rainbow trout viscera silage.

Amino acid	As % of total	As g kg^{-1} dry matter
Ala	6.68	24.3
Arg	6.74	24.6
Asp	10.26	37.4
Cys	0.0	0.0
Glu	14.19	51.7
Gly	8.36	30.5
His	2.72	9.9
ILe	4.99	18.2
Leu	7.27	26.5
Lys	7.47	27.2
Met	1.56	5.7
Phe	4.15	15.1
Pro	5.55	20.2
Ser	5.98	21.8
Thr	5.36	19.5
Tyr	2.83	10.3
Val	5.89	21.5

Table 6-3 Growth performance data (mean ± SEM).

	FA	SL	SH	R
Feed conversion and growth				
FCR	1.78 ± 0.45	1.97 ± 0.32	1.66 ± 0.29	1.78 ± 0.39
SGR (% d^{-1})	0.93 ± 0.07	0.97 ± 0.07	0.77 ± 0.03	1.01 ± 0.10
Male fish				
Jones CF	55.2 ± 0.99	56.2 ± 0.82	55.6 ± 0.98	55.6 ± 0.76
Weight (g)	87.8 ± 4.39^{a}	87.4 ± 2.66^{a}	75.8 ± 2.13^{b}	93.0 ± 3.20^{a}
Length (mm)	179.0 ± 2.79^{a}	175.2 ± 2.23^{ab}	169.8 ± 2.14^{b}	179.3 ± 2.00^{a}
Height (mm)	49.6 ± 0.70^{ab}	48.8 ± 0.95^{ab}	46.9 ± 0.71^{b}	51.0 ± 1.36^{a}
Female fish				
Jones CF	57.5 ± 0.47	58.4 ± 0.68	56.9 ± 0.89	56.9 ± 1.10
Weight (g)	52.2 ± 2.18	49.1 ± 1.57	55.0 ± 1.67	53.8 ± 1.33
Length (mm)	148.8 ± 1.80^{a}	144.1 ± 1.36^{b}	149.5 ± 2.24^{a}	149.4 ± 1.40^{a}
Height (mm)	40.7 ± 0.82^{ab}	40.1 ± 0.64^{a}	42.9 ± 0.74^{c}	42.4 ± 0.68^{bc}
Male : Female ratio				
	1.0 : 1	0.83 : 1	1.09 : 1	0.89 : 1

Abbreviations: FCR – Feed conversion ratio; SGR – Specific growth rate; Jones CF – Jones condition factor

Note: FCR and SGR values are calculated over the whole trial using combined data for male and female fish. Data for male and female fish and male : female ratio as determined at the end of the trial (Sampling 2). Values within each row which share a common superscript do not differ significantly.

Table 6-4 Haematological data (mean ± SEM)

	FA	SL	SH	R
Sampling 1				
Leukocytes (10^5 mm^{-3})	1.65 ± 0.13	1.41 ± 0.24	1.51 ± 0.29	1.09 ± 0.11
Erythrocytes (10^6 mm^{-3})	2.39 ± 0.19	2.41 ± 0.14	2.53 ± 0.05	2.51 ± 0.05
Hematocrit (%)	35.4 ± 2.09	32.8 ± 0.73	32.8 ± 1.46	34.6 ± 2.54
Sampling 2				
Leukocytes (10^5 mm^{-3})	0.78 ± 0.10	0.81 ± 0.07	0.76 ± 0.10	0.68 ± 0.06
Erythrocytes (10^6 mm^{-3})	2.80 ± 0.14	2.44 ± 0.18	2.73 ± 0.15	2.42 ± 0.13
Hematocrit (%)	32.3 ± 4.10	33.4 ± 1.86	39.0 ± 4.73	40.0 ± 1.94

Table 6-5 Non-specific immune parameters (mean ± SEM).

	FA	SL	SH	R
Sampling 1				
Phagocytic activity	0.97 ± 0.04[a]	1.18 ± 0.04[b]	1.06 ± 0.04[ab]	1.00 ± 0.06[a]
Lysozyme (µg ml^{-1})	15.0 ± 3.81	13.4 ± 4.32	18.8 ± 3.95	10.3 ± 4.90
Immunoglobulin (mg ml^{-1})	2.73 ± 1.19	1.44 ± 0.80	1.38 ± 0.48	2.94 ± 1.24
Total protein (mg ml^{-1})	18.3 ± 0.86	18.8 ± 0.64	18.4 ± 0.57	20.8 ± 0.60
Sampling 2				
Lysozyme (µg ml^{-1})	10.5 ± 4.19	14.2 ± 5.74	17.4 ± 4.84	17.5 ± 4.16
Immunoglobulin (mg ml^{-1})	4.66 ± 0.53	3.29 ± 0.89	4.31 ± 0.69	2.14 ± 0.99
Total protein (mg ml^{-1})	20.1 ± 0.17	20.2 ± 0.60	20.4 ± 2.06	21.4 ± 0.78

Note: Values within each row which share a common superscript do not differ significantly.

Table 6-6 Intestinal morphology (mean ± SEM) as characterised at the end of the trial.

	FA	SL	SH	R
Proximal intestine				
Fold length (µm)	316 ± 15.0	308 ± 33.7	309 ± 52.7	269 ± 23.3
Fold width (µm)	129 ± 7.55	122 ± 5.18	128 ± 6.81	121 ± 6.08
L:W	2.48 ± 0.18	2.55 ± 0.34	2.39 ± 0.38	2.21 ± 0.12
IP (µm)	24955 ± 1357	24685 ± 1856	20830 ± 2389	23875 ± 1281
Mid intestine				
Fold length (µm)	213 ± 11.1	202 ± 31.3	202 ± 13.8	192 ± 18.4
Fold width (µm)	105 ± 5.08	110 ± 14.0	88.2 ± 5.98	101 ± 5.65
L:W	2.02 ± 0.07	1.83 ± 0.13	2.33 ± 0.25	1.92 ± 0.21
IP (µm)	14979 ± 2780	11840 ± 2366	13991 ± 3390	11899 ± 1920
Distal intestine				
Fold length (µm)	166 ± 17.2	176 ± 18.5	164 ± 21.7	194 ± 13.0
Fold width (µm)	108 ± 9.70	104 ± 5.59	104 ± 12.7	117 ± 8.15
L:W	1.54 ± 0.10	1.71 ± 0.19	1.59 ± 0.08	1.67 ± 0.07
IP (µm)	7314 ± 962	8694 ± 1084	7066 ± 891	8357 ± 874

Abbreviations: L:W - Length to width ratio; IP - inner perimeter

Figures

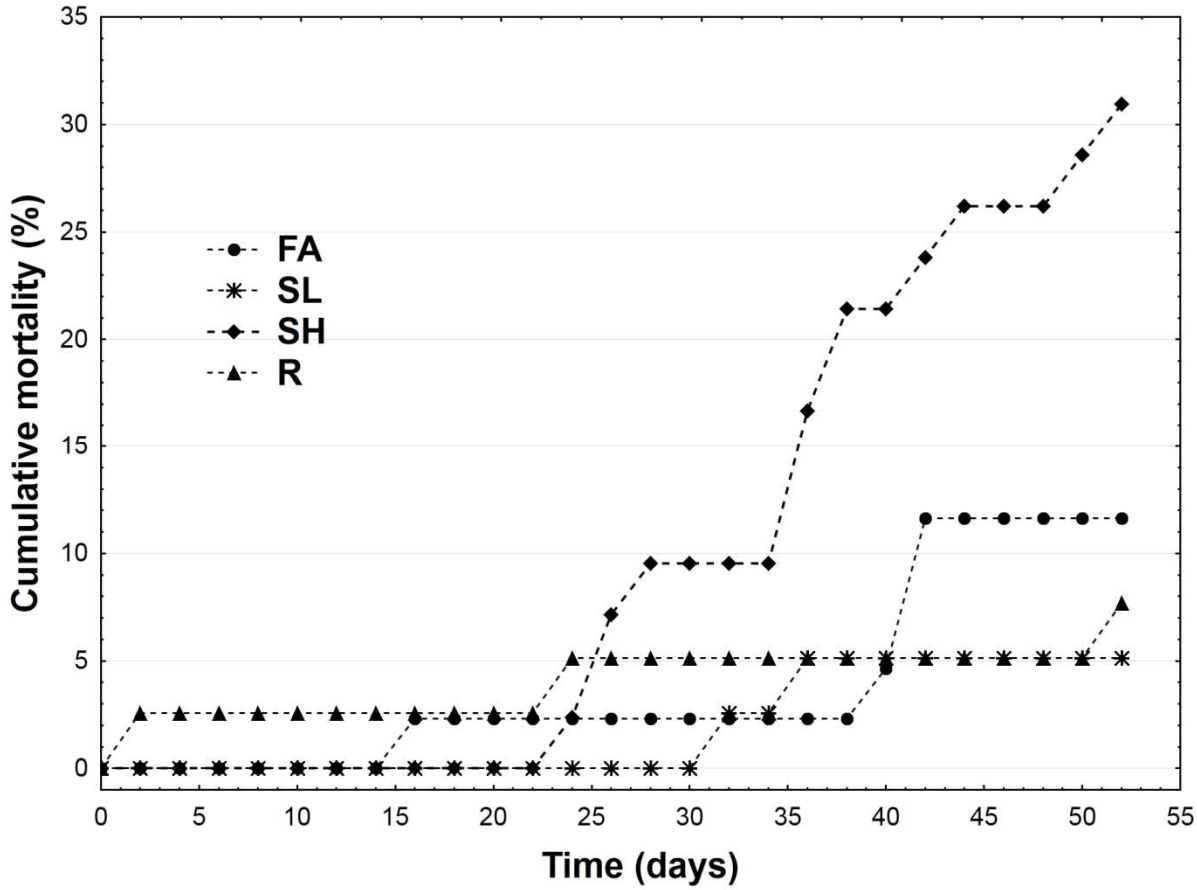

Figure 6-1 Cumulative mortality over the course of the trial, for all treatments. Values are expressed as % of the total number of animals in each treatment at trial initiation.

Chapter 7: Comparison of dietary fish protein hydrolysates in Mozambique tilapia

"All there is to thinking is seeing something noticeable which makes you see something you weren't noticing which makes you see something that isn't even visible." Norman Maclean, A River Runs Through It

"If you sit on the doorstep long enough, I daresay you will think of something." J.R.R. Tolkien, The Hobbit

This chapter is yet to be submitted for review

The work described in this chapter addresses aim III, and makes the following novel contributions:

1) It is demonstrated that production performance of Mozambique tilapia is significantly different in treatments incorporating hydrolysed proteins from different raw material origins, and that the dietary hydrolysed protein inclusion level plays an important role in determining the effects on production performance.
2) The study generated new data that will provide important guidance to feed manufacturers regarding the suitability of hydrolysed fish proteins as feed ingredients for the Mozambique tilapia, a species for which little information is available regarding the nutritional effects of fish protein hydrolysates, but which is relevant to the South African aquaculture industry.
3) All three dietary hydrolysed proteins evaluated can serve as good feed ingredients, as demonstrated by high growth rates and lack of adverse effects on animal health status or intestinal morphology.

Summary of authors' contributions

Goosen, N.J.: Responsible for trial planning, experimental design, feed formulation, sourcing of commercial hydrolysates, and feed preparation; completed daily trial operation duties; responsible for sampling of animals for production performance, immunology, haematology and intestinal morphology; completed the analysis of immunity, haematology and intestinal morphology; did statistical analysis of data; responsible for writing draft manuscript. Estimated % contribution to the work: 80 %

De Wet, L.F.: Assisted with feed formulation, took part in general discussion surrounding the project. and provided feedback on the final manuscript. Estimated % contribution to the work: 10 %

Görgens, J.F.: took part in general discussion surrounding the project and provided feedback on the final manuscript. Estimated % contribution to the work: 10 %

Comparison of hydrolysed proteins from different raw materials in diets for Mozambique tilapia *Oreochromis mossambicus*

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Running head: Hydrolysed proteins in feeds for Mozambique tilapia

Key words:

comparative growth study; condition factor; fishmeal replacement; fish protein hydrolysates; fish silage; intestinal morphology; non-specific immunity; production performance

Abbreviations

ANOVA – analysis of variance; FCR – feed conversion ratio; FPH – fish protein hydrolysate; H – height; IG – serum immunoglobulin; Jones CF – Jones condition factor; L – length; mg – milligram; ml – millilitre; SE – standard error of the mean; SGR – specific growth rate; t – time; TP – serum total protein; W – weight; Wid – width; µg – microgram

Abstract

The study aimed to compare three fish protein hydrolysates (FPH) from different origins as dietary ingredients for Mozambique tilapia *Oreochromis mossambicus* (according to aim III of the study). The ingredients were: self-prepared fish silage from rainbow trout viscera, and two commercial FPH products from shrimp and mixed marine by-products. Each ingredient was further included at two dietary levels: a low level where hydrolysed protein contributed 20 $g \cdot kg^{-1}$ protein to the diet and a high level where protein contribution was 40 $g \cdot kg^{-1}$. It was found that all three FPH products sustained high specific growth rates in excess of 2.5 % of body weight per day at both inclusion levels, and that there were no negative impacts on production performance, animal health or intestinal gross morphology compared to the control. Further, there were no significant differences in specific growth rate, feed conversion, dress-out %, non-specific immunity parameters or intestinal morphology between any treatments. However, low inclusion of FPH from mixed marine origin (treatment HCL) showed significantly higher growth than high inclusion of self-prepared silage (treatment SH) through significantly higher final animal length and height; a simultaneous significant decrease in Jones Condition Factor in treatment HCL might be explained by a change in animal body composition. It is concluded that all FPH products were acceptable feed ingredients at the inclusion levels investigated; however differences in animal growth seen between treatments HCL and SH might be the result of the different inclusion levels employed, and the FPH raw material origin. Further, interpretation of animal condition factor should also take into account supplementary trial data, as differences between treatments might not signify differences in animal well-being, but rather changes in body composition.

Introduction

Fish protein hydrolysates (FPH) from fish and shellfish origin have been proposed as partial or total substitutes for fishmeal as protein source in aquaculture diets for finfish (Aksnes, et al., 2006b; El-Sayed, 1998; Nwanna, et al., 2004; Ramasubburayan, et al., 2013; Soltan, et al., 2008). When produced from processing by-products, FPH can contribute to the sustainability of the aquaculture sector through decreased reliance on wild caught fish for sourcing of feed ingredients (Naylor, et al., 2009). Apart from acting as protein source, some FPH exhibit additional functionally beneficial or bio-active effects when incorporated in formulated diets, e.g. increased intestinal enzyme activity, improved disease resistance and survival, stimulation of the non-specific immunity, increased levels of insulin like growth factor in blood plasma and stimulation of feed intake (Bui, et al., 2014; Cahu, 1999; Chotikachinda, et al., 2013; Kotzamanis, et al., 2007; Kousoulaki, et al., 2013; Liang, et al., 2006; Tang, et al., 2008; Zheng, et al., 2012). Additionally, improved growth performance upon dietary FPH inclusion has also been widely reported in various finfish species (Bui, et al., 2014; Goncalves, et al., 1989; Refstie, et al., 2004; Zheng, et al., 2012; 2013).

FPH products have also been evaluated specifically in tilapia species. Tilapias are a globally important group of aquaculture species and significant amounts of fishmeal are used to produce formulated feeds for them (Bostock, et al., 2010; FAO, 2010). As in other finfish, FPH products have been evaluated as protein source and potential replacers of fishmeal (Cavalheiro, et al., 2007; El-Hakim, et al., 2007; Fagbenro, et al., 1994; Leal, et al., 2010; Plascencia-Jatomea, et al., 2002) and soybean meal (Hernández, et al., 2013), as immune stimulant (Goosen et al. 2014b) or to determine the effects of FPH inclusion on intestinal digestive enzyme activity (Santos, et al., 2013). In fishmeal replacement studies, it was generally found that FPH can replace a portion of the fishmeal in formulated tilapia diets without negatively impacting production performance (refer to Table 3-1).

FPH are produced from a range of raw materials and currently a number of different commercial FPH products are available for inclusion in aquafeeds. However, comparative studies where FPH from different origins are evaluated simultaneously are lacking (Bui, et al., 2014). As the various FPH products differ according to raw materials and production processes (Kristinsson and Rasco, 2000a; b), comparative studies are required for the proper selection of optimal feed ingredients. Feed ingredients, however, should not only be selected for the ability to provide good animal growth, but also for their ability to ensure good animal health status. It is therefore necessary to ensure that dietary FPH inclusion does not cause decreased health status (e.g. depressed immune function) in experimental animals (Kiron, 2012).

The aim of the study was therefore to do a comparative growth study where three different FPH products were evaluated (each at two dietary inclusion levels) for effects on production performance. Any effects on health status of the FPH products were monitored by way of blood and non-specific immunity parameters and liver weight, while effects on the digestive tract were monitored by way of the gross intestinal morphology.

Materials and Methods

Experimental design, system and animals

Three different hydrolysed protein products were evaluated at two dietary inclusion levels in a feeding trial and compared to a control diet, resulting in a total of 7 treatments. The three hydrolysates used in the trial were: rainbow trout viscera silage prepared according to methods previously described by Goosen and co-workers (2014b), a commercial shrimp by-product hydrolysate (Actipal HP1, Aquativ, France) and a commercial hydrolysate from mixed marine by-products (Actipal HC2, Aquativ, France). The diets were formulated such that the protein contribution in the low inclusion diets amounted to 20 $g \cdot kg^{-1}$ final feed while the high inclusion diets contained 40 $g \cdot kg^{-1}$ protein from the protein hydrolysates. Diets were formulated to be iso-proteic and iso-energetic (refer to Table 7-1). The high inclusion level of silage was determined from a previous investigation using rainbow trout silage and *Oreochromis mossambicus* (Goosen et al. 2014b), where 285 g wet silage per kg feed was shown to inhibit animal growth, and this inclusion level was therefore taken to be the maximum for the current trial.

Six replicates were used per treatment, with 15 fish initially stocked per tank (90 fish per treatment). The experimental animals were all male, sex-reversed Mozambique tilapia *Oreochromis mossambicus* from a single cohort, with a mean initial weight ranging from 10.3 g to 11.0 g, with no significant differences between the respective treatments (refer to Table 7-2). The experimental system used was a heated, indoor recirculating system at Welgevallen Experimental Farm, at Stellenbosch University, South Africa. The volume of experimental tanks was 80 litres, and continuous aeration was supplied. Water temperatures were 26 ºC – 30 ºC for the duration of the trial. Fish were fed a ration of approximately 3 % of body weight per day, in three equal portions daily.

As indicators of production performance, fish growth rate, feed conversion, animal weight, length, height, the Jones condition factor (Jones CF) and the dress-out % at final sampling were determined. Fish health status was characterised as a combination of non-specific immunity parameters (serum lysozyme, total protein and immunoglobulin

concentration), red blood cell volume (haematocrit values), and the hepatosomatic index (liver weight as a percentage of body weight). The intestinal gross morphology of the mid-intestine was monitored as an indication of intestinal changes that might result from the different dietary ingredients.

The feeding trial was run for a duration of 61 days, and had three samplings: one at initiation, Sampling 1 (34 days after trial initiation) and Sampling 2 (at the conclusion of the trial). At Sampling 1, all fish in each tank were weighed and measured, and blood was withdrawn from one randomly chosen animal per tank to determine non-specific immunity parameters and red blood cell volume. Sampled animals were removed from the experimental tanks and were not replaced. At Sampling 2, the same parameters were measured as at Sampling 1; additionally, the fish sampled for measuring blood parameters were slaughtered to determine dress-out % and liver weight, while the same animals' intestinal tracts were used for determination of gross intestinal morphology.

Diet preparation and analysis

Rainbow trout silage was prepared and de-oiled as described previously (Goosen, et al., 2014a; Goosen, et al., 2014c). A commercial tilapia diet using fishmeal and soybean meal as main protein sources (AquaNutro Finfish Starter, NutroScience, Malmesbury, South Africa; proprietary formulation) was used as basal diet for all treatments. Diets were prepared by mixing all ingredients with the basal diet and a specified amount of water to form moist paste suitable for extrusion. Dietary mixtures were subsequently extruded at 70 °C and dried in a ventilated drying oven at a temperature of 55 °C. The amount of water added prior to extrusion was adjusted to take into account the water content of the various protein hydrolysates. The proximate composition of the feeds were determined using standard AOAC analyses (AOAC, 2003); for diet formulations and proximate compositions of diets refer to Table 7-1.

Experimental and analytical procedures

Animals were only handled after being anaesthetized with AQUI-S (Lower Hutt, New Zealand). All experimental and analytical procedures were approved by Stellenbosch University Ethics Committee (certificate number: SU_ACUM_00018).

Production performance parameters

Production performance was characterised by computing specific growth rate (SGR), feed conversion ratio (FCR) and the Jones Condition Factor (Jones CF). The respective

formulae used were SGR = 100*[ln(W_f/W_i)/(t), with units of grams and days for *W* and *t*, where W_f denotes the final mean animal weight and W_i initial mean weight; FCR = (W_f - W_i)/feed given, with animal weight and feed given in units of grams; Jones CF = 1000* W/L^2H, with *W* in grams and *L* (animal length) and *H* (animal height) in units of cm, according to Jones, et al. (1999). Dress-out % is defined as the carcass weight after animals were gutted and gilled, and was expressed as the percentage of initial weight.

Animal health status

At each sampling, blood was collected from the caudal veins of sampled animals for determination of non-specific immunity parameters, using sterile 27 gauge syringes. The blood serum was recovered for the determination of lysozyme, total protein and immunoglobulin concentration, as described previously (Goosen, et al., 2014c). Additional blood was sampled to determine the haematocrit values using glass capillaries in a haematocrit centrifuge. At Sampling 2, the liver weight of each slaughtered animal was recorded to determine the hepatosomatic index.

Intestinal morphology

Gross intestinal morphology of the mid intestine of experimental animals was monitored according to the methods described previously (Goosen, et al., 2014c).

Statistical analysis

All data were analysed by way of one-way ANOVA, with a significance level of $P < 0.05$. Tukey's post-hoc test was applied to determine which means differed if ANOVA analysis indicated the existence of significant differences between means. All data analysis was performed with the Statistica software package (version 11, StatSoft, Inc.).

Results

Production performance

All diets were accepted readily by the experimental animals and no mortalities occurred during the trial. Production performance parameters as evaluated at the end of the trial (Sampling 2) are summarised in Table 7-2 and Figure 7-1.

SGR values ranged from 2.59 ± 0.14 d^{-1} (mean ± standard error) for treatment SH to 2.84 ± 0.11 d^{-1} in treatment HCL; however there were no statistically significant differences between any treatments for SGR. There were also no statistically significant differences in FCR, dress-out % (Table 7-2) or final animal weight (Figure 7-1 B) between any treatments. The highest FCR value was 1.60 ± 0.23 for treatment SH, while the lowest was seen in treatment HCL (1.22 ± 0.05). Dress-out % and final animal weight of all treatments were between 78.8 % - 82.9 % and 53.7 g – 60.9 g respectively.

There were no significant differences between any of the treatments and the control for final animal length, height or Jones CF (Figure 7-1). Treatment HCL had statistically significant higher animal length and height than treatment SH (5.1 % and 6.8 % increase in length and height respectively), while Jones CF for treatment HCL was significantly lower than that of treatment SL (by 4.9 %) and SH (by 4.0 %). There were no significant differences in any of the production performance parameters or animal measurements at Sampling 1 (data not shown).

Animal health status

Table 7-3 reports the different parameters used to evaluate the health status of the animals. There were no statistically significant differences at Sampling 1 or Sampling 2 between any of the treatments for any of the parameters evaluated. At Sampling 1, serum lysozyme concentration was between 16.7 $\mu g \cdot ml^{-1}$ – 27.4 $\mu g \cdot ml^{-1}$, total protein concentrations between 20.4 $mg \cdot ml^{-1}$ – 21.8 $mg \cdot ml^{-1}$, immunoglobulin levels were 1.34 $mg \cdot ml^{-1}$ – 3.21 $mg \cdot ml^{-1}$ and haematocrit values between 39.9 % - 43.3 %. The liver weights of animals were not evaluated at Sampling 1. At Sampling 2, lysozyme concentrations were 14.5 $\mu g \cdot ml^{-1}$ – 20.9 $\mu g \cdot ml^{-1}$, total protein 21.7 $mg \cdot ml^{-1}$ – 23.2 $mg \cdot ml^{-1}$, immunoglobulin 1.87 $mg \cdot ml^{-1}$ – 2.47 $mg \cdot ml^{-1}$, haematocrit values 40.0 % - 42.8 % and hepatosomatic index values 1.95 % - 2.27 %. Visual inspection of internal organs revealed no apparent differences (e.g. abnormally coloured livers or intestinal fat build-up) between treatments that warranted further investigation.

Intestinal morphology

Measurements of the gross intestinal structure for animals in the different treatments are presented in Table 7-4. There were no statistically significant differences between any of the measured gross morphological features. The values of mean inner perimeter length of intestines were between 9731 µm – 11151 µm, mean outer perimeter 5356 µm – 6290 µm, mean fold length 129 µm – 165 µm, mean fold width 102 µm – 117 µm, mean mucosa thickness 29.0 µm – 43.3 µm and the ratio of fold length to fold width (L:Wid) ranged from 1.30 to 1.68.

Discussion

Commercial FPH products from a variety of raw material origins are available for feed formulation, including hydrolysates from krill, marine finfish by-products and shrimp processing by-products. Nutritional analysis provides essential information regarding the suitability of feed ingredients for dietary inclusion for particular species. However, when ingredients have similar nutritional profiles, comparative studies that evaluate ingredients simultaneously under the same conditions are required to select the optimal ingredient. Therefore the current study investigated three FPH products and monitored effects on animal growth, health and intestinal morphology.

All three FPH products served as good feed ingredients at the inclusion levels evaluated, and sustained high growth rates in excess of 2.5 % increase in bodyweight per day (Table 7-2). The various FPH products contributed to the protein content of the experimental diets and successfully substituted a portion of fishmeal. In studies with Nile tilapia (*Oreochromis niloticus*), total replacement of fishmeal was achieved with fish and shrimp head silage without significant negative impacts of growth (Cavalheiro, et al., 2007; El-Hakim, et al., 2007). However, in another study shrimp head silage replacing more than 15% of fishmeal lead to significantly decreased growth (Plascencia-Jatomea, et al., 2002), while shrimp silage included at 6% of the final diet (corresponding to 20% fishmeal replacement) had no significant reduction in production performance (Leal, et al., 2010). In the current trial, total FPH inclusion level was restricted, as previous studies using rainbow trout silage in diets for *Oreochromis mossambicus* showed growth depression at dietary protein contribution of 40 $g \cdot kg^{-1}$ (Goosen et al. 2014b). Due to the restricted inclusion level of silage and the experimental design where each FPH was to contribute equivalent levels of protein to the final diet, the maximum inclusion level of the FPH products was not established in the current trial.

Production performance between treatments was not equivalent. The trial showed that the combination of FPH type and dietary inclusion level can significantly affect production performance parameters. The low inclusion level of mixed-origin hydrolysate (HCL) resulted in fish with significantly higher total animal length and height compared to the high silage treatment (SH) and can therefore be said to have exhibit better growth (Figure 7-1). Final animal weights and SGR in treatment HCL were also higher than in treatment SH, although differences were not significant. However, the differences might have become statistically significant if the trial was run for a longer period which more closely represents a full grow-out cycle. The reason for higher growth in treatment HCL than SH might be because the high silage inclusion was affecting growth rate. An equivalent silage inclusion level has been shown in a previous trial to negatively impact growth in *Oreochromis mossambicus* (Goosen et al. 2014b). In a comparative FPH evaluating three hydrolysates from different origins at a single inclusion level in red seabream, it could not be shown that there were significant differences in production performance (Bui, et al., 2014); however only two of the ingredients significantly improved performance compared to the control.

There were no indications that the FPH products had any negative impacts on animal health status or intestinal morphology. Humoral non-specific immune parameters and haematocrit values were measured at both samplings and served as indicators of health status at the two different sampling times. The lack of significant differences between treatments serves as indication that the different dietary ingredients did not affect animal health negatively. Further, at Sampling 2, neither the hepatosomatic index nor the gross intestinal morphology differed significantly between treatments, further indicating that dietary FPH supplementation had no negative impacts on fish health or the digestive system. FPH also had no effect on hepatosomatic index in Atlantic salmon (Espe, et al., 2012). It is known that dietary FPH inclusion can result in increased levels of some humoral immune parameters in various finfish species (Bui, et al., 2014; Liang, et al., 2006; Tang, et al., 2008), although no increases were seen in the current trial. Increased phagocytic activity (a measure of cellular non-specific immunity) has been reported before in *O. mossambicus* upon dietary fish silage supplementation (Goosen et al. 2014b), but effects on cellular immunity were not investigated in the current trail.

Caution should be used when interpreting differences in fish condition as measured with the use of condition factors. The significantly lower Jones CF found in treatment HCL when compared to both silage containing treatments, indicates that there were significant differences in the relative bodily proportions of the animals in the two treatments. However, the practical significance of these differences is difficult to interpret in the present trial. The conventional interpretation is that fish with a higher condition factor are in better physiological condition and general health (Jones, et al., 1999; Richter, et al., 2000).

However, it is also acknowledged that the relationship between condition factor and the physiological status of fish is not always clear (Sutton, et al., 2000). Applying the conventional interpretation to the data of the current trial, the conclusion might be reached that fish in treatment HCL were in worse condition than those in treatments SL and SH, a conclusion which is clearly not true from growth parameter and immunity data. In fact, fish in treatment HCL were significantly longer and higher than those in treatment SH and had numerically higher final weights than both silage containing diets. Conclusions of the trial outcome should therefore not be based on condition factor data alone, but should also take into account the additional data gathered.

The decrease in Jones CF seen for treatment HCL compared to treatments SL and SH could be explained by a change in body composition as a result of FPH supplementation. An alternative explanation for the change in condition factor that is linked to changes in body composition is discussed by Richter, et al. (2000). The various condition factors are essentially a calculation of fish density from animal weight and a combination of animal length and height, depending on the specific condition factor. In the case of the Jones CF, the conventional interpretation where fish condition increases with increasing condition factors is applied by the authors (Jones, et al., 1999). However, in the case where condition factor only varies according to fish density, an increase in fish condition should decrease the condition factor as water is replaced by less dense components like fat and protein in the body with a fairly constant amount of ash (Richter, et al., 2000). In hydrolysed protein studies, significant changes in carcass composition were found in Nile tilapia (El-Hakim, et al., 2007; Leal, et al., 2010), rainbow trout (Aksnes, et al., 2006a) and carp larvae (Carvalho, et al., 1997) upon dietary supplementation of different levels, size-fractions, and types of protein hydrolysates. The effect of FPH supplementation on body composition was not studied in the current trial.

Conclusion

Growth performance indicated that all protein hydrolysates evaluated were acceptable feed ingredients for total protein contribution levels from the hydrolysates as high as 40 $g \cdot kg^{-1}$. However, growth performance of treatments was not equivalent, and might be a result of hydrolysate raw material origin and dietary inclusion level. There were further no negative impacts on animal health status or gross intestinal morphology from any of the treatments. The results obtained for the Jones Condition Factor highlight the possible difficulties in interpretation of condition factor data when there are significant differences between treatments; it is recommended that conclusions not be made based on condition factor data alone, but that supplementary trial data should also be taken into consideration.

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Tables

Table 7-1 Formulations and proximate composition of experimental diets

	Diet formulations						
Ingredient (g·kg^{-1})	**SL**	**SH**	**HPL**	**HPH**	**HCL**	**HCH**	**C**
Basal diet	805	712	940	940	902	876	940
Fishmeal	30	0	30	0	38	9	60
Trout silage	165	288	-	-	-	-	-
HP	-	-	30	60	-	-	-
HC	-	-	-	-	60	115	-
Proximate composition (g·kg^{-1})							
Crude protein	471	472	474	466	474	470	462
Crude fat	64	59	61	62	62	60	59
Moisture	59	62	54	54	54	56	59
Ash	112	109	112	116	116	115	115
Carbohydrates [a]	294	298	299	302	294	299	305
Gross energy (MJ·kg^{-1})	18.7	18.6	18.8	18.7	18.7	18.7	18.5

[a] – Determined by difference

Table 7-2 Summary of production performance parameters. Data are presented as mean ± standard error (SE).

	SL	SH	HPL	HPH	HCL	HCH	C
W_i (g)	10.3 ± 0.26	10.9 ± 0.35	11.0 ± 0.29	10.7 ± 0.30	10.7 ± 0.33	11.0 ± 0.33	11.0 ± 0.36
SGR (d^{-1})	2.81 ± 0.08	2.59 ± 0.14	2.66 ± 0.12	2.71 ± 0.08	2.84 ± 0.11	2.67 ± 0.13	2.67 ± 0.13
FCR	1.35 ± 0.04	1.60 ± 0.23	1.42 ± 0.07	1.37 ± 0.04	1.22 ± 0.05	1.51 ± 0.11	1.47 ± 0.08
Dress-out %	82.5 ± 1.07	81.9 ± 0.80	82.7 ± 0.62	83.2 ± 1.20	82.9 ± 0.81	82.6 ± 0.76	78.8 ± 3.88

W_i – Initial weight

Table 7-3 Indicators of fish health and immunity. Data are presented as mean ± SE.

	SL	SH	HPL	HPH	HCL	HCH	C
Sampling 1							
Lysozyme (µg·ml^{-1})	20.6 ± 2.78	25.3 ± 4.35	27.4 ± 6.58	19.8 ± 3.8	22.4 ± 3.70	16.7 ± 2.60	17.6 ± 2.52
TP (mg·ml^{-1})	20.4 ± 0.94	20.4 ± 0.25	21.5 ± 1.53	21.5 ± 1.15	21.8 ± 1.29	21.0 ± 0.97	21.0 ± 1.14
IG (mg·ml^{-1})	1.73 ± 0.94	1.34 ± 0.56	3.21 ± 1.10	1.75 ± 0.70	1.55 ± 0.54	2.11 ± 0.80	3.00 ± 1.35
Haematocrit (%)	41.4 ± 1.55	43.3 ± 2.47	41.7 ± 1.08	41.1 ± 1.32	40.1 ± 1.58	41.0 ± 2.17	39.9 ± 1.44
Sampling 2							
Lysozyme (µg·ml^{-1})	14.5 ± 2.46	20.9 ± 4.30	20.1 ± 3.30	16.9 ± 2.20	19.5 ± 3.09	18.5 ± 2.21	16.9 ± 2.81
TP (mg·ml^{-1})	22.2 ± 0.51	23.2 ± 0.19	22.2 ± 0.46	22.7 ± 0.82	21.9 ± 0.31	21.7 ± 0.60	23.1 ± 0.38
IG (mg·ml^{-1})	1.87 ± 0.45	2.13 ± 0.24	1.96 ± 0.52	2.45 ± 0.58	2.47 ± 0.30	1.94 ± 0.78	2.05 ± 0.41
Haematocrit (%)	41.3 ± 1.76	42.8 ± 2.87	41.3 ± 1.20	41.8 ± 1.33	40.0 ± 1.86	41.5 ± 2.50	40.6 ± 1.50
Hepatosomatic index (%)	2.13 ± 0.27	2.27 ± 0.25	2.08 ± 0.17	2.19 ± 0.25	1.95 ± 0.18	2.25 ± 0.14	1.96 ± 0.25

Abbreviations: TP – total protein; IG – immunoglobulin

Table 7-4 Summary of data of intestinal morphology (mean ± SE). All values are reported in units of µm, except the L:Wid which is a dimensionless quantity.

	SL	SH	HPL	HPH	HCL	HCH	C
Inner perimeter	9731 ± 958	10150 ± 717	10524 ± 1273	10704 ± 1027	11151 ± 1296	10570 ± 1447	11234 ± 1428
Outer perimeter	5932 ± 533	5757 ± 422	5590 ± 668	6198 ± 377	5764 ± 266	5356 ± 271	6290 ± 589
Villi length	147 ± 17.8	144 ± 12.8	154 ± 24.0	139 ± 9.30	165 ± 24.2	160 ± 20.7	129 ± 17.6
Villi width	110 ± 8.75	113 ± 6.83	117 ± 10.1	104 ± 8.65	112 ± 11.2	108 ± 19.0	102 ± 18.4
L:Wid	1.38 ± 0.21	1.30 ± 0.13	1.34 ± 0.22	1.35 ± 0.08	1.50 ± 0.16	1.68 ± 0.32	1.31 ± 0.09
Mucosa width	39.5 ± 4.16	34.5 ± 2.33	43.3 ± 7.00	29.0 ± 4.23	33.0 ± 4.32	41.5 ± 8.85	37.4 ± 3.52

L:Wid – Ratio of villi length to villi width.

Figures

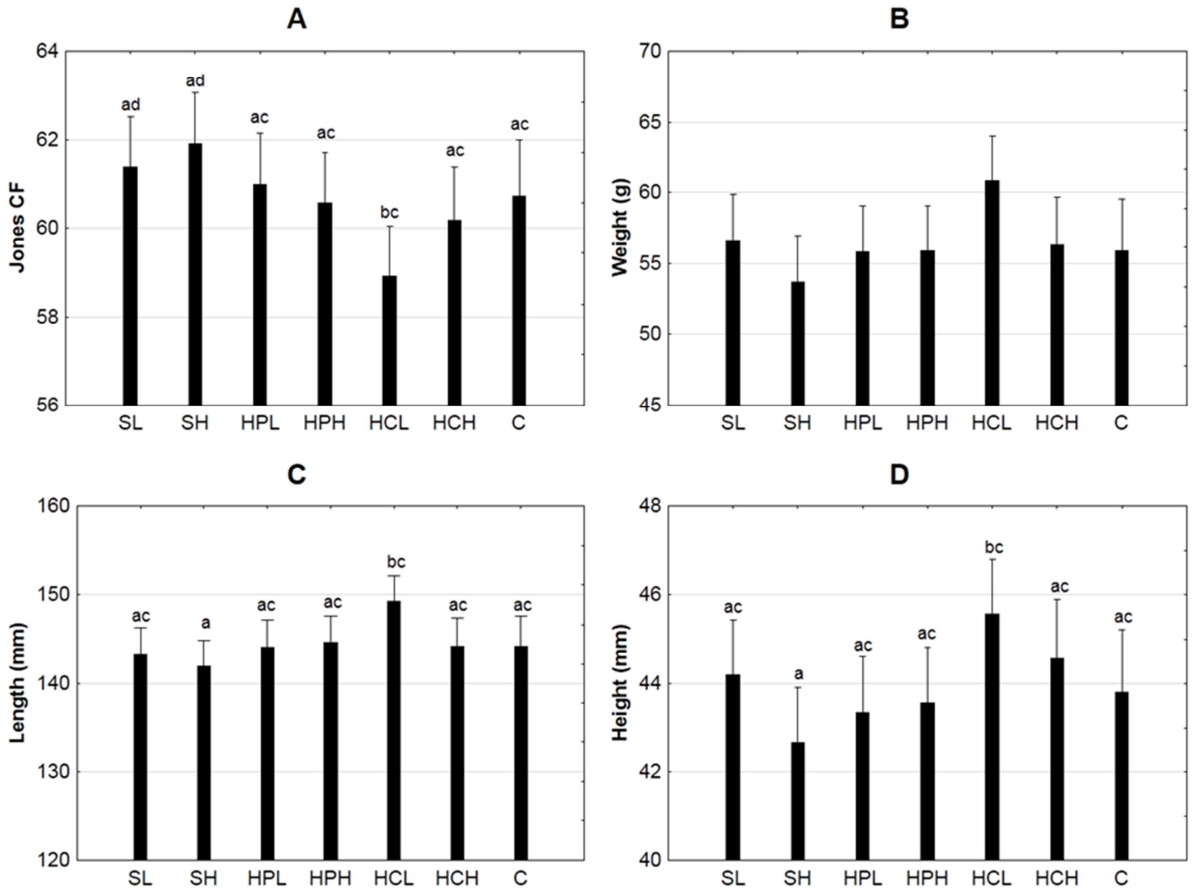

Figure 7-1 Jones CF (A), animal weight (B), length (C) and height (D) as measured at the conclusion of the trial. Values are shown as mean ± 95% confidence intervals. Common text superscripts indicate that means do not differ significantly.

Chapter 8: Fish silage oil in diets for South African abalone

"There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after." J.R.R. Tolkien, The Hobbit

This chapter is also published in the form of a journal article:

Goosen, N.J., De Wet, L.F., Görgens, J.F., 2014. Rainbow trout silage oil as immunity enhancing feed ingredient in formulated diets for South African abalone *Haliotis midae*. Aquaculture. 430, 28 - 33.

Abstract

In order to address aims I and II of the study, this chapter specifically aimed to determine the bio-active properties and effects on production performance of silage oil recovered from rainbow trout processing waste in the South African abalone. Bio-activity was quantified through cellular and non-cellular non-specific immunity, and concentration of circulating hemocytes. Results indicated that cellular non-specific immunity was significantly enhanced through dietary silage oil addition; however, this was accompanied by significant decreases in production performance (lower growth rates and higher feed conversion). Silage oil supplementation had no negative impacts on final foot muscle proximate composition, fatty acid profile or feed water stability.

The work described in this chapter makes the following novel contributions:

1) For the first time it is shown in any abalone species, that dietary lipids can enhance abalone immune function.
2) Significant increases in immunity are accompanied by decreased production performance, rendering rainbow trout silage oil ineffective as protein sparing feed ingredient.
3) It is shown that dietary silage oil supplementation did not result in changes to the foot muscle fatty acid profile or proximate composition.
4) The fatty acid profile of the foot muscle of cultured South African abalone is reported.

Summary of authors' contributions

Goosen, N.J.: Responsible for majority of trial planning, experimental design, feed formulation, sourcing of preparation of experimental feed ingredients; prepared epxerimental feeds; responsible for sampling of animals for production performance, immunology and foot muscle fatty acid composition; analysed immunity parameters; completed water stability determination; performed statistical analysis of data; wrote draft manuscript and incorporated comments of co-authors before submission. Estimated % contribution to the work: 80 %

De Wet, L.F.: Assisted with securing experimental facilities, and with feed formulation and experimental design. Assisted with interpretation of final data and reviewed the completed manuscript. Took part in general discussion surrounding the project. Estimated % contribution to the work: 10 %

Görgens, J.F.: Assisted with interpretation of final data and reviewed the completed manuscript. Took part in general discussion surrounding the project. Estimated % contribution to the work: 10 %

Rainbow trout silage oil as immunity enhancing feed ingredient in formulated diets for South African abalone *Haliotis midae*

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Key Words

fish silage, sustainability, polyunsaturated fatty acids, protein replacement, water stability, muscle composition

Abbreviations

SGR – specific growth rate; FCR – feed conversion ratio; SD – standard deviation; RFI – relative feed intake; PBS – phosphate buffered saline; FAME – fatty acid methyl esters; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids

Abstract

This study was conducted to evaluate the effects of rainbow trout silage oil on the immune function, growth performance and foot muscle fatty acid profile and proximate composition of South African abalone *Haliotis midae*. Rainbow trout silage oil was prepared from farmed rainbow trout processing waste, included at 25 $g \cdot kg^{-1}$ in a formulated diet and compared to a control diet in a 153 day growth trial. It was found that rainbow trout silage oil addition significantly improved abalone cellular immune function by increasing hemocyte phagocytic activity from 48.8% in the control diet to 65.2% in the silage oil diet. However, production performance was negatively impacted: specific growth rate (SGR) and final animal weight were decreased 17% and 5.6% respectively, and feed conversion ratio (FCR) increased by 25.1%. Foot muscle fatty acid profile and proximate composition were unaffected. It is concluded that dietary fish silage oil inclusion can improve cellular immune function in *Haliotis midae*, but that the optimal inclusion level should be determined in order to negate the negative effects on production efficiency.

Introduction

Little is known about the potential effects of dietary lipids on the immune function of abalone. Dietary lipids in formulated abalone diets are a source of important nutrients like essential fatty acids that are required for growth, gonad maturation and immune function (Dang, et al., 2011; Mai, et al., 1995; Nelson, et al., 2002; Van Barneveld, et al., 1998). In finfish and shellfish aquaculture, it is accepted that dietary lipids play an important role in immune function (Kiron, 2012; Trichet, 2010). Dietary lipids can influence animal immunity via different mechanisms. Lipids are an important source of energy to an organism (Kiron, 2012) and contribute to energy required for proper immune function and response (Martin, et al., 2010). Lipids are also a source of n-3 fatty acids that are known to play an essential role in the immune function of fish (Trichet, 2010). It is therefore also possible that these polyunsaturated fatty acids (PUFA) could influence the immunity of abalone.

Fish silage oil recovered from fish processing waste is a potential abalone feed ingredient as it can serve as a good source of essential fatty acids and it has the advantage of being a more sustainably produced feed ingredient than conventional fish oil using wild caught fish (Goosen, et al., 2014; Naylor, et al., 2009; Vidotti, et al., 2011). Production of conventional fish oil from wild caught fish is not envisaged to

increase in future, due to the stagnation of output from capture fisheries (FAO, 2012) and the concerns related to the sustainability of oil production from wild caught fish (WWF, 2012). The utilisation of feed ingredients derived from fish processing waste in abalone feeds will improve the sustainability of the feed (ASC, 2012; Naylor, et al., 2009), as it does not contribute to increased fishing pressure.

Silage oil inclusion in abalone diets can potentially lower dietary protein requirements, by contributing dietary energy. Excess protein in diets is not utilised for growth, but as a source of energy (Chaitanawisuti, et al., 2011; Zhou, et al., 2007). Protein is one of the more expensive nutrients to supply in feed formulations (Guerreiro, et al., 2014; Singh, et al., 2006) and lowering the protein level in abalone diets without affecting production performance will have economic advantages to the abalone feed industry. Decreased feed protein content can also improve general husbandry conditions in production systems. High dietary protein intake has been identified as possible contributing factor to bloating experienced during episodes of increased water temperatures, which might lead to mortalities (Green, et al., 2011a; Kirkendale, et al., 2010). Deamination of amino acids during catabolism further leads to deterioration of water quality in culture systems through ammonia excretion (Chaitanawisuti, et al., 2011; Singh, et al., 2006), which is suspected to be a contributing factor to bloating (Matthias Wessels, personal communication).

Oil addition to abalone diets could influence final product quality through alteration of muscle proximate composition or fatty acid profile. As tissue lipids strongly influences meat flavour (Britz and Hecht, 1997) and abalone diet composition is able to affect abalone body composition and fatty acid profile (Dunstan, et al., 1996; Mateos, et al., 2011; Xu, et al., 2011), it is important to monitor the lipid level and composition of the abalone meat to determine any possible adverse effects of silage oil inclusion on foot muscle composition.

Little work has been done on the potential effects of dietary lipids on the immune function of abalone, therefore the study aimed to determine the effect of dietary fish silage oil derived from processing waste on the immune function of the South African abalone *Haliotis midae*, and to monitor production parameters and changes in muscle lipid content and composition.

Materials and Methods

Experimental design

The inclusion of silage oil in abalone diets was evaluated in a 153 day growth trial between August 2011 and February 2012 on Wild Coast Abalone farm, Eastern Cape, South Africa. Two diets were compared: a control diet that consisted of a commercial, proprietary formulation (AquaNutro Abalone Grower, NutroScience, Malmesbury, South Africa) and an experimental diet which consisted of the control diet supplemented with 25 $g \cdot kg^{-1}$ rainbow trout silage oil. Silage oil inclusion level was chosen to ensure that the final crude fat content of the diet would be below that which was previously shown to be growth inhibiting in *Haliotis midae* (70 - 100 $g \cdot kg^{-1}$) (Britz and Hecht, 1997; Green, et al., 2011b).

Each treatment was replicated 6 times by feeding each diet to six individual baskets. At the conclusion of the trial, 3 animals per treatment were randomly selected from different baskets (n = 3) for determination of fatty acid profile and proximate composition of abalone foot muscle. Non-specific immunity was determined by sampling one animal from each replicate basket (n = 6).

Diet preparation

The two diets were prepared through cold extrusion at 70 °C and dried in a drying oven at 55 °C. Diet composition and proximate analysis are given in Table 8-1. The control diet contained higher crude protein than the silage oil diet (406 $g \cdot kg^{-1}$ vs. 389 $g \cdot kg^{-1}$) and lower crude fat (33 $g \cdot kg^{-1}$ vs. 58 $g \cdot kg^{-1}$), but approximately the same gross energy (16.8 $MJ \cdot kg^{-1}$ and 16.9 $MJ \cdot kg^{-1}$)

The silage oil was recovered from trout silage prepared from farmed rainbow trout viscera sourced from a local fish processor. Briefly, fish silage was prepared through addition of 25 $g \cdot kg^{-1}$ formic acid (85%) to rainbow trout viscera and allowed to hydrolyze at ambient temperature (15 °C - 25 °C). The floating silage oil was recovered through manual decantation after 3 days, stabilised through addition 1 $ml \cdot litre^{-1}$ of butylated hydroxytoluene/ butylated hydroxyanisole mixture as antioxidant (Oxipet L, Bitek) and stored in plastic containers at 4 °C for 14 days until diet preparation. The fatty acid profile of the silage oil is given in Table 8-4.

Experimental system and procedures

Experiments were conducted under the same conditions as experienced by the on-growing operations of the commercial farm. Animals were housed in plastic baskets in culturing tanks at commercial stocking densities, and fed to apparent satiation according to on-farm protocols. The experimental system was continuously supplied with unfiltered seawater as extracted from the ocean and culturing tanks were continuously aerated. Animals were subjected to a natural photoperiod. Initial and final animal weights were determined as the mean of 30 randomly selected animals from each basket. Initial mean animal weights ± standard deviation (SD) were 125.7 g ± 5.1.

The production parameters evaluated were animal weight, specific growth rate (SGR), feed conversion ratio (FCR) and relative feed intake (RFI). SGR was calculated as $SGR = 100*[\ln(W_{i+1}/W_i)/(t_{i+1} - t_i)]$, with units of grams and days for W and t respectively, at sampling time i. FCR was calculated as follows: $FCR = (W_{i+1} - W_i)/(\text{feed given})$; the FCR values calculated are viewed as an effective on-farm feed conversion due to inability to recover uneaten feed. RFI is determined as $RFI = (\text{feed given})/[W_f + W_i)/2]$, with W_f and W_i the final and initial mean animal weight in grams respectively. Animals were fed according to apparent requirements by experienced operators and uneaten feed was removed.

Measurement of non-specific immunity

Non-specific immunity of abalone was determined by evaluation of the concentration and phagocytic activity of circulating hemocytes, and measuring total protein and lysozyme concentrations of hemolymph. Hemolymph was collected from the pedal sinus using sterile 29 gauge syringes; collection was done immediately after capture of each animal. Upon collection, hemolymph samples were divided into two aliquots of which one was frozen immediately until analysis of total protein and lysozyme levels could be performed. The remaining fresh hemolymph was used for determination of the concentration of circulating hemocytes and phagocytic activity of hemocytes.

Phagocytic activity of hemocytes was determined by the method used by Dang et al. (2011). Yeast was prepared for the assay by autoclaving 4% Congo red (Sigma) and 4% baker's yeast (NCP, Modderfontein, South Africa) in phosphate buffered saline (PBS) at 121 °C for 20 minutes. The suspension was centrifuged and washed 4 times with PBS and re-suspended in synthetic seawater (40 $g \cdot litre^{-1}$ sea-salts, Sigma,

dissolved in reverse osmosis water). Phagocytic rate was determined by spreading 50 µl of fresh hemolymph on a glass slide and incubating at room temperature for 10 minutes to allow hemocytes to adhere to the slide. After adhesion, remaining hemolymph was poured off the slide, 50 µl of yeast suspension was added to adhered hemocytes and incubated for 30 minutes at room temperature. Phagocytosis was terminated through washing slides with synthetic seawater and fixing hemocytes using 10% (w/w) formaldehyde solution in synthetic seawater for 20 minutes. Phagocytosis was quantified by manually counting at least 100 hemocytes per slide under a light microscope and expressing the number of hemocytes containing yeast as a percentage of the total number of hemocytes counted.

The concentration of circulating hemocytes was determined through manual cell counts of undiluted hemolymph under a light microscope.

Total protein and lysozyme concentration of hemolymph were determined from frozen samples. Hemolymph was thawed at room temperature, centrifuged at 14 000 rpm for 10 minutes to remove cells and debris and the supernatant was transferred to new Eppendorf tubes. Total protein concentration was determined using the linearized Bradford assay of Zor and Selinger (1996) using bovine serum albumin standard. Lysozyme concentration was determined with the method of Sankaran and Gurnani (1972).

Proximate analysis of feeds and abalone muscle

Standard analyses were used to determine the proximate composition of feeds and abalone foot muscle. Crude protein was calculated as 6.25×N, and nitrogen was determined according to method 992.15 (AOAC, 1992). Crude fat was determined according to Lee et al. (1996) while moisture (AOAC, 2002b) and ash (AOAC, 2002a) were done according to AOAC methods.

Fatty acid analysis of silage oil and abalone muscle

Lipids were extracted from abalone foot muscle according to the method of Folch et al. (1957) and fatty acid profiles were characterized using gas chromatography. Prior to gas chromatography injection, oils were transmethylated with a 19:1 methanol:sulphuric acid mixture. An internal standard of heptadecanoic acid (C17:0, 98%, Sigma Cat no. H3500) and a standard fatty acid methyl ester (FAME) mixture (Supelco 37 Component FAME mix, Cat no. 47885-U, Bellefonte, USA) were used. The FAMEs are identified by comparison of the retention times to those of the standard FAME mixture. Sample volume injected was 1 µl. The gas chromatograph is

a Thermo Finnigan Focus GC (Thermo Electron S.p.A., Milan, Italy), fitted with BPX70 column, length 60 m and inner diameter 0.25 mm (SGE International, Victoria, Australia); carrier gas was hydrogen at 20 ml·min^{-1} with a split of 20:120; initial and final temperatures were 60 °C and 120 °C respectively, with a rate of increase of 1.7 °C·min^{-1}. Injector and detector temperatures were 220 °C and 260 °C respectively.

Measurement of relative feed water stability

The relative water stability of feeds was determined according to an accelerated stability test method utilised by the Feed Technology Group, University of Stellenbosch, whereby excessive aeration of a specific amount of feed is employed to determine physical integrity of feed when submerged in water. Feed samples were weighed into wire mesh containers and immersed in individual testing chambers. Each testing chamber is aerated individually. Testing chambers were filled with fresh water and the feeds were immersed and aerated for 18 hours, after which the remaining feed was recovered and dried overnight at 55 °C. Water stability is defined as the percentage of dry feed remaining after 18 hours of immersion and is viewed as the physical stability of the feed, as the testing procedure did not enable the determination of nutrient leaching from the feed. Six replicates were used per treatment.

Statistical analysis

As only two treatments were compared, all data were analysed using the t-test in Statistica version 11 software (StatSoft, Inc.). Differences were viewed to be significant for $p < 0.05$.

Results

Immune function

Figure 8-1 shows phagocytic activity of hemocytes (A), concentration of circulating hemocytes (B), concentration of hemolymph lysozyme (C) and total protein concentration (D). Phagocytic activity was significantly higher for NC+SO (48.8% and 65.2% for the control and treatment NC+SO respectively), while there were no significant differences in any of the other non-specific immunity parameters. Hemocyte concentration was 2.31 x $10^6 \cdot ml^{-1}$ for the control and 2.73 x $10^6 \cdot ml^{-1}$ for treatment NC+SO; hemolymph lysozyme concentration was 1.51 $\mu g \cdot ml^{-1}$ and 1.40 $\mu g \cdot ml^{-1}$ and total protein concentration was 8.09 $mg \cdot ml^{-1}$ and 8.64 $mg \cdot ml^{-1}$ for the control and treatment NC+SO respectively.

Production performance parameters

The results of production performance parameters are summarised in Table 8-2. There were significant differences in final animal weight, SGR and FCR between treatments, while no differences were observed in RFI of the animals. Final animal weight and SGR were 5.6% and 17.2% lower respectively for treatment NC+SO when compared to the control, while FCR for treatment NC+SO was 25.1 % higher.

Muscle proximate composition and fatty acid profile

There were no differences in proximate composition of abalone foot muscle between treatments (Table 8-3). The control treatment comprised of 205 ± 7.3 $g \cdot kg^{-1}$ (mean ± SD) crude protein, 7.1 ± 1.5 $g \cdot kg^{-1}$ crude fat and 695 ± 31.2 $g \cdot kg^{-1}$ moisture, while the silage oil treatment comprised 199 ± 5.3 $g \cdot kg^{-1}$ crude protein, 6.8 ± 1.0 $g \cdot kg^{-1}$ crude fat and 708 ± 33.1 $g \cdot kg^{-1}$ moisture. The muscle fatty acid profiles for both treatments are shown in Table 8-4; the only fatty acid for which statistically significant differences between treatments were seen was for C18:1n9*trans* where the amount was 0.43% ± 0.04 in the control 0.35% ± 0.02 in the silage oil treatment. Saturated fatty acids (SFA) comprised 44.1% ± 5.06 and 45.7% ± 2.69 of total fatty acids in abalone foot muscle in the control and treatment NC+SO respectively, monounsaturated fatty acids (MUFA) comprised 23.3% ± 3.24 and 23.0% ± 2.89 and polyunsaturated fatty acids (PUFA) comprised 32.2% ± 1.79 and 31.0% ± 2.1 in the control and silage oil treatment respectively.

Feed water stability

Figure 8-2 shows the results for relative water stability of feeds determined under accelerated conditions. There was no significant difference between the treatments and the mean ± SD was 54.3% ± 7.6 in the control and 53.6% ± 13.1 for treatment NC+SO.

Discussion

Fish silage was prepared from farmed rainbow trout processing waste. The silage oil was recovered, included in a formulated feed for South African abalone and evaluated as feed ingredient in a growth trial on a commercial abalone farm. The effects of dietary silage oil inclusion in South African abalone diets were quantified and compared to a control diet by evaluating the effects on the non-specific immunity, production performance parameters and foot muscle fatty acid profile and proximate composition of the experimental animals.

It is reported that the cellular immune function of abalone can be enhanced by dietary supplementation of rainbow trout silage oil, as demonstrated by the significantly increased phagocytic activity of hemocytes in the silage oil diet (Figure 8-1A). Decreased immune function can increase the susceptibility of abalone to disease (Cheng, et al., 2004; Travers, et al., 2008) with possible economic losses to producers; therefore good immune function of cultured animals is of paramount importance to the abalone industry. Increased phagocytic activity of hemocytes is a good indication of increased immune status, as hemocytes play a central role in both the cellular and non-cellular (humoral) immune function of abalone (Hooper, et al., 2007; Travers, et al., 2008). The humoral immune parameters measured were not affected by dietary silage oil addition. The hemolymph lysozyme concentration was found to be low in the experimental animals (approximately 1.5 $\mu g \cdot ml^{-1}$ for both treatments), which is in agreement with the findings of Shuhong and co-workers (2004) who found similarly low levels of lysozyme in *Haliotis diversicolor supertexta*.

The increased cellular immune function resulting from rainbow trout silage oil supplementation can possibly be explained through the provision of dietary fatty acids that play a role in enhancing abalone immune function. Some polyunsaturated fatty acids like n-3 PUFA are known to play a role in the immune function of fish (Trichet, 2010). The silage oil contained high levels of polyunsaturated fatty acids (36.1% ± 0.59 of total fatty acids), and 23.1% of total fatty acids consisted of n-3 PUFA. It is also

known that the fatty acid composition of phagocytic cells are important in fish (Montero, et al., 2003). It is possible that the fatty acid profile of hemocytes could have been altered by dietary silage oil addition, which in turn might have improved phagocytic ability.

Addition of rainbow trout silage oil to the formulated diet of South African abalone could not successfully decrease feed protein content, as production performance parameters were negatively impacted as a result of oil supplementation. The exact reason for the decreased production performance was not established in this study. It has been suggested that dietary oil could interfere with digestive processes through decreased nutrient uptake or reduced digestibility (Green, et al., 2011b; Van Barneveld, et al., 1998). The decreased production performance might also be due to the creation of an imbalance in the relative amounts of certain dietary fatty acids due to silage oil supplementation. The ratio of dietary linoleic acid to eicosapentaenoic acid significantly affected the growth in juvenile *Haliotis discus hannai* (Xu, et al., 2011). However, this was not investigated in the current trial. Another possible reason for the impaired production performance might be the decreased crude protein content of the feed, resulting from nutrient dilution by silage oil addition. However, in the current trial, the feed crude protein content in treatment NC+SO decreased by only 17 $g \cdot kg^{-1}$ after silage oil addition, but this corresponded to significant decreases in production performance (SGR decreased by 17.2%, final animal weight by 5.6% and FCR increased by 25.1% in the silage oil diet), which is inconsistent with the relatively minor decrease in feed protein content.

The trial data indicate that the interrelation between immune function, production efficiency and dietary feed intake in abalone is complex. This fact has also been noted in a previous study which monitored immune function and growth subsequent to algal diet supplementation (Dang, et al., 2011). In the current trial an improvement in cellular immune function coincided with a decrease in production performance as a result of silage oil supplementation in the diet. Improvement in cellular immunity is beneficial to producers, but the impaired production efficiency is not. From a producer's perspective, it is therefore necessary to find a balance where cellular immunity can be improved without significantly impairing production efficiency. It is possible that decreasing the silage oil inclusion level could achieve this, but needs to be investigated further.

There was no indication from data that the experimental animals altered their feed intake to compensate for a change in gross energy content of the diet. The calculated gross energy value of treatment NC+SO was slightly higher than for the control diet (16.9 $MJ \cdot kg^{-1}$ for treatment NC+SO and 16.8 $MJ \cdot kg^{-1}$ in the control), yet the

RFI (measuring the feed intake relative to animal weight) did not differ (Table 8-2). Abalone are thought to regulate their feed intake according to energy requirements and this was also demonstrated in greenlip abalone (Stone, et al., 2013); however it could not be shown in the current trial. A similar observation was made by Britz and Hecht (1997) in *H. midae*, who found that experimental animals did not decrease their feed intake when fed diets with higher energy content, although the authors did observed that the large variations in feed intake may have masked any trends. In the current study, uneaten feed could not be recovered to determine a more accurate estimate of actual feed intake and similar to the study of Britz and Hecht (1997), may have precluded the detection of differences in feed intake. It is further also possible that the magnitude in the increase in dietary crude energy (0.1 $MJ \cdot kg^{-1}$ or approximately 0.6%) in treatment NC+SO was not large enough for animals to significantly alter feed intake.

Silage oil addition had no negative impacts on the composition of abalone foot muscle. Neither the proximate composition nor the fatty acid profile of abalone foot muscle was affected by the additional dietary silage oil.

The largest proportion of the fatty acids found in the foot muscle of cultured *H. midae* consisted of SFA. Very little information is available on the fatty acid composition of *H. midae*, especially for cultured animals. Results of the current study do, however, correlate well with the soft tissue fatty acid composition of juvenile wild caught *H. midae* (Knauer, et al., 1995). SFA comprised 44.1% and 45.7% for the control and silage oil supplemented diets respectively, which is similar to the SFA content of 42.5% found in the wild caught animals. PUFA levels were 31.0% and 32.2% in the current study and 34.4% in the wild abalone, with the remainder of the fatty acids in each case comprising MUFA. In both wild and cultured *Haliotis rubra*, the largest proportion of fatty acids were also found to be SFA (Su, et al., 2004).

Results of the relative water stability tests confirm that no detrimental effects on feed water stability resulted from silage oil addition. Decreased feed availability to the animals as a result of disintegration of feed can therefore be discounted as the reason for the poorer production performance of the oil supplemented feed. Due to the accelerated nature of the testing method, the relative water stability results do not reflect the actual water stability of the feeds in commercial production systems and can therefore only be employed to compare the relative water stability of two or more feeds. Leaching of nutrients from feed was not determined.

The relatively larger animals used in the current trial (approximately 125 g initial weight) utilise dietary lipid less efficiently than smaller animals of up to 50 g. Production parameters were significantly impacted in the current trial at total dietary lipid level of 58 $g \cdot kg^{-1}$, which (based on published data from previous investigations in

H. midae) was thought to be below the maximum acceptable dietary inclusion level. Two other studies on *H. midae* found that levels of 100 $g \cdot kg^{-1}$ for animals of 0.2 g and 7.8 g mean weight (Britz and Hecht, 1997) and 70 $g \cdot kg^{-1}$ for animals up to 50 g mean weight (Green, et al., 2011b) were the threshold where dietary lipid levels became excessive. Abalone have comparatively low lipase levels in the digestive tract (Britz, et al., 1996; Garcìa-Esquivel and Felbeck, 2006; Knauer, et al., 1996) and it has been found that the South African abalone does not utilize dietary lipids efficiently as energy source above a certain threshold level (Green, et al., 2011b). In other abalone species, the lipase activity in juvenile *Haliotis rubra* was relatively high, and it was suggested that juveniles may be more efficient than older animals in digesting and utilization of dietary lipids (Johnston, et al., 2005). This aspect deserves further investigation in *H. midae*.

In conclusion, the study showed that dietary rainbow trout silage oil inclusion can enhance cellular immunity in *Haliotis midae*, but that production performance (SGR and FCR) is negatively impacted at total dietary lipid content of 58 $g \cdot kg^{-1}$. Dietary silage oil had no adverse effects on animal feed intake and feed water stability, and had no negative impacts on muscle proximate composition or muscle fatty acid profile. Future research should determine optimal rainbow trout silage oil inclusion levels for different size classes of animals, in order to establish whether the negative effects on production performance can be negated through establishing appropriate inclusion levels, without sacrificing the positive effects that silage oil has on immunity of *Haliotis midae*.

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Tables

Table 8-1 Diet formulations and proximate composition of experimental diets. NC - negative control; NC+SO - silage oil supplemented diet.

Diet formulation (g·kg^{-1})		
Ingredients	**NC**	**NC+SO**
Basal diet	1000	975
Trout silage oil	-	25
Total	1000	1000
Proximate composition (g·kg^{-1})		
Crude protein	406	389
Crude fat	33	58
Crude fibre	26	25
Moisture	83	85
Ash	83	81
Carbohydrates*	369	362
Gross energy (MJ·kg^{-1})	16.8	16.9

* Determined by difference

Table 8-2 Summary of production performance data. All data are presented as mean ± SD. Values within the same row with differing superscripts are significantly different ($p < 0.05$). NC - negative control diet; NC+SO - silage oil supplemented diet.

Performance parameter	**NC**	**NC+SO**
$W_{initial}$ (g)	125.7 ± 5.1	125.7 ± 5.1
W_{final} (g)	163.9 ± 9.3[a]	154.7 ± 2.0[b]
SGR (d^{-1})	0.169 ± 0.01[a]	0.140 ± 0.02[b]
FCR	2.19 ± 0.07[a]	2.74 ± 0.28[b]
RFI (g feed·g animal $weight^{-1}$)	0.56 ± 0.03	0.58 ± 0.02

Abbreviations: W - animal weight; SGR - specific growth rate; FCR - feed conversion ratio; RFI - relative feed intake.

Table 8-3 Proximate composition of abalone foot muscle 'as is'. Data are presented as mean ± SD (n = 3). NC - negative control diet; NC+SO - silage oil supplemented diet.

Component (g·kg^{-1})	**NC**	**NC+SO**
Crude fat	7.1 ± 1.5	6.8 ± 1.0
Crude Protein	205 ± 7.3	199 ± 5.3
Ash	16.4 ± 1.4	18.4 ± 2.5
Moisture	695 ± 31.2	708 ± 33.1
Dry matter	305 ± 31.2	292 ± 33.1
Carbohydrates*	76.5 ± 24.2	67.8 ± 30.3

* Determined by difference

Table 8-4 Fatty acid profiles of silage oil and abalone foot muscle. Data are presented as mean (% of total fatty acids) ± SD, with n = 3 for foot muscle data. Different superscripts in the same row indicate significant differences between treatments for foot muscle data.

Fatty acid	Rainbow trout silage oil	Abalone foot muscle	
		NC	NC+SO
C12:0	0.02 ± 0.004	ND	ND
C14:0	1.34 ± 0.09	0.93 ± 0.76	1.04 ± 0.56
C15:0	0.09 ± 0.0	0.48 ± 0.13	0.46 ± 0.09
C16:0	24.2 ± 0.46	22.3 ± 4.79	23.4 ± 2.48
C18:0	1.2 ± 0.01	17.8 ± 1.67	19.0 ± 1.9
C20:0	0.09 ± 0.001	0.63 ± 0.28	0.55 ± 0.13
C21:0	0.02 ± 0	0.37 ± 0.26	0.26 ± 0.05
C22:0	0.1 ± 0.002	1.6 ± 1.3	1.0 ± 0.4
C24:0	0.02 ± 0.001	ND	ND
C14:1	0.02 ± 0.002	ND	ND
C15:1	0.02 ± 0.001	ND	ND
C16:1	11.7 ± 0.07	3.0 ± 0.75	2.8 ± 0.20
C18:1n9*cis*	25.1 ± 0.16	19.5 ± 3.3	19.6 ± 2.7
C18:1n9*trans*	0.03 ± 0.0004	0.43^{a} ± 0.04	0.35^{b} ± 0.02
C20:1	0.02 ± 0.0003	0.45 ± 0.41	0.27 ± 0.06
C22:1n9	0.03 ± 0.0003	0.41 ± 0.36	0.25 ± 0.05
C24:1	0.09 ± 0.001	ND	ND
C18:2n6*cis*	12.2 ± 0.20	5.2 ± 0.9	4.9 ± 1.0
C18:2n6*trans*	0.04 ± 0.0	ND	ND
C18:3n6	0.31 ± 0.003	1.18 ± 0.55	0.75 ± 0.16
C18:3n3	0.6 ± 0.01	2.9 ± 0.18	3.0 ± 0.52
C20:2	0.10 ± 0.001	1.8 ± 0.89	1.4 ± 0.11
C20:3n6	0.11 ± 0.002	2.2 ± 0.44	2.4 ± 0.33
C20:3n3	0.011 ± 0.0	0.64 ± 0.69	0.28 ± 0.07
C20:4n6	0.3 ± 0.01	1.8 ± 0.59	1.6 ± 0.26
C20:5n3	0.09 ± 0.001	0.74 ± 0.23	0.82 ± 0.25
C22:2	0.01 ± 0.0	ND	ND
C22:5n3	11.4 ± 0.22	11.4 ± 2.92	12.9 ± 2.49
C22:6n3	11.0 ± 0.17	4.4 ± 3.05	2.9 ± 0.41
SFA**	27.0 ± 0.54	44.1 ± 5.06	45.7 ± 2.69
MUFA***	36.9 ± 0.12	23.3 ± 3.24	23.0 ± 2.89
PUFA#	36.1 ± 0.59	32.2 ± 1.79	31.0 ± 2.1
PUFA:SFA	1.39 ± 0.05	0.74 ± 0.13	0.68 ± 0.07
(n-6):(n-3)	0.56 ± 0.002	0.51 ± 0.04	0.49 ± 0.08

Abbreviations: ND: Not detected; ** Saturated fatty acids; *** Monounsaturated fatty acids; # Polyunsaturated fatty acids

Figures

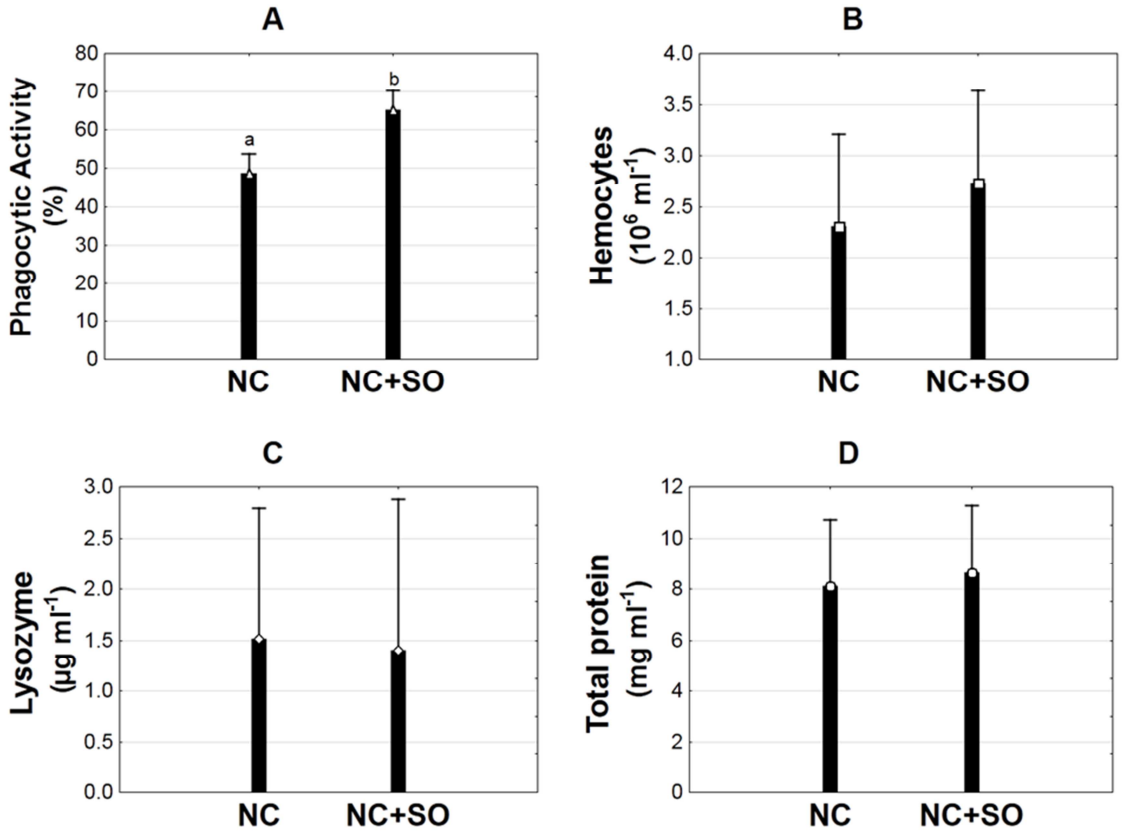

Figure 8-1 Immunity parameters: Phagocytic activity of hemocytes (A) and concentration of circulating hemocytes (B) lysozyme (C) and total protein (D) in the hemolymph. Data are presented as mean ± 95% confidence intervals (n = 6). Differing text superscripts denote statistically significant differences between means. NC - negative control diet; NC + SO - silage oil enriched diet.

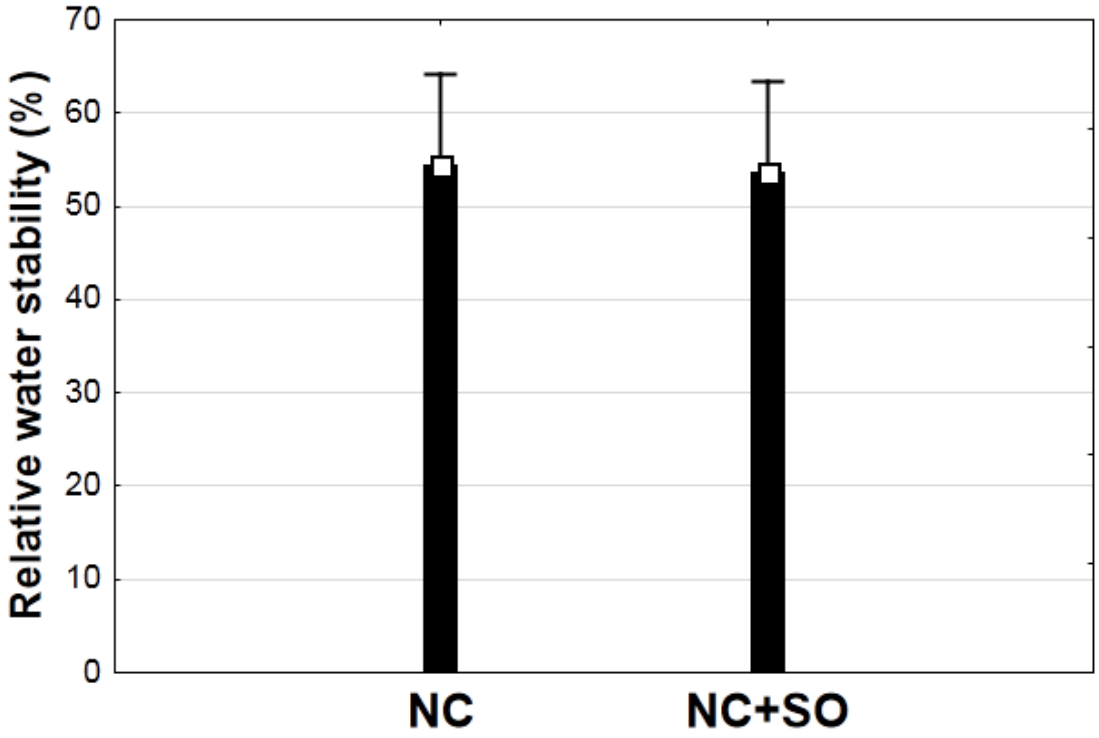

Figure 8-2 Relative feed water stability after 18 hours of submersion. Data are presented as mean ± 95% confidence intervals (n = 6). NC - negative control diet; NC + SO - silage oil enriched diet.

Chapter 9: Fish protein hydrolysates in diets for South African abalone

"I am haunted by waters." Norman Maclean, A River Runs Through It.

This chapter is also published in the form of a journal article:

Goosen, N.J., De Wet, L.F., Görgens, J.F., 2014. The effects of protein hydrolysates on the immunity and growth of the abalone *Haliotis midae*. Aquaculture. 428 - 429, 243 - 248.

Abstract

In accordance with aims I, II and III of the study, the work reported in this chapter investigated bio-active properties and effects on production performance of chemical fish silage in diets for South African abalone, and compared it to a commercial fish protein hydrolysate. The hydrolysed fish proteins were also included at two dietary levels, and the high inclusion level was further fed using a constant and alternating feeding strategy. It was found that the commercial hydrolysate significantly improved cellular non-specific immunity, but only at the low inclusion level; fish silage did not show the same improvement. Both low and high inclusion of the commercial hydrolysate significantly improved growth rate, but only under constant feeding. An intermittent feeding strategy did not improve and immunity or production performance. It was further found that hydrolysed fish protein can negatively impact on feed water stability, but that different effects are found according to the type and inclusion level of the hydrolysate.

The work reported in this chapter contributes the following novel findings:

1) For the first time it is shown in an abalone species that dietary fish protein hydrolysates can significantly enhance the non-specific cellular immune function.
2) Dietary hydrolysed protein from chemical fish silage and commercial fish protein hydrolysate resulted in significant differences in production performance.
3) Differences in production performance are correlated to the differences in hydrolysed protein properties and effects on feed stability.
4) An intermittent feeding strategy of fish protein hydrolysate containing diets did not result in any improvements in immunity or production performance.

Summary of authors' contributions

Goosen, N.J.: Responsible for: majority of trial planning, experimental design, feed formulation, sourcing of commercial feed ingredients and preparation of experimental feed ingredients; preparation of epxerimental feeds; sampling of animals for production performance and immunology; analysis of immunity parameters; completion of feed water stability tests; statistical analysis of data; wroting of draft manuscript and incorporation of comments of co-authors before submission. Estimated % contribution to the work: 80 %

De Wet, L.F.: Assisted with securing experimental facilities, and with feed formulation and experimental design. Assisted with interpretation of final data and reviewed the completed manuscript. Took part in general discussion surrounding the project. Estimated % contribution to the work: 10 %

Görgens, J.F.: Assisted with interpretation of final data and reviewed the completed manuscript. Took part in general discussion surrounding the project. Estimated % contribution to the work: 10 %

The effects of protein hydrolysates on the immunity and growth of the abalone *Haliotis midae*

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Key Words

South African abalone; Hydrolysed proteins; fish silage; water stability; phagocytosis; immune stimulation

Abbreviations

DOH – Degree of hydrolysis; LSD – Least significant difference; DWG – daily weight gain; FCR – feed conversion ratio; RFI – relative feed intake

Abstract

Dietary hydrolysed proteins have been shown to stimulate the non-specific immunity of various finfish species, while the potential for immune stimulation of these feed ingredients in abalone has not been investigated. The immune-stimulating potential of two hydrolysed protein sources (self-prepared fish silage, and a commercial fish protein hydrolysate) at two dietary inclusion levels in abalone diets was therefore measured in the South African abalone *Haliotis midae,* using animals of initial mean weight of 123 g – 128 g. Diets containing the high inclusion levels were also fed in two feeding regimes: continuous feeding, or phase feeding, where the hydrolysed protein diets were alternated monthly with the control diet. In low inclusion diets, hydrolysate inclusion contributed 6 $g \cdot kg^{-1}$ protein in the final diet, while in high inclusion diets this was increased to 18 $g \cdot kg^{-1}$. It was found that the low inclusion level of the commercial hydrolysate significantly increased the cellular immunity through increasing the phagocytic activity of hemocytes by 18% compared to the control diet, while none of the other diets showed any significant differences compared to the control. Both inclusion levels of the commercial hydrolysed protein significantly improved daily weight increase of animals. Phase feeding had no positive impacts on immunity or production performance. The inclusion of both types of hydrolysed proteins sources significantly decreased the water stability of feeds compared to the control, except at the lowest inclusion level of the commercial hydrolysate. It is concluded that the use of dietary hydrolysed proteins can lead to improved cellular immunity and growth in abalone, however it is important to determine appropriate inclusion levels to prevent negative impacts on feed water stability and production performance.

Introduction

The potential benefits to abalone derived from the functional and bio-active properties of enzymatically hydrolysed proteins included in diets are not well described. In *Haliotis fulgens*, dietary inclusion of autolysed abalone viscera increased feed attraction (Viana, et al., 1994), and successfully served as dietary protein source and fishmeal replacement (Guzmán and Viana, 1998; Viana, et al., 1996). The use of hydrolysed proteins in finfish diets has been reported widely, where both autolysed proteins (fish silage) and protein hydrolysates prepared with added enzymes have been employed successfully in aquafeeds, with improved production performance reported in eel, African catfish, Atlantic salmon, large yellow croaker and red seabream (Bui, et al., 2014; Goncalves, et al., 1989; Refstie, et al., 2004; Soltan, et al., 2008; Tang, et al., 2008). The reported biological effects included the improvement of disease resistance and survival in European sea bass larvae (Cahu, 1999; Kotzamanis, et al., 2007), stimulation of the non-specific immunity in Japanese sea bass, large yellow croaker and red seabream (Bui, et al., 2014; Liang, et al., 2006; Tang, et al., 2008), increased intestinal enzyme activity in European sea bass larvae (Kotzamanis, et al., 2007) and an alteration of the spatial gene expression of a possible di- and tri-peptide transporter in Atlantic cod (Bakke, et al., 2010).

Hydrolysed dietary proteins have been linked to stimulation of the non-specific immunity in finfish (Bui, et al., 2014; Liang, et al., 2006; Tang, et al., 2008), although not reported in abalone. Improved immunity in abalone could have benefits to producers, as a good immune response is critically important in ensuring good disease resistance (Gopalakrishnan, et al., 2009; Hooper, et al., 2007). However, continuous feeding of dietary immune stimulants to slow-growing abalone could be counterproductive and increase production costs, as is known in finfish where continual feeding of immune stimulants over an extended period of time can lead to decreased efficacy of immune stimulants (Dr. Galina Jeney, Hungarian Research Institute for Fisheries, Aquaculture and Irrigation, personal communication). Further, in abalone it has also been hypothesized that over-stimulation of immune functions could lead to impaired growth (Hooper, et al., 2010).

Suitable inclusion levels of hydrolysed proteins need to be established in abalone diets, as high inclusion of hydrolysed fish proteins in finfish diets can lead to decreased growth, presumably as a result of excessive amounts of free amino acids and short chain peptides (Espe, et al., 1992). Decreased growth upon high inclusion of hydrolysed protein has been reported in African catfish (Soltan, et al., 2008), rainbow trout (Guzel, et al., 2011), carp fingerlings (Ramasubburayan, et al., 2013) and sea bass larvae (Cahu, 1999;

Kotzamanis, et al., 2007). The threshold where dietary inclusion level of hydrolysed fish proteins in abalone diets causes decreased growth has not been reported.

In addition to inclusion levels of hydrolysed proteins in abalone diets, the potential impact on water stability of feeds also requires consideration. Abalone feeds must maintain water stability during long periods of submerging, due to slow feeding of abalone. Leaching of soluble ingredients or physical disintegration of feed will result in direct economic losses and may have adverse impacts on water quality (Kirkendale, et al., 2010). However, the water solubility of hydrolysed fish proteins is higher than for the corresponding intact proteins (Kristinsson and Rasco, 2000) and inclusion of hydrolysed proteins could therefore compromise the water stability of feeds (Guzmán and Viana, 1998).

The study aimed to evaluate the effect of two hydrolysed protein products on immune function and production parameters of South African abalone *Haliotis midae*, at two dietary inclusion levels and using different feeding regimes, and to determine the effects of hydrolysed proteins on the water stability of abalone feeds.

Materials and methods

Experimental design

The experiment consisted of seven treatments (refer to Table 9-1), each replicated 6 times. The treatments comprised of a control diet (C) that was prepared using a basal diet (AquaNutro Abalone Grower, NutroScience, Malmesbury, South Africa) supplemented with 25 $g \cdot kg^{-1}$ trout silage oil, two diets with a low (SL) and high (SH) level of self-prepared wet rainbow trout viscera silage (endogenous enzyme hydrolysate), and two diets with a low (HPL) and high (HPH) level of a commercially available hydrolysate (enzymatically hydrolysed product; ACTIPAL HP 1, AQUATIV, France) – all fed continuously for the duration of the 153 day experimental period. The diets containing the higher levels of silage (SH) and hydrolysate (HPH) were duplicated in two treatments that employed alternating feeding regimes (30-day intervals) with the control diet. As the current trial formed part of a larger investigation, the control diet had to be supplemented with trout silage oil in order to achieve iso-nutrient formulations over the entire investigation.

Inclusion level of fish silage proteins was determined such that the crude protein component of the fish silage comprised 6 $g \cdot kg^{-1}$ of the final feed (after drying) for the low inclusion level, and 18 $g \cdot kg^{-1}$ for the high inclusion level. Feeds in which the commercial protein hydrolysate were incorporated, were formulated to be iso-proteic and iso-energetic to

the corresponding silage diets. See Table 9-2 for diet formulations and proximate composition.

Ingredient and diet preparation

Fish silage was prepared from rainbow trout viscera as described previously (Goosen, et al., 2014). Floating oil was recovered from the silage after 3 days through manual decantation and antioxidant (1 ml·litre^{-1} butylated hydroxytoluene and butylated hydroxyanisole mixture, Oxipet L, Bitek) was added. Recovered oil was stored in airtight plastic containers at 4 °C and utilised in diet preparation 12 days post silage preparation. Silage was de-oiled with a centrifugal dairy separator (Elecrem Model 1, Elecrem, France). The degree of protein hydrolysis (DOH) in the silage was calculated using the trichloroacetic acid (TCA) precipitation method of Hoyle and Merrit (1994). Equal volumes of a 20% (w/v) TCA solution in distilled water and de-oiled silage were mixed at room temperature and incubated for 1 hour. The mixtures were centrifuged for 5 minutes at 14000 rpm, supernatant was sampled and analyzed for total nitrogen using the Kjeldahl method (AOAC, 2003). The DOH value was expressed as the percentage of non-precipitated nitrogen to total sample nitrogen and was found to be 90.9%.

To prepare the diets, the two hydrolysed proteins were mixed with the oil and basal diet and a specified amount of water was added to create a paste suitable for extrusion; the amount of water added to the silage diets before extrusion was decreased to take into account the water already added as part of the silage. The commercial hydrolysed protein was in powder form and no adjustment was made to the water added. All diets were prepared through extrusion at 70 °C, followed by drying in a ventilated oven at 55 °C. The final diets were presented as flat rectangular flakes with approximate dimensions of 20 mm x 30 mm. The commercial diet used in the control also served as the basal diet for all experimental diets. Proximate composition of the experimental feeds were determined according to standard AOAC methods (AOAC, 2003) and is shown in Table 9-2.

Experimental system and procedures

All feeding trials were conducted at Wild Coast Abalone Farm, Haga-Haga, South Africa, according to commercial culture conditions for South African abalone *Haliotis midae*. Experimental animals were housed in plastic baskets under natural photoperiod, fed once daily to apparent satiation by experienced personnel, and uneaten feed was removed. The system had continuous supply of aeration and unfiltered seawater as extracted from the nearby ocean. Mean animal weight was determined at trial initiation and termination, as the mean weight of 30 randomly selected animals from each basket. Initial mean animal weight

ranged between 123 g and 128 g, without any statistically significant differences between treatments (refer to Table 9-3). At the conclusion of the trial, one animal from each basket was also sampled for the determination of immune function.

Daily weight gain (DWG), feed conversion ratio (FCR), relative feed intake (RFI) and total mortalities were evaluated as indicators of production performance of each treatment. DWG was expressed as $mg \cdot animal^{-1} \cdot day^{-1}$ between sampling time *i* and *i + 1* and was calculated as $DWG = 1000 \times (W_{i+1} - W_i)/(t_{i+1} - t_i)$ with *W* and *t* having units of grams and days respectively. Feed conversion ratio (FCR) was calculated as $FCR = (W_{i+1} - W_i)/(\text{feed given})$ with the feed given measured in grams; the calculated FCR values are considered to be the effective on-farm feed conversion. RFI was calculated as $RFI = (\text{feed given})/(W_m)$ where W_m denotes the mean animal weight in each container during the growing period.

Measurement of non-specific immunity

Non-specific immunity of abalone was characterised through quantification of cellular parameters (hemocyte counts and phagocytic activity) and non-cellular parameters (total protein and lysozyme concentrations of hemolymph). At the end of the trial, hemolymph was collected immediately after animal collection, through pedal sinus puncture and extracting hemolymph using sterile 29 gauge syringes. Samples were divided into two aliquots upon collection: one was frozen immediately and stored for laboratory analysis of total protein and lysozyme. The fresh hemolymph aliquot was used to quantify the phagocytic activity of hemocytes and to count the number of circulating hemocytes.

Phagocytic activity of hemocytes was determined by the method used by Dang, et al. (2011). Congo red (Sigma) and baker's yeast (NCP, Modderfontein, South Africa) were mixed (4% inclusion of yeast and dye) in phosphate buffered saline (PBS) and autoclaved at 121 °C for 20 minutes. The autoclaved yeast was recovered through centrifugation, re-suspended and washed 4 times in PBS and finally re-suspended in synthetic seawater (40 $g \cdot litre^{-1}$ sea-salts, Sigma, dissolved in reverse osmosis water). The rate of phagocytosis of hemocytes was determined by spreading 50 µl fresh hemolymph on a glass slide and allowing hemocytes to adhere to the slide for 10 minutes at room temperature. The remaining hemolymph was then poured off, 50 µl of the yeast suspended in synthetic seawater was added to the hemocytes and the glass slides were then incubated for 30 minutes. After incubation, the slides were washed with synthetic seawater and hemocytes were fixed with 10% (w/w) formaldehyde in synthetic seawater solution, for 20 minutes. Phagocytosis was quantified by cell counts using a light microscope. At least 100 hemocytes were counted, and phagocytosis was expressed as the percentage of hemocytes

that contained yeast cells. Circulating hemocytes were determined through manual cell counts under a light microscope using undiluted hemolymph.

For measurement of total protein and lysozyme concentration of hemolymph, frozen samples were thawed at room temperature and centrifuged at 14000 rpm for 10 minutes to remove intact cells and possible debris. The supernatant was then transferred to clean Eppendorf tubes. Total protein concentration was quantified according to the linearized Bradford assay (Zor and Selinger, 1996) using bovine serum albumin standard. Lysozyme concentration was determined with the method of Sankaran and Gurnani (1972) as described previously (Goosen, et al., 2014).

Measurement of water stability of feeds

Water stability tests of abalone feed were completed in accordance with the standard protocol of the Feed Technology Group, University of Stellenbosch (Viljoen and De Wet, 2009). It should be noted that the method does not detect leaching of specific nutrients, but is a measure of the physical resistance of the feed against disintegration. The water stability is defined as the percentage of feed remaining (on a dry weight basis) after 18 hours of submersion. Each feed was evaluated for water stability in 6 randomly allocated testing chambers. A set amount of feed (20 g) was weighed into a wire mesh container and submerged into a separate testing chamber in an apparatus containing 100 individual chambers. Each testing chamber was aerated individually with an air stone in order to provide accelerated feed disintegration when compared to commercial production systems. After 18 hours of submersion, wire mesh containers were removed and the remaining feed was dried at 60 °C for 8 hours.

Statistical analysis

All data were analysed by one-way ANOVA using Statistica version 10 software (StatSoft, Inc.). Differences were viewed to be significant for $P < 0.05$. If ANOVA indicated that some means differed significantly, Fisher's least significant difference (LSD) post-hoc test was employed to determine which means differed.

Results

Production parameters

Results of production performance parameters are shown in Table 9-3. There were no significant differences in final animal weight, but DWG and FCR differed significantly between some treatments. DWG of treatments HPL and HPH were respectively 11% and 14% higher than the control, while none of the other treatments differed from the control. Both treatments HPL and HPH had significantly higher DWG than treatments SL and SH, but neither treatment differed from SH, Phase. FCR values of treatments SH and HPH, Phase were significantly higher than that of the control by 27% and 19% respectively, while none of the other treatments differed significantly from the control. Treatments SH and HPH, Phase also had significantly higher FCR values than treatment HPL.

All treatments had significantly higher RFI values than that of the control. Both treatments incorporating low inclusion levels of hydrolysed proteins (SL and HPL) had significantly lower RFI levels than treatments incorporating high levels of hydrolysed proteins (treatments SH and HPH), and treatments employing phase feeding (SH, Phase and HPH, Phase).

Few mortalities occurred during the trial: four in the control and two in treatment SL.

Water stability of feeds

The water stability of the different feeds is shown in Figure 9-1. Diets SL, SH and HPH all showed a significant decrease in water stability compared to the control by 55%, 92% and 38% respectively, while diet HPL did not differ from the control. Water stability for treatment SH was also significantly lower than for treatments HPL and HPH by 91% and 87% respectively, while treatments SL and SH did not differ significantly from one another.

Immunity

The results of the immune measurements are shown in Table 9-4. No significant differences between treatments were seen for the lysozyme concentration or total protein content in the hemolymph, or circulating hemocyte concentration. Significant differences were found in the phagocytic activity of hemocytes, with treatment HPL increasing phagocytic activity by 18% compared to the control. No other treatments differed significantly from the control. Phagocytic activity in treatment HPL was significantly higher than in all other treatments except treatment SL.

Discussion

The trial demonstrated that the inclusion of dietary hydrolysed proteins can enhance the cellular immune function in *Haliotis midae*. This is confirmed through the significant increase in phagocytic activity observed for treatment HPL compared to the control (Table 9-4). The use of hydrolysed proteins as immune stimulants in abalone aquaculture would broaden the range of possible supplementations available to producers. Hydrolysed proteins would thus compliment other dietary additives that have been shown to stimulate immune response in abalone species, e.g. probiotics enhanced immunity and disease resistance of *H. midae* (Macey and Coyne, 2005) and *H. discus hannai* (Jiang, et al., 2013), dietary fish silage oil increased phagocytic activity of *H. midae* (results currently under review), and a variety of macro- and microalgal supplements had both positive and negative effects on immune parameters of *H. laevigata* (Dang, et al., 2011). Pyridoxine supplementation to diets for *H. discus hannai* led to significantly increased phagocytic and phenoloxidase activity (Chen, et al., 2005).

The increased phagocytic activity seen in treatment HPL might be a result of immune stimulating peptides present in the hydrolysed protein incorporated into the feed. It has been shown that low molecular weight peptides found in a fish protein hydrolysate increased the activity of phagocytic cells (Bøgwald, et al., 1996). Improvements in immune parameters were also reported for dietary supplementation of hydrolysed protein in finfish species (Bui, et al., 2014; Liang, et al., 2006; Tang, et al., 2008), but the exact mechanism of working was not determined. Other positive effects e.g. improved growth and feed efficiency of protein hydrolysates in diets for turbot and Japanese flounder have also been attributed to the low molecular weight component of protein hydrolysates (Zheng, et al., 2012; 2013). A lack of immune stimulation by the rainbow trout silage containing diets could be related to the final hydrolysate properties. The efficacy of hydrolysed proteins are partially dependent on the peptide size in the hydrolysate (Murray, et al., 2003) which might have differed in the autolysed rainbow trout silage and industrially produced protein hydrolysate.

Increasing the hydrolysate inclusion above that used in the low inclusion level diets, did not result in increased immune stimulation. Phagocytic activity in treatment SH was significantly lower than for SL, while that of HPH was also significantly lower than treatment HPL (Table 9-4). However, in finfish studies increasing hydrolysate inclusion levels increased total antioxidative capacity in turbot (Zheng, et al., 2013), while increasing dietary hydrolysate inclusion from 5 % to 15 % in Japanese sea bass also increased lysozyme activity and complement hemolytic activity; however dietary inclusion level did not affect phagocytic activity (Liang, et al., 2006). In the current study, the reason for the significantly decreased phagocytic activity in the high inclusion diets compared to the low inclusion diets

could not be linked to over stimulation of the immune system, as there were no statistically significant differences in phagocytic activity between treatments SH and SH, Phase, and HPH and HPH, Phase. It is possible that the significantly decreased water stability of the high inclusion diets could be responsible for the significantly decreased phagocytosis compared to the corresponding low inclusion diets. Decreased water stability could lead to decreased intake of dietary components that enhance phagocytosis.

All measured production performance parameters, except mortalities, were affected by the inclusion of hydrolysed proteins in the test diets (Table 9-3). DWG was affected positively by inclusion of the commercial hydrolysed protein, irrespective of the inclusion level, but silage inclusion did not improve DWG at either inclusion level. Phase feeding had no effect on DWG. In previous hydrolysed protein studies in abalone, significantly higher growth was seen for diets that incorporated heated and unheated silage for *Haliotis fulgens* (Viana, et al., 1996); however the silage diets were compared to a lower protein, kelp diet and can therefore not be said to have improved growth due to hydrolysed protein content. In another study, both heated and unheated silage significantly decreased growth when compared to a commercial control (Viana, et al., 1999), but this might be due to the lower protein and higher lipid content in the silage containing diets compared to the commercial reference diet. High dietary lipid levels are known to decrease abalone production performance (Green, et al., 2011). In other aquaculture species, improved growth rates as a result of dietary protein hydrolysates were observed in both finfish (Berge and Storebakken, 1996; Bui, et al., 2014; Liang, et al., 2006; Ramasubburayan, et al., 2013; Refstie, et al., 2004) and shrimp *Penaeus vannamei* (Nguyen, et al., 2012)

Hydrolysed protein inclusion significantly affected the water stability of the experimental feeds. High inclusion of both hydrolysed proteins decreased the water stability of feeds (Figure 9-1). Trout silage inclusion also resulted in significantly decreased water stability compared to the corresponding inclusion level of commercial hydrolysed protein. The lower water stability found at the higher inclusion level of each type of hydrolysed protein can be explained by the increased solubility of hydrolysed proteins compared to the original protein substrates that were hydrolysed. Espe and co-workers (1999) noted the importance of the solubility and the inclusion level of protein hydrolysates in aquaculture diets. The enzymatic hydrolysis process results in an increase in the solubility of the native proteins (Kristinsson and Rasco, 2000), which may then leach from the feeds when fed (Guzmán and Viana, 1998; Santos, et al., 2013; Viana, et al., 1996). The difference in water stability between the different types of hydrolysates might be explained by the differences in production methods and raw materials. Production methods and the control of hydrolysis conditions heavily influence the properties of the resultant hydrolysed proteins (Kristinsson and Rasco, 2000; Shahidi, et al., 1995). As the silage was prepared through autolysis

employing the endogenous digestive enzymes present in the fish waste, the hydrolysed proteins present in the silage will differ from the commercial hydrolysed protein produced under controlled conditions.

Water stability is critically important in abalone feeds as it can negatively impact animal production performance, system water quality and feeding costs (Flemming, et al., 1996; Guzmán and Viana, 1998; Knauer, et al., 1993). The impact on production performance was seen in the current study where a significant decrease in feed water stability in treatment SH was the probable cause of a significant (27%) increase in FCR (Table 9-3). Non-significant increases in FCR were also seen in all other treatments where the water stability of the feeds was lower than that of the control. The exception was treatment HPL, which showed a non-significant decrease in FCR of 4.4% compared to the control treatment, and a feed water stability that did not differ from the control. Unfortunately, uneaten feed could not be recovered during trials, which prevented more accurate estimates of true feed intake and feed conversion.

Decreased feed stability may also have contributed to increased RFI values compared to the control diet, seen for all treatments (Table 9-3). High inclusion levels of hydrolysed protein (treatments SH and HPH) corresponded to decreased water stability and significantly increased RFI values, compared to the low inclusion level of the corresponding hydrolysed protein. Animals in the production systems were fed according to apparent satiation; decreased water stability might have created the wrongful impression that animals were consuming more of the feed, while feeds were in actual fact lost due to disintegration.

There were no benefits to immunity or production efficiency by employing an intermittent feeding strategy in the current trial (Tables 8-3 and 8-4). For both treatments SH, Phase and HPH, Phase, none of the immune parameters were significantly increased when compared to either the control or the corresponding continuous feeding treatment. There were also no improvements in FCR, RFI or weight gain by the intermittent feeding treatments. Moreover, in treatment HPH, Phase a significant decrease in DWG was found compared to treatment HPH, and FCR increased significantly compared to the control. The decrease in production performance in an intermittent treatment might be explained by the fact that abalone might go through an adaptation period when fed new diets, as was seen in previous work on *H. midae* (Goosen, 2007). The reason why this is not also reflected in the data for treatment SH, Phase is unclear.

The effect of trout silage on immunity of *H. midae* should be evaluated using diets that do not contain trout silage oil, in order to establish whether trout silage can contribute to immune stimulation. In the current trial, treatment SL did not differ significantly from the control treatment, however statistical post-hoc LSD analysis revealed that treatment SL was close to the threshold of statistical significance ($P = 0.07$; data not shown). All treatments

including the control contained dietary trout silage oil, which has been shown to significantly increase phagocytic activity of *H. midae* (results currently under review). The non-significant increase in phagocytic activity observed in treatment SL in the current trial might be significant when evaluated in the absence of trout silage oil.

To conclude, it has been shown that the inclusion of the commercial hydrolysed protein product in formulated diets for *H. midae* can lead to improved immune function and weight increase. Hydrolysed proteins should be utilised at appropriate inclusion levels in abalone diets, as excessive inclusion can cause impaired production performance as a result of decreased feed stability. An intermittent feeding strategy of the high inclusion level also had no positive impacts on immunity or production performance.

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Tables

Table 9-1 Summary of experimental diets and the combination of diets fed in each treatment.

Diet / ingredient	Inclusion level	Feeding regime	Diets fed	Treatment
Reference	N/A	Constant	C	C
Rainbow trout silage	Low	Constant	SL	SL
	High	Constant	SH	SH
	High	Phase	SH + C	SH, Phase
Commercial hydrolysate	Low	Constant	HPL	HPL
	High	Constant	HPH	HPH
	High	Phase	HPH + C	HPH, Phase

Table 9-2 Diet formulation and proximate composition.

Ingredient ($g \cdot kg^{-1}$)	C	SL	SH	HPL	HPH
Abalone grower	975	920	833	968	950
Trout silage oil	25	24	15	25	20
Trout silage (wet)	0	56	152	0	0
HP1 (dry)	0	0	0	7	30
Total	1000	1000	1000	1000	1000

Proximate composition of experimental diets ($g \cdot kg^{-1}$)					
Moisture	86	89	79	77	71
Ash	79	79	80	84	82
Crude fat	58	62	58	63	57
Crude protein	389	392	405	395	404
Carbohydrates[a]	388	378	378	381	386
Gross energy ($MJ \cdot kg^{-1}$)	18.2	18.3	18.4	18.4	18.5

a – Determined by difference

Table 9-3 Summary of production parameters. Data are presented as mean ± SD. Superscripts with common letters indicate means do not differ significantly.

	C	SL	SH	HPL	HPH	SH, Phase	HPH, Phase
W_i (g)	125 ± 3.61	123 ± 2.74	126 ± 9.19	123 ± 7.67	128 ± 14.2	124 ± 5.98	125 ± 5.07
W_f (g)	155 ± 2.03	152 ± 3.20	156 ± 8.62	156 ± 7.03	162 ± 12.6	155 ± 6.01	157 ± 5.38
DWG (mg·animal^{-1}·day^{-1})	195 ± 19.2^{a}	189 ± 13.6^{a}	192 ± 17.8^{a}	216 ± 16.7^{bc}	223 ± 21.4^{b}	204 ± 15.1^{ab}	193 ± 10.4^{ac}
FCR	2.74 ± 0.28^{ad}	2.96 ± 0.32^{abd}	3.49 ± 0.50^{c}	2.62 ± 0.32^{d}	3.14 ± 0.54^{ac}	3.14 ± 0.37^{ac}	3.26 ± 0.29^{bc}
RFI (g feed·g animal weight^{-1})	0.58 ± 0.02^{a}	0.62 ± 0.03^{b}	0.72 ± 0.04^{c}	0.62 ± 0.03^{b}	0.73 ± 0.03^{c}	0.70 ± 0.04^{c}	0.73 ± 0.03^{c}
Total mortalities	4	2	0	0	0	0	0

Abbreviations: W_i - initial animal weight; W_f - final animal weight; DWG – daily weight gain; FCR – feed conversion ratio; RFI – relative feed intake.

Table 9-4 Summary of immune function data. Data are presented as mean ± SD. Superscripts with common letters indicate means do not differ significantly.

	C	SL	SH	HPL	HPH	SH, Phase	HPH, Phase
Lysozyme (µg·ml^{-1})	3.12 ± 1.53	1.95 ± 1.75	1.82 ± 1.91	0.24 ± 0.31	1.37 ± 1.33	3.61 ± 0.28	0.96 ± 0.77
Total protein (mg·ml^{-1})	8.64 ± 2.43	8.02 ± 2.01	8.67 ± 1.88	7.5 ± 2.34	9.06 ± 1.9	8.23 ± 0.94	7.65 ± 1.88
Phagocytosis (%)	65.2 ± 7.08^{acg}	72.1 ± 3.25^{ad}	62.9 ± 7.67^{bce}	76.9 ± 2.93^{d}	65.7 ± 3.44^{aef}	67.4 ± 4.16^{acf}	59.3 ± 12.7^{beg}
Hemocytes (10^6·ml^{-1})	2.73 ± 1.28	3.13 ± 1.50	1.83 ± 1.13	6.18 ± 2.63	3.24 ± 2.87	4.03 ± 4.39	4.81 ± 3.52

Figures

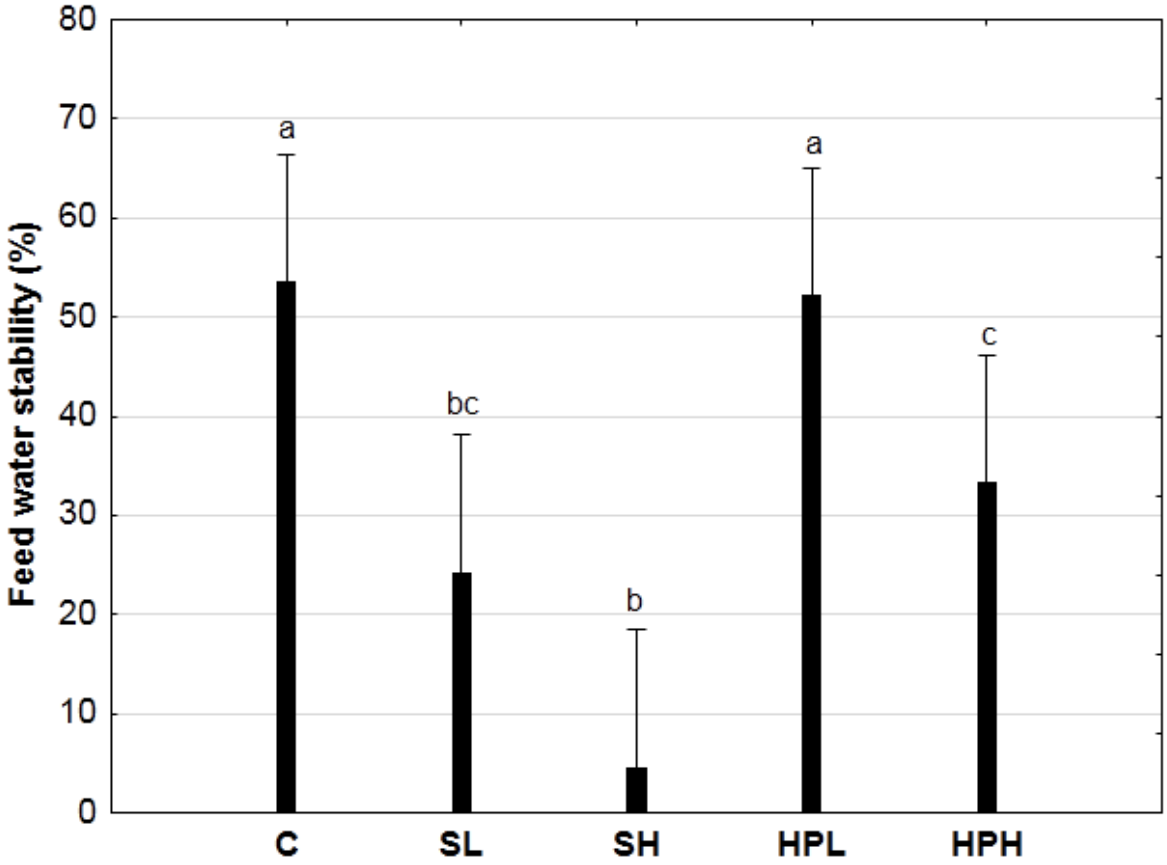

Figure 9-1 Water stability of the different diets after 18 hours of aeration. Data are presented as mean ± 95% confidence intervals. Superscripts with common letters do not differ significantly.

Chapter 10: Formic acid in diets for South African abalone

"I remembered the old doctor, - 'It would be interesting for science to watch the mental changes of individuals, on the spot.' I felt I was becoming scientifically interesting." Joseph Conrad, Heart of Darkness

"'If the others heard me talking out loud they would think that I am crazy,' he said aloud. 'But since I am not crazy, I do not care.'" Ernest Hemingway, The Old Man and the Sea

This chapter is yet to be submitted for review

The work described in this chapter addresses aim IV, and makes the following novel contributions:

1) Formic acid supplementation did not affect production performance in treatments containing trout silage oil or commercial hydrolysed fish proteins and can therefore be eliminated as the agent responsible for differences in production performance.
2) A novel mode of action of formic acid in formulated abalone diets is demonstrated, namely an increase in feed water stability.
3) The increased feed water stability is correlated to improved production performance in treatments containing raw, unprocessed trout viscera.
4) The formic acid did not significantly impact on bacterial load of the formulated feed.

Summary of authors' contributions

Goosen, N.J.: Responsible for trial planning, experimental design, feed formulation, sourcing of commercial hydrolysates, and feed preparation; completed daily trial operation duties; responsible for sampling of animals for production performance, immunology, haematology and intestinal morphology; completed the analysis of immunity, haematology and intestinal morphology; did statistical analysis of data; responsible for writing draft manuscript. Estimated % contribution to the work: 80 %

De Wet, L.F.: Assisted with feed formulation, took part in general discussion surrounding the project and provided feedback on the final manuscript. Estimated % contribution to the work: 10 %

Görgens, J.F.: took part in general discussion surrounding the project and provided feedback on the final manuscript. Estimated % contribution to the work: 10 %

Formic acid mitigates the decrease in production performance in abalone fed diets containing unprocessed trout viscera

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Running title:

Formic acid limits production performance decrease

Key words:

Organic acid; water stability; South African abalone; processing by-products

Abstract

In a South African abalone *Haliotis midae* L. growth trial, formic acid was included as control in formulated diets that contained feed ingredients originating from fish processing by-products. Four diets were evaluated with and without formic acid (yielding a total of 8 experimental diets): a commercial reference diet, one containing trout silage oil, one containing unprocessed trout viscera and another containing a commercial hydrolysed protein. It was found that the inclusion of unprocessed trout viscera negatively impacted production performance of experimental animals, and the effect could be attributed to significantly decreased water stability of the feed. The inclusion of 2 g kg^{-1} formic acid partially mitigated the negative effects of the inclusion of unprocessed viscera, through significantly improved feed water stability, but the mechanism through which formic acid improves feed water stability is unknown. Formic acid had no significant antimicrobial effect in the viscera containing diet. No improvement in production performance or feed water stability was found upon formic acid supplementation in any of the other diets, which may be related to the relatively low inclusion rate of formic acid compared to previous studies.

Introduction

Dietary organic acids and their salts have been shown to improve production performance of various aquaculture species. Supplementation of aquafeeds with organic acids or organic acid salts led to production performance improvements in rainbow trout (De Wet, 2005), Arctic charr (Ringø, 1991), tilapia (Hassaan, et al., 2014; Ramli, et al., 2005), beluga (Khajepour and Hosseini, 2012) and abalone (De Wet, et al., 2012; Goosen, et al., 2011), and feed intake was increased in shrimp (Da Silva, et al., 2013).

Organic acids exert their effects during several stages of the feed manufacturing, feeding and digestive processes. These substances contribute to feed hygiene and decreased pathogen exposure through their antimicrobial properties. Especially the short chain organic acids are known to be antimicrobial (Franco, et al., 2005; Partanen and Mroz, 1999), are used to control pathogens in animal feeds (Koyuncu, et al., 2013) and have been shown to lead to decreased microbial loads in the intestinal tract of animals (Ng, et al., 2009) or altered the composition of intestinal microflora (Zhou, et al., 2009). Specific action of certain organic acids against known pathogens in aquaculture has also been reported (Adams and Boopathy, 2013). Certain organic acids can lead to improved nutrient digestibility, as reported for phosphorous digestibility in juvenile yellowtail (Sarker, et al., 2012), beluga (Khajepour and Hosseini, 2012) and rainbow trout (Pandey and Satoh, 2008). Most organic acids can further serve directly as sources of energy in the metabolism of animals (Lückstädt, 2008).

In recent trials, different hydrolysed proteins and oils derived from processing by-products were evaluated in formulated diets for the South African abalone *Haliotis midae* L. (Goosen, et al., 2014a; b). Formic acid containing fish silage formed part of the trial, therefore control diets incorporating formic acid were included to distinguish between the effects of formic acid and the feed ingredients evaluated. The effects of formic acid on the production performance of *H. midae*, incorporated in combination with different processing waste derived feed ingredients, are reported here.

Materials and methods

Experimental design

For the current work, four formulated diets (each with and without formic acid) were considered, yielding a total of 8 diets. The four diets were a commercial reference diet A0, which consisted of a formulated abalone diet (AquaNutro Abalone Grower, NutroScience, Malmesbury, South Africa) and three diets in which the commercial diet was supplemented with different ingredients originating from processing by-products, namely trout silage oil (diet A2), minced, unprocessed trout viscera (diet A4) and a commercial hydrolysed shrimp protein (ACTIPAL HP 1, AQUATIV, France) designated diet A6. A breakdown of the experimental diets and the nomenclature employed is given in Table 10-1. Diet formulations and proximate analyses are given in Table 10-2. Result of growth trials for diets without formic acid or unprocessed viscera (diets A0, A2 and A6) have been reported previously (Goosen, et al., 2014a; b), but are included in the current work for comparison purposes.

As organic acids have been shown to improve the growth of *H. midae* in a previous study (Goosen, et al., 2011), it was necessary to include formic acid containing control diets in the current trial to eliminate formic acid as the active component. The formic acid inclusion level (2 g kg^{-1}) in diets A1, A3, A5 and A7 was therefore chosen to be equivalent to that which would be included along with the fish silage in the larger trial (Goosen, et al., 2014a). The inclusion level of the unprocessed trout viscera in diet A4 was calculated so that the total lipid level would not exceed 60 g kg^{-1} in the final diet. Trout silage oil inclusion in diets A2 and A6 were chosen to replicate the amount of lipid added to diet A4 as a result of the viscera inclusion.

The diets were evaluated in a growth trial using *H. midae* as experimental species under commercial rearing conditions. Each diet was fed to six randomly allocated baskets.

Feed preparation

A commercial formulated abalone diet (AquaNutro Abalone Grower, NutroScience, Malmesbury, South Africa) served as basal diet for all treatments. Rainbow trout viscera were collected from Three Streams Smokehouse, Franschhoek, South Africa. A portion of the viscera was used to prepare fish silage as described previously (Goosen, et al., 2014c), and a portion was frozen at -20 °C. Three days

after ensiling, floating oil was recovered from the fish silage through manual decantation and antioxidant (1 ml litre^{-1} butylated hydroxytoluene and butylated hydroxyanisole mixture, Oxipet L, Bitek) was added. The oil was stored at 4 °C in airtight plastic containers until use.

Immediately prior to diet preparation, the frozen viscera were minced and antioxidant was added at 0.5 ml kg^{-1}. All diets were prepared by mixing feed ingredients and the basal diet (refer to Table 10-2 for diet formulations), followed by extrusion at 70 °C and drying at 55 °C. Liquid formic acid (85 %, Protea Chemicals, South Africa) was added to the diets A1, A3, A5 and A7 during ingredient mixing, at an inclusion of 2 g kg^{-1}.

Only the proximate composition of the diets not containing formic acid were determined, as the formic acid inclusion level was assumed to be sufficiently low so as not to significantly affect diet composition. The proximate composition (Table 10-2) was determined according to standard AOAC methods (AOAC, 2003).

Growth trials

Growth trials were conducted at Wild Coast Abalone Farm, Haga-Haga, South Africa, using South African abalone *Haliotis midae*. All procedures and management practices were done according to commercial culture practices. Animals were fed daily to apparent satiation by experienced operators, and were housed in plastic baskets. Initial and final mean animal weight was determined as the mean weight of 30 randomly selected animals from each basket (Table 10-3).

Production performance was assessed by computing daily weight gain (DWG), feed conversion ratio (FCR), relative feed intake (RFI) and total mortalities. DWG (mg·animal^{-1}·day^{-1}) between sampling time *i* and *i + 1* was calculated as DWG = $1000\times(W_{i+1} - W_i)/(t_{i+1} - t_i)$ with *W* and *t* having units of grams and days respectively. Feed conversion ratio (FCR) is expressed as the wet animal weight gain per unit dry feed given, while RFI is expressed as the weight of dry feed given per unit animal weight.

Feed water stability

The water stability of the experimental feeds were evaluated according to a standard protocol of the Feed Technology Group, University of Stellenbosch (Viljoen

and De Wet, 2009) as described by (Goosen, et al., 2014a). Water stability tests were replicated 6 times for each diet.

Feed microbiology

In order to establish possible reasons for the significant growth differences observed between treatments A4 and A5, feed samples from these treatments were analysed for total viable bacteria using microbial plate counts. This was done by grinding 1 g of feed and suspending in 9 mL sterile saline solution. One mL of the suspension was then plated out in triplicate onto nutrient agar (meat extract 1 g L^{-1}, yeast extract 2 g L^{-1}, peptone 5 g L^{-1}, sodium chloride 8 g L^{-1} and agar 15 g L^{-1}). The plates were incubated at 26 °C for 48 hours to determine total colony forming units (CFU).

Statistical analysis

All data were analysed by way of one-way ANOVA with the Statistica software package (version 11, StatSoft, Inc.), followed by Tukey's post-hoc test if ANOVA analysis indicated significant differences between means. Differences were seen to be significant when $P < 0.05$.

Results

The results for total mortalities, RFI and final animal weights are summarised in Table 10-3. There were statistically significant differences in final animal weight, with animals in treatment A4 weighing significantly less than in all other treatments by between 8.4 % - 15.5 %, while none of the other treatments differed from one another. RFI values for treatments A4 and A5 were both significantly higher than all other treatments, and RFI for treatment A4 was also significantly higher than treatment A5 by 15.7 %. RFI for treatment A3 was significantly lower than for treatments A6 and A7, while there were no differences found between treatments A0, A1, A2, A6 and A7.

Figure 10-1 shows results of DWG (A) and FCR (B) achieved during the growth trial, and the water stability of the experimental feeds (C). DWG of treatment A4 was significantly lower than that of all other treatments by 35.9 % - 51.6 %, while that of treatments A2 and A5 were also significantly lower than A0. There were no significant differences between treatments A0, A1, A3, A6 and A7. The FCR of treatment A4 was

significantly higher than all other treatments by 70.7% - 195 %. FCR values of treatment A5 was significantly higher than A0, A1, A2, A3 and A6, but did not differ from A7, while treatments A0, A1, A2, A3, A6 and A7 did not differ significantly. The water stability of feed A4 was extremely poor and no feed remained after 18 hours of submersion in any of the testing chambers. The water stability of feed A5 was significantly lower than that of all other feeds, and no differences were found between feeds A0, A1, A2, A3, A6 and A7.

The microbial counts detected no significant difference in total colony forming units (CFU) between feeds A4 and A5. Total CFU (mean ± standard error) was 303 ± 20.3 CFU ml^{-1} for feed A4 and 287 ± 29.6 CFU ml^{-1} for feed A5.

Discussion

Four diets were evaluated both with and without formic acid supplementation in a growth trial with *Haliotis midae*. Three of the diets contained feed ingredients originating from by-products of processing operations. The effects of formic acid supplementation on production performance and the water stability of the experimental feeds were evaluated.

The inclusion of untreated trout viscera in formulated abalone diets adversely affected the production performance of animals. Final animal weight and DWG were significantly lower in treatment A4 than all other treatments, while RFI and FCR for treatment A4 were significantly higher than that of all other treatments (refer to Table 10-3 and Figure 10-1). The decreased production performance can be attributed to the poor water stability of feed A4 (Figure 10-1 C). Poor water stability will lead to a decrease in the time available for the animals to feed, which will limit actual feed intake and lead to feed losses. Acceptable water stability of abalone feeds is critically important to commercial operations, as feeds need to remain stable for prolonged periods of submersion to provide sufficient feeding time for this slow feeding species. Decreased water stability of feeds can lead to nutrient losses, impaired water quality in production systems and direct economic losses due to increased feeding costs (Flemming, et al., 1996; Guzmán and Viana, 1998; Kirkendale, et al., 2010; Knauer, et al., 1993).

Formic acid supplementation partially mitigated the negative effects on production performance as a result of dietary unprocessed viscera inclusion. Final mean animal weight and DWG were significantly higher for treatment A5 when

compared to A4, while RFI and SGR for treatment A5 were significantly lower than for A4 (Table 10-3 and Figure 10-1). The addition of formic acid to the diet containing unprocessed trout viscera, however, could not restore production performance to the level obtained in treatments A2 and A3 (diets A2 and A3 are the appropriate control diets to use for comparison, as they are formulated on an iso-nutrient basis with diets A4 and A5; the commercial diets, A0 and A1, contain lower levels of crude fat and higher crude protein; refer to Table 10-2). Both RFI and FCR were significantly higher for treatment A5 compared to treatments A2 and A3, while DWG and final mean animal weight did not differ.

The improvement in production performance of treatment A5 compared to treatment A4 can be attributed to the improvement of the water stability resulting from formic acid addition. When treatments A5 and A4 are compared, Figure 10-1 and Table 10-3 show that formic acid addition significantly improved the water stability of diet A5 compared to A4, and that the improved water stability coincided with significantly improved DWG and mean final animal weight, and significantly decreased FCR and RFI. It is unlikely that the antimicrobial properties of formic acid contributed to the improved production performance, as no significant differences in microbial numbers in diets A5 and A4 could be detected (refer to section 4). The exact mechanism by which formic acid improved the water stability of feed A5 compared to A4 is not known, but improved water stability of feeds is a mode of action not previously reported in literature.

Formic acid supplementation to the diets that did not contain unprocessed trout viscera had no effect on production parameters or feed water stability. There were no significant differences in final mean animal weight, DWG, RFI, SGR or water stability when treatment A0 is compared with A1, A2 with A3, or A6 with A7. The lack of significant effects of formic acid in these diets might be related to the relatively low inclusion level of 2 g kg^{-1}. In a previous investigation, significantly increased growth of *H. midae* was found upon dietary inclusion of mixtures of organic acids and organic acid salts (Goosen, et al., 2011); however the total organic acid inclusion level in that particular investigation was 20 g kg^{-1}, which is an order of magnitude higher than what was used in the current study. Consequently it is possible that any gains in production performance resulting from 2 g kg^{-1} formic acid inclusion to diets not containing unprocessed trout viscera might have been too small to detect.

In conclusion it has been shown that the supplementation of 2 g kg^{-1} formic acid to a formulated abalone diet that contained unprocessed trout viscera, significantly

improved abalone production performance through improved water stability of the feed. However, formic acid supplementation was not able to fully mitigate the negative effects of unprocessed trout viscera inclusion in the current investigation, as the formic acid supplemented viscera diet led to poorer production performance than a corresponding iso-nutrient diet that did not contain viscera. Further research is required to determine if formic acid can contribute to improved production performance in combination with other feed ingredients in abalone diets, and to determine the exact mechanism by which formic acid increased the water stability of the viscera containing diet.

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Tables

Table 10-1 – Summary of the experimental design and treatment abbreviations used in the investigation.

Diet	Diet abbreviation
Commercial reference	A0
Reference + FA	A1
Reference + Trout oil	A2
Reference + Trout oil + FA	A3
Reference + Trout viscera	A4
Reference + Trout viscera + FA	A5
Reference + Protein hydrolysate	A6
Reference + Protein hydrolysate +FA	A7

Abbreviations: FA – formic acid

Table 10-2 Formulation and proximate composition of experimental diets.

Ingredient (g·kg^{-1})	**A0**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**
Basal diet	1000	998	975	973	946	944	968	966
Trout silage oil	-	-	25	25	-	-	25	25
Trout viscera	-	-	-	-	54	54	-	-
HP1 (dry)	-	-	-	-	-	-	7	7
Formic acid	-	2	-	2	-	2	-	2
Total	1000	1000	1000	1000	1000	1000	1000	1000
Proximate composition (g·kg^{-1})								
Crude protein	406	ND	389	ND	392	ND	395	ND
Crude fat	33	ND	58	ND	64	ND	63	ND
Moisture	83	ND	85	ND	81	ND	77	ND
Ash	83	ND	81	ND	80	ND	84	ND
Carbohydrates[a]	395	ND	387	ND	383	ND	381	ND
Gross energy (MJ·kg^{-1})	16.8	-	18.2	-	18.4	-	18.4	-

Abbreviations: HP1 – Commercial protein hydrolysate; ND – Not determined

Table 10-3 Summary of selected production performance parameters. Data are presented as mean ± standard error

	A0[A]	**A1**	**A2[A]**	**A3**	**A4**	**A5**	**A6[B]**	**A7**
W_0 (g)	127 ± 2.65	122 ± 2.33	125 ± 1.48	124 ± 2.85	124 ± 1.80	129 ± 2.11	123 ± 3.13	127 ± 3.99
W_f (g)	164 ± 3.80^{a}	158 ± 2.05^{a}	155 ± 0.83^{a}	156 ± 2.43^{a}	142 ± 2.56^{b}	157 ± 2.95^{a}	156 ± 2.87^{a}	159 ± 2.05^{a}
RFI (g g $^{-1}$)	0.56 ± 0.01^{ae}	0.56 ± 0.01^{ae}	0.58 ± 0.01^{ade}	0.54 ± 0.01^{a}	0.86 ± 0.02^{b}	0.73 ± 0.02^{c}	0.62 ± 0.01^{ef}	0.63 ± 0.01^{df}
Total mortalities	1	2	4	2	3	6	0	0

Abbreviations: W_0 – Initial animal weight; W_f –Final animal weight; RFI – Relative feed intake

A – Growth data previously reported in (Goosen, et al., 2014b)

B – Growth data previously reported in (Goosen, et al., 2014a)

Figures

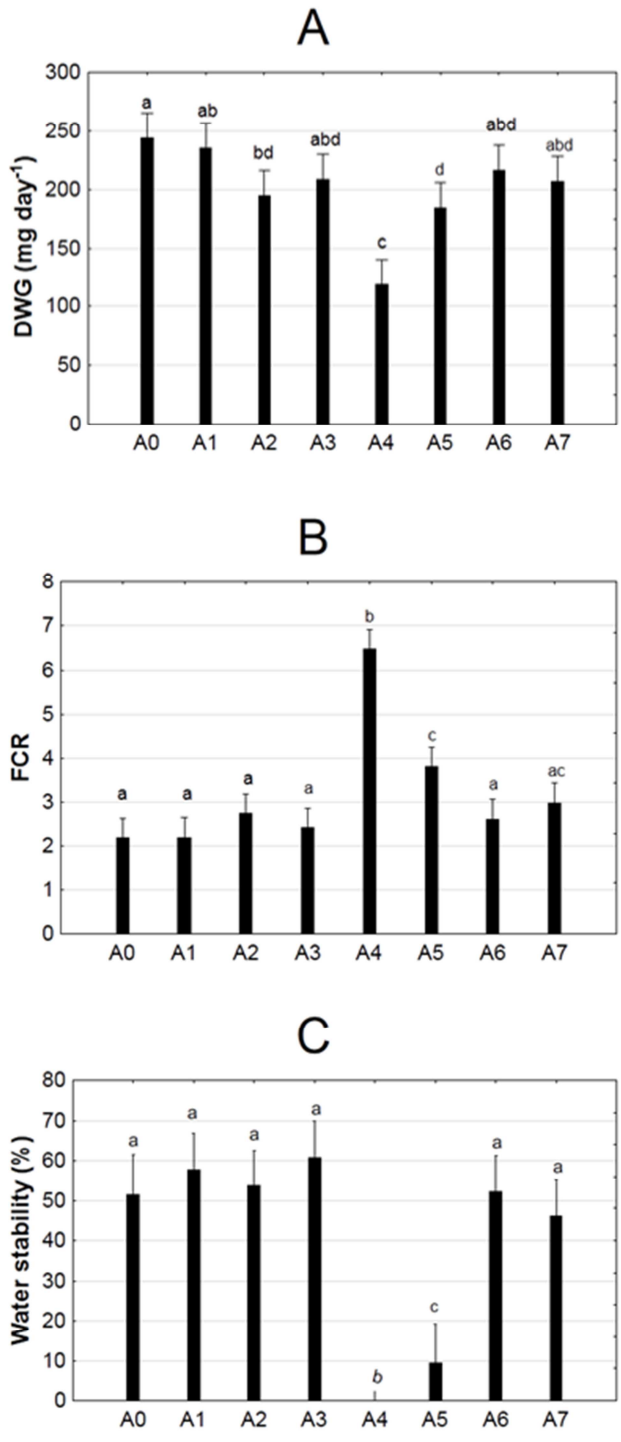


Figure 10-1 Results of daily weight gain (A), feed conversion ratio (B) and feed water stability (C).

Chapter 11: Conclusions

"Eventually, all things merge into one, and a river runs through it." Norman Maclean, A River Runs Through It

This study has demonstrated that chemical ensiling of fish processing waste can be employed as a simple processing method to produce aquafeed ingredients that exhibit beneficial bio-activities when included in aquafeeds. These beneficial effects are likely to increase the economic value of the waste derived feed ingredients, and can thereby contribute to the economic viability of further processing of fish processing waste and by-products. The utilisation of fish processing waste for the production of aquafeed ingredients further decreases the dependence on dedicated capture fisheries to provide feed ingredients for the aquaculture industry, and thereby serves to improve the sustainability of the aquafeed manufacturing industry.

It was found that the lipid component of chemical fish silage resulted in favourable physiological response when supplemented to formulated aquafeeds. In both Mozambique tilapia and South African abalone, the dietary silage oil resulted in significantly improved cellular non-specific immunity. In Mozambique tilapia, the silage oil increased overall survival rate and resulted in good fillet fatty acid profile, without affecting production performance (Chapter 5). In South African abalone production performance was negatively impacted, which could be related to the dietary inclusion level of the silage oil (Chapter 8).

The hydrolysed protein component of fish silage was found to improve cellular non-specific immunity in Mozambique tilapia, with simultaneous improvements in overall survival, independent of the formic acid used for ensiling (Chapter 6). In the South African abalone there were indications that fish silage may improve cellular non-specific immunity, but results were not conclusive, possibly due to the inclusion of fish silage oil in the treatment (Chapter 9). In both species, production performance and stimulation of the non-specific immunity were dependent on dietary fish silage inclusion levels: in both Mozambique tilapia and South African abalone excessive silage inclusion resulted in decreased growth rates. In the case of Mozambique tilapia, decreased growth is attributed to negative metabolic effects of high dietary levels of free amino acids and short chain peptides; in trials with the South African abalone the feed water stability was significantly decreased at the high level of silage inclusion, which might be responsible for the decreased growth.

Dietary supplementation of fish silage and controlled hydrolysis FPH resulted in differences in growth performance in both species, and also in differences in physiological effects in the South African abalone. Dietary controlled hydrolysis FPH resulted in statistically significant increases in non-specific cellular immune function in abalone, while

improvements resulting from fish silage supplementation were not significant. Controlled hydrolysis FPH supplementation further resulted in statistically significant higher growth rates than fish silage diets (Chapter 9). In Mozambique tilapia, no differences in bio-active properties were detected, although a controlled hydrolysis FPH showed improved growth compared to fish silage at high inclusion (Chapter 7). The differences in FPH performance is most likely a result of the different raw material origins and dietary inclusion levels.

Potential effects of formic acid used in chemical silage preparation have been eliminated, and a new, previously unknown effect has been described. In Mozambique tilapia it was shown that formic acid is not responsible for the improved non-specific immunity, or the decreased growth in diets containing high silage inclusion (Chapter 6). Formic acid might contribute to the anti-microbial action of fish silage oil in the intestine of Mozambique tilapia, and might further contribute to the alteration of gross intestinal morphology which was observed (Chapter 5). A novel mechanism of working of formic acid has been described in abalone diets containing raw trout viscera, where feed supplementation with formic acid resulted in significantly improved water stability, with consequent significant improvements in animal growth rate (Chapter 10).

The results and findings of the work could be valuable to the fish processing, aquafeed and aquaculture production sectors. Within the context of utilisation of fish processing waste, it is unlikely that ensiling of processing waste will replace current fishmeal manufacturing from waste; rather, silage preparation could play an important role in utilising previously unutilised raw materials and in so doing, increase the overall utilisation of fish processing waste. Ensiling of fish processing waste is therefore seen as an alternative to waste disposal in cases where alternative processing is not a viable option, and includes waste disposal by land processors and fisheries at sea. The main advantage of the ensiling method is that it could be employed by processors as a simple waste management strategy that simultaneously generates additional revenue.

Aquafeed manufacturers can benefit from the bio-active properties of fish silage and fish silage oil. The specialist bio-active effects of these ingredients described in this study can enable aquafeed producers to increasingly shift toward the production of functional aquafeeds that exhibited specific, beneficial bio-active effects. Being waste derived products, fish silage and fish silage oil will have the additional benefit to producers of enlarging the list of potential aquafeed ingredients without increasing demand for ingredients from wild capture fisheries. Aquaculture producers can derive benefit of functional feeds through improved production performance and animal health, which could improve profitability.

There are certain challenges associated with the industrial utilisation of fish silage and fish silage oil in aquafeeds. The aqueous component of fish silage contains high levels

of water, which can translate to high transportation costs to feed manufacturing facilities if no concentration of the silage is performed beforehand. There is also a limitation on the maximum amount of fish silage that can be included as ingredient in aquafeeds, as excessive amounts of highly hydrolysed protein has been confirmed to negatively impact production performance in various species. This puts an additional constraint on feed formulation when employing fish silage, and could lead to formulation challenges. Fish silage prepared from oily fish species should preferably be de-oiled: high levels of oil in fish silage can result in non-homogenous mixing, as the oil and aqueous layers are immiscible; further, oil oxidation can lead to nutritional degradation of the oil, resulting in a nutritionally inferior feed ingredient.

The number of treatment replicates employed in the feeding trials and the subsequent analyses and assays, may have limited the ability to detect statistically significant differences between treatments for some important parameters. The reasons for the limitations in the number of replicates have been discussed in Chapter 2; nonetheless, it is acknowledged that an increased number of treatment replicates could have resulted in better detection of statistically significant differences between treatments in trials. However, within the limitations of the study the use of an increased number of replicates was not feasible. Conversely, the number of replicates employed during trials were sufficient to detect important differences between treatments for some parameters; as such the number of replicates per treatment are not thought to be a major limitation of the study, although the replicates could be increased in future investigations to increase the certainty of the results.

Future research should include the following aspects:

I. Determine the optimum dietary inclusion level of silage oil in formulated diets for the South African abalone by conducting a dose dependence feeding trial where fish silage oil is included in a formulated diet in increasing levels. This should be done in an effort to mitigate the negative effects of silage oil inclusion on production performance, without sacrificing the improvement in cellular immune function.

II. Evaluate fish silage as dietary immune stimulant in the South African abalone in the absence of silage oil. Due to the immune-stimulatory effects of silage oil, it is possible that immune stimulation by fish silage could have been masked. This can be accomplished by performing a feeding trial and incorporating fish silage, but not silage oil, in the experimental feeds, and

quantifying the effects on the non-specific immunity of the experimental animals.

III. Determine the digestibility of the protein and lipid components of fish silage in the two studied species. In order to obtain the actual biologic availability of these nutrients, digestibility trials are required. Digestibility can be determined using both *in vitro* and *in vivo* methods; however, *in vivo* methods by way of feeding trials are deemed more reliable in aquaculture and future work should be based on these methods.

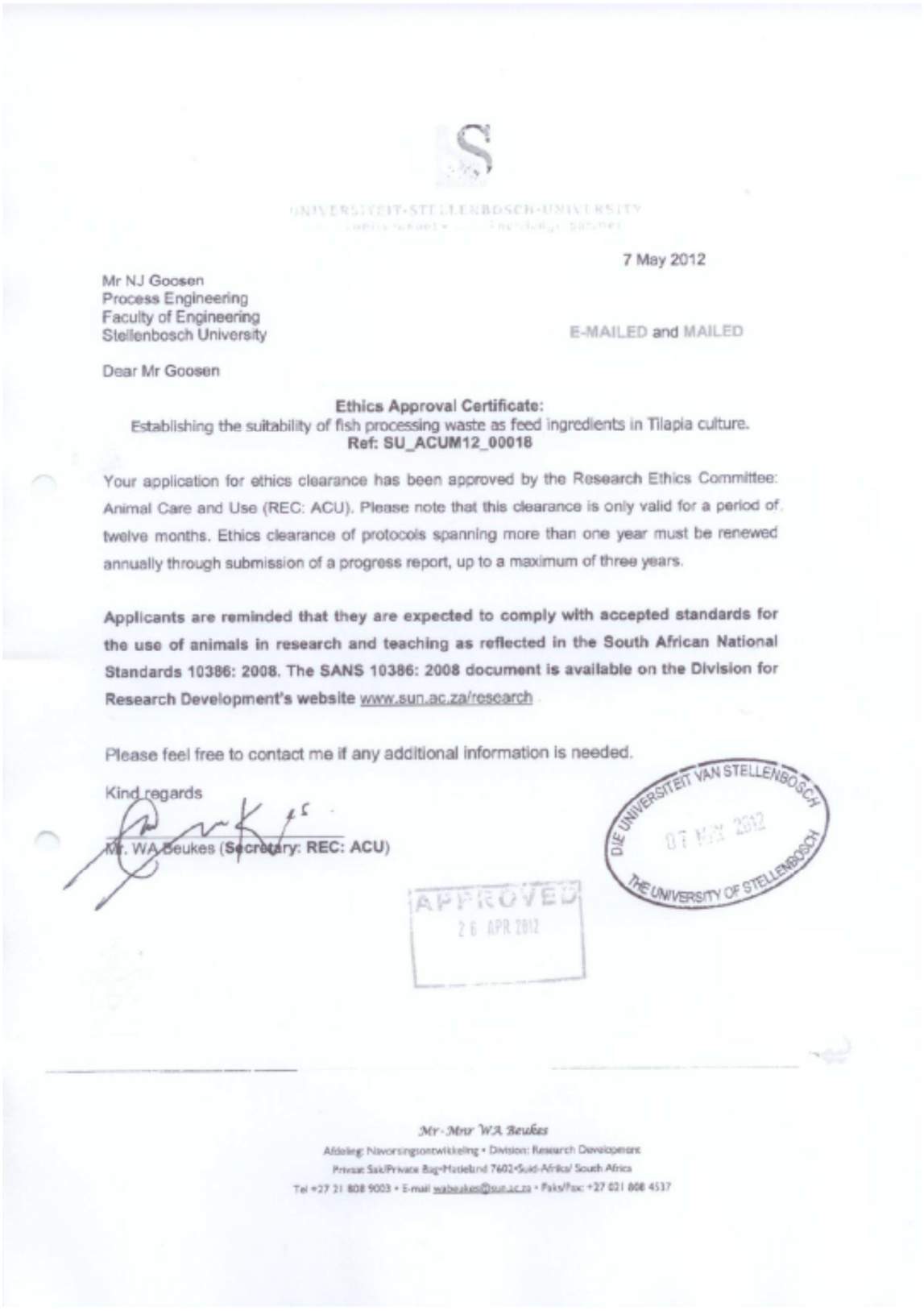
IV. Investigate the effects of dietary FPH and/or silage oil on gene expression. It has been shown that feed ingredients can alter gene expression in animals, which alters physiological processes and pathways; if the effect of specific dietary ingredients on gene expression can be determined, these could be manipulated to benefit aquaculture production. Work of this nature will include a practical feeding trial, however the majority of the work will be laboratory based to quantify gene expression.

V. Establish the effects of silage production conditions and downstream processing conditions on the final product characteristics. Final product quality will affect aquaculture production efficiency, and is largely determined by raw material quality and processing conditions. If larger scale technology is applied for fish silage preparation and downstream processing (e.g. drying), a sound knowledge of how processing affects product quality is required. Feeds incorporating fish silage and fish silage oil should be prepared using industrial or pilot scale feed manufacturing equipment to simulate practical feed manufacturing conditions. Feeds can be evaluated through chemical tests e.g. amino acid and oil quality analyses, or through feeding trials to evaluate animal production performance and nutrient digestibility.

"Let us hear the conclusion of the whole matter: Fear God and keep His commandments, for this is man's all." Ecclesiastes 12:13

Appendix A: Ethical clearance

Electronic copy of the ethical clearance certificate required for all tilapia trials, issued by the Research Ethics Committee, Stellenbosch University.



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Appendix C: Silage preparation method

All batches of silage and silage oil that were utilised in the various feeding trials of the study (Chapters 5 through 10), were prepared in the following way:

Viscera obtained from farmed rainbow trout were collected from a South African fish processor (3 Streams Smokehouse, Franschhoek) on the same day that the animals were slaughtered, and ensiled in 25 litre plastic containers through the addition of 2.5 weight % formic acid (85 % purity). Silage was always prepared from batches of viscera weighing between 17 kg and 20 kg. The formic acid and viscera were thoroughly mixed by hand, after which the plastic containers were placed indoors to allow protein hydrolysis to proceed at ambient temperatures (15 °C - 25 °C). The silage was stirred once per day by hand for the first three days post ensiling, taking care not to mix the free floating oil and the aqueous phase. Free floating oil was harvested by hand from the silage three days after ensiling took place, and a commercially available antioxidant (butylated hydroxytoluene / butylated hydroxyanisole mixture, Oxipet L, Bitek) was added at the ratio of 1 ml antioxidant mixture per litre of oil, according to manufacturer recommendations. The harvested oil was stored in 2.5 litre plastic containers at 4 °C until used for diet preparation.

The remaining silage was stirred and allowed to hydrolyse further until the batch was de-oiled. Due to the separation of the oil and aqueous phases during protein hydrolysis, de-oiling of the silage was necessary to avoid possible non-homogenous distribution of the aqueous and oil phases, which may in turn have resulted in non-homogenous experimental feeds if used in feed preparation. The silage employed in Chapter 6, Chapter 7 and Chapters 9 and 10 were de-oiled 37 days, 14 days and 6 days post ensiling respectively. The time until silage was de-oiled was dictated by the scheduling and availability of feed preparation equipment and the availability of commercial feeds that formed the basis of the experimental diets. Silage was de-oiled using a commercial centrifugal dairy creamer (Elecrem Model 1, Elecrem, 54 Avenue des Pépinières, Parc Médicis 94260, Fresnes, France). De-oiled silage was transferred to clean 25 litre containers and left at ambient temperature until used in the preparation of the experimental feeds. The oil fraction recovered during the de-oiling process was discarded.

Appendix D: Statistical supplement

Table D-1: Supplementary statistical data for results reported in Chapter 5: Tables 5-3 to 5-5 and Figure 5-2.

Parameter	Number of treatments	n	F	P-value
Table 5-3				
FCR	2	5	0.73	0.82
SGR	2	5	0.51	0.96
Jones CF (Male)	2	11/13[a]	8.70	0.01
Weight (Male)	2	11/13	0.39	0.54
Length (Male)	2	11/13	0.02	0.90
Height (Male)	2	11/13	2.52	0.13
Jones CF (Female)	2	7/12[b]	1.14	0.30
Weight (Female)	2	7/12	0.69	0.42
Length (Female)	2	7/12	1.98	0.18
Height (Female)	2	7/12	0.62	0.44
Table 5-4				
FL (Pr)	2	5	0.65	0.44
FW (Pr)	2	5	0.65	0.44
IP (Pr)	2	5	1.64	0.24
OP (Pr)	2	5	3.84	0.09
FL : FW (Pr)	2	5	0.01	0.93
IP: OP (Pr)	2	5	0.40	0.55
FL : OP (Pr)	2	5	0.79	0.40
FL (Mid)	2	5	15.5	0.004
FW (Mid)	2	5	1.37	0.28
IP (Mid)	2	5	5.57	0.046
OP (Mid)	2	5	0.59	0.46
FL : FW (Mid)	2	5	14.3	0.005
IP: OP (Mid)	2	5	7.10	0.029
FL : OP (Mid)	2	5	9.57	0.015
FL (Dis)	2	5	0.68	0.43

Parameter	Number of treatments	n	F	P-value
FW (Dis)	2	5	1.05	0.34
IP (Dis)	2	5	0.004	0.94
OP (Dis)	2	5	1.61	0.24
FL : FW (Dis)	2	5	0.002	0.96
IP: OP (Dis)	2	5	0.17	0.69
FL : OP (Dis)	2	5	0.02	0.89
Table 5-5				
Erythrocytes (S1)	2	5	0.10	0.76
Leukocytes (S1)	2	5	0.01	0.92
Hematocrit (S1)	2	5	0.18	0.68
Phagocytic activity (S1)	2	5	19.7	0.002
Lysozyme (S1)	2	5	0.62	0.53
Immunoglobulin (S1)	2	5	0.002	0.97
Total protein (S1)	2	5	1.96	0.22
Erythrocytes (S2)	2	5	0.64	0.46
Leukocytes (S2)	2	5	1.38	0.29
Hematocrit (S2)	2	5	0.61	0.47
Lysozyme (S2)	2	5	0.004	0.95
Immunoglobulin (S2)	2	5	0.53	0.51
Total protein (S2)	2	5	1.57	0.26
Figure 5-2				
CFU: feed	2	3	10.2	0.03
CFU: intestine (S1)	2	3	39.8	0.003
CFU: intestine (S2)	2	3	42.8	0.003

Abbreviations: n – number of observations; F – test statistic; P – value - probability value; FCR – feed conversion ratio; SGR – specific growth rate; Jones CF – Jones condition factor; FL – fold length; FW – fold width; IP – inner perimeter; OP – outer perimeter; Pr – proximate intestine; Mid – mid intestine; Dis – distal intestine; S1 – Sampling 1; S2 – Sampling 2; CFU – colony forming units

Notes:

[a] – Control: 11 observations; Silage oil: 13 observations

[b] – Control: 7 observations; Silage oil: 12 observations

Table D-2: Supplementary statistical data for results reported in Chapter 6: Tables 6-3 to 6-6.

Parameter	Number of treatments	n	F	P-value
Table 6-3				
FCR	4	5	0.11	0.96
SGR	4	5	1.33	0.31
Jones CF (Male)	4	13/15/12/15[a]	0.22	0.88
Weight (Male)	4	13/15/12/15	4.84	0.005
Length (Male)	4	13/15/12/15	3.41	0.02
Height (Male)	4	13/15/12/15	2.97	0.04
Jones CF (Female)	4	13/18/11/17[b]	0.81	0.49
Weight (Female)	4	13/18/11/17	2.40	0.08
Length (Female)	4	13/18/11/17	2.86	0.045
Height (Female)	4	13/18/11/17	3.51	0.02
Table 6-4				
Leukocytes (S1)	4	5	1.26	0.32
Erythrocytes (S1)	4	5	0.34	0.80
Hematocrit (S1)	4	5	0.51	0.68
Leukocytes (S2)	4	5	0.56	0.65
Erythrocytes (S2)	4	5	1.35	0.30
Hematocrit (S2)	4	5	1.89	0.18
Table 6-5				
Phagocytic activity (S1)	4	5	3.92	0.04
Lysozyme (S1)	4	5	0.66	0.59
Immunoglobulin (S1)	4	5	0.87	0.48
Total protein (S1)	4	5	3.12	0.06
Lysozyme (S2)	4	5	1.93	0.19
Immunoglobulin (S2)	4	5	1.29	0.33
Total protein (S2)	4	5	4.22	0.74

Parameter	Number of treatments	n	F	P-value
Table 6-6				
Fold length (Pr)	4	5	0.39	0.76
Fold width (Pr)	4	5	0.35	0.79
L:W (Pr)	4	5	0.29	0.83
IP (Pr)	4	5	1.14	0.37
Fold length (Mid)	4	5	0.19	0.90
Fold width (Mid)	4	5	1.24	0.33
L:W (Mid)	4	5	1.35	0.30
IP (Mid)	4	5	0.36	0.78
Fold length (Dis)	4	5	0.66	0.59
Fold width (Dis)	4	5	0.50	0.69
L:W (Dis)	4	5	0.39	0.76
IP (Dis)	4	5	0.67	0.59

Abbreviations: n – number of observations; F – test statistic; P – value - probability value; FCR – feed conversion ratio; SGR – specific growth rate; Jones CF – Jones condition factor; L:W – length to width ratio; IP – inner perimeter; OP – outer perimeter; Pr – proximate intestine; Mid – mid intestine; Dis – distal intestine; S1 – Sampling 1; S2 – Sampling 2; FA – formic acid containing treatment; SL – low silage treatment; SH – high silage treatment; R – reference diet.

Notes:

[a] – FA: 13 observations; SL: 15 observations; SH: 12 observations; R: 15 observations

[b] – FA: 13 observations; SL: 18 observations; SH: 11 observations; R: 17 observations

Table D-3: Supplementary statistical data for results reported in Chapter 7: Tables 7-2 to 7-4 and Figure 7-1.

Parameter	Number of treatments	n	F	P-value
Table 7-2				
W_i	7	6	0.69	0.66
SGR	7	6	0.61	0.72
FCR	7	6	1.26	0.30
Dress-out %	7	6	0.90	0.50
Table 7-3				
Lysozyme (S1)	7	6	0.97	0.46
TP (S1)	7	6	0.21	0.97
IG (S1)	7	6	0.62	0.71
Haematocrit (S1)	7	6	0.42	0.86
Lysozyme (S2)	7	6	0.55	0.77
TP (S2)	7	6	1.37	0.25
IG (S2)	7	6	0.24	0.96
Haematocrit (S2)	7	6	0.21	0.97
Hepatosomatic index (S2)	7	6	0.35	0.90
Table 7-4				
Inner perimeter	7	6	0.20	0.98
Outer perimeter	7	6	0.49	0.81
Villi length	7	6	0.42	0.86
Villi width	7	6	0.16	0.98
L:Wid	7	6	0.51	0.79
Mucosa width	7	6	0.89	0.51
Figure 7-1				
Jones CF	7	6	2.69	0.02
Weight	7	6	1.74	0.11
Length	7	6	2.26	0.04
Height	7	6	2.18	0.04

Abbreviations: n – number of observations; F – test statistic; P – value - probability value; W_i – initial animal weight; FCR – feed conversion ratio; SGR – specific growth rate; TP – total protein; IG – immunoglobulin; L;Wid – length to width ratio; Jones CF – Jones condition factor; S1 – Sampling 1; S2 – Sampling 2.

Table D-4: Supplementary statistical data for results reported in Chapter 8: Tables 8-2 and 8-3 and Figures 8-1 and 8-2.

Parameter	Number of treatments	n	F	P-value
Table 8-2				
$W_{initial}$	2	6	0.29	0.60
Wfinal	2	6	5.56	0.04
SGR	2	6	13.8	0.004
FCR	2	6	21.4	0.001
RFI	2	6	1.50	0.25
Table 8-3				
Crude fat	2	3	0.07	0.80
Crude Protein	2	3	1.41	0.30
Ash	2	3	1.46	0.29
Moisture	2	3	0.25	0.64
Dry matter	2	3	0.25	0.64
Carbohydrates*	2	3	0.15	0.72
Figure 8-1				
Phagocytic activity	2	6	26.2	0.0004
Hemocytes	2	6	0.55	0.47
Lysozyme	2	6	0.02	0.89
Total protein	2	6	0.11	0.75
Figure 8-2				
Relative water stability	2	6	0.02	0.90

Abbreviations: n – number of observations; F – test statistic; P – value - probability value; $W_{initial}$ – initial animal weight; W_{final} – final animal weight; SGR – specific growth rate; FCR – feed conversion ratio; RFI – relative feed intake.

Table D-5: Supplementary statistical data for results reported in Chapter 9: Tables 9-3 and 9-4 and Figure 9-1.

Parameter	Number of treatments	n	F	P-value
Table 9-3				
W_i	7	6	0.33	0.92
W_f	7	6	1.08	0.40
DWG	7	6	3.27	0.01
FCR	7	6	3.67	0.006
RFI	7	6	28.5	0.000
Table 9-4				
Lysozyme	7	6	1.76	0.18
Total protein	7	6	0.51	0.80
Phagocytosis	7	6	4.52	0.002
Hemocytes	7	6	1.68	0.16
Figure 9-1				
Feed water stability	5	6	26.2	0.0004
Hemocytes	5	6	0.55	0.47
Lysozyme	5	6	0.02	0.89
Total protein	5	6	0.11	0.75

Abbreviations: n – number of observations; F – test statistic; P – value - probability value; W_i – initial animal weight; W_f – final animal weight; DWG – daily weight gain; FCR – feed conversion ratio; RFI – relative feed intake.

Table D- 6: Supplementary statistical data for results reported in Chapter 10: Table 10-3 and Figure 10-1.

Parameter	Number of treatments	n	F	P-value
Table 10-3				
W_0	8	6	0.77	0.62
W_f	8	6	5.81	0.000
RFI	8	6	84.5	0.000
Figure 10-1				
DWG	8	6	42.1	0.000
FCR	8	6	13.2	0.000
Water stability	8	6	25.5	0.000

Abbreviations: n – number of observations; F – test statistic; P – value - probability value; W_0 – initial animal weight; W_f – final animal weight; RFI – relative feed intake; DWG – daily weight gain; FCR – feed conversion ratio