The effects of xylanase and arabinoxylan-oligosaccharides on the growth performance, non-specific immunity, hindgut microbial diversity and hindgut short-chain fatty acid production of African catfish, *Clarias gariepinus*

by

Stephan Johann Gericke

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Supervisor: Dr Khalid Salie

Co-supervisor: Dr Neill Goosen

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Declaration

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Summary

Aquaculture is one of the fastest growing food-producing industries in the world, however, this industry is still highly dependent and relies more on the production of fishmeal and fish oil resources from marine capture fisheries than any other food producing industry. This trend has increased over the last few decades despite the stagnant production of capture-based fisheries. With the aim of becoming more sustainable, aquaculture feed producers have started to incorporate plant-protein ingredients as alternatives for fishmeal and fish oil. The principal challenge facing formulators when incorporating high levels of plant ingredients in aquafeeds is how to eliminate or even exploit the accompanying antinutritional factors, such as non-starch polysaccharides, while improving the low nutrient quality and digestibility of the plant ingredients. The aim of the study was to contribute to the global trend towards more sustainable aquaculture feeds by investigating the effect of two feed additives (endo-1,4-β-xylanase and an arabinoxylan-oligosaccharide-containing compound) to potentially improve the performance of formulated diets containing high levels of plant ingredients fed to African catfish, *Clarias gariepinus*. Liquid endo-xylanase (Nutrase Xyla) was obtained through Nutrex Belgium, while AXOS were thermochemically produced, through the method of steam explosion, from brewer's spent grains, which is generally regarded as a waste product. In order to achieve the study's aim, specific objectives were formulated and include the determination of i) a suitable inclusion level for both functional feed additives, as well as their effect on the ii) production performance parameters, iii) selected humoral non-specific immunity parameters, iv) hindgut microbial diversity, and v) hindgut short-chain fatty acid concentration of *C. gariepinus* fed highly plant-based diets. The study was comprised out of two independently run 91-day feedings trials, viz. xylanase and AXOS trials. Both trials consisted of four dietary treatments (a control and three test treatments) with each treatment replicated six times and receiving six randomly placed, mixed sex *C. gariepinus* at the start of each trial. The three test treatments of the xylanase trial each contained xylanase inclusion levels of 100, 150 and 200 PPM, respectively, while the test treatments of the AXOS trial each contained an AXOS-containing component at 0.3, 0.6 and 1.2%, respectively. Results from the xylanase trial showed that dietary xylanase was able to significantly decrease (P=0.041) the Shannon's microbial diversity index of the xylanase 200 treatment compared to the control treatment. The control and xylanase 150 treatments also had a significantly higher Shannon's diversity score compared to the pre-treatment group (sampled at Day 0). Furthermore, the control and xylanase 150 treatments had a significantly higher (P=0.050) Simpson's diversity index compared to the pre-treatment group. During the course of the trial, dietary AXOS supplementation showed to significantly increase the immunoglobulin levels of fish fed the AXOS 0.6 and AXOS 1.2 treatments compared to the control and AXOS 0.3 treatment groups.

At the end of the trial, the AXOS 1.2 treatment had a significantly higher (P=0.004) immunoglobulin level compared to all the other treatments. The study concluded that dietary xylanase was able to significantly decrease the hindgut microbial diversity of *C. gariepinus* based on a dose-dependent manner. The absence of any negative significant effect of the AXOS-containing compound on the growth and fillet composition proved that AXOS can be thermochemically produced from a waste product without the presence of semi-antinutritional factors. Additionally, AXOS significantly increased the immunoglobulin levels of *C. gariepinus* based on a dose-dependent manner. Overall, the supplementation of xylanase and AXOS in fishmeal-free diets of *C. gariepinus* may have promising potential as functional feed additives that may directly enhance the innate immunity of fish through interacting with the gut-associated lymphoid tissue or indirectly through the modulation of the hindgut microbiota.

Opsomming

Akwakultuur is een van die vinnigste groeiende voedselproduksiebedrywe in die wêreld. Hierdie bedryf is egter steeds hoogs afhanklik van en berus meer op die produksie van vismeel- en visoliehulpbronne uit mariene vangsgebaseerde visserye as enige ander voedselproduksiebedryf. Hierdie tendens het die afgelope paar dekades toegeneem ten spyte van die stagnante produksie van mariene vangsgebaseerde visserye. Met die doel om meer volhoubaar te wees, het produsente van akwakultuurvoere begin met die opneming van plant-proteïen bestanddele as alternatiewe vir vismeel en visolie. Die vernaamste uitdaging vir formulateerders wanneer hoë vlakke van plantaardige bestanddele by akwakultuurvoere ingesluit word, is hoe om die gepaardgaande antinutrisionele faktore, soos nie-stysel polisakkariede, te elimineer of selfs te ontgin, terwyl die lae voedingskwaliteit en verteerbaarheid van die plantbestanddele verbeter word. Die doel van die studie was om by te dra tot die wêreldwye tendens na volhoubare akwakultuurvoere deur die effek van twee toevoegingstowwe (endo-1,4-β-xylanase en 'n arabinoxilan-oligosakkariedbevattende verbinding) te ondersoek om die prestasie van ‘n geformuleerde dieet met hoë vlakke van plantaardige bestanddele wat gevoer word aan Afrika-katvis, *Clarias gariepinus,* te verbeter. Xylanase en arabinoxylan-oligosakkariede (AXOS) het soortgelyke mikrobiese- en immunomodulatoriese eienskappe alhoewel hul doeltreffendheid ten opsigte van hierdie eienskappe in visvoere nog nie bewys is nie. Vloeibare endo-xylanase (Nutrase Xyla) is verkry deur Nutrex Belgium, terwyl AXOS termochemies van broueryse spandeerprodukte geproduseer is (algemeen beskou as 'n afvalproduk). Ten einde die doel van die studie te bereik, is spesifieke doelwitte geformuleer en sluit die bepaling van i) 'n geskikte insluitingvlak vir beide funksionele toevoegingstowwe in, asook hul effek op die ii) produksie prestasie parameters, iii) geselekteerde humorale nie-spesifieke immuniteit parameters, iv) agterderm mikrobiese diversiteit, en v) agterderm kortketting vetsuurkonsentrasie van *C. gariepinus* wat hoogs plantgebaseerde diëte gevoer is. Die studie het bestaan uit twee onafhanklike lopende 91-dae voedingsproewe, nl. xylanase en AXOS proewe. Beide proewe het bestaan uit vier dieetbehandelings ('n kontrole en drie toetsbehandelings) met elke behandeling wat ses keer herhaal is en wat ses willekeurig geplaasde gemengde seks *C. gariepinus* aan die begin van elke proefbeurt ontvang het. Die drie toetsbehandelings van die xylanase-proefneming het elk xylanase-insluitingsvlakke van onderskeidelik 100, 150 en 200 PPM bevat, terwyl die toetsbehandelings van die AXOS-proef elk 'n AXOS-bevattende komponent teen 0,3, 0,6 en 1,2% bevat het. Uit die xylanase-proefneming is bevind dat die xylanase dieet die Shannon mikrobiese diversiteitsindeks van die xylanase 200-behandeling aansienlik kon verlaag (P=0,041) in vergelyking met die kontrolebehandeling. Die kontrole en xylanase 150 behandelings het ook 'n aansienlike hoër Shannon diversiteitstelling behaal in vergelyking met

die voorbehandelingsgroep (op Dag 0 gemonster). Verder het die kontrole en xylanase 150 behandelings 'n aansienlike hoër (P=0.050) Simpson diversiteitsindeks in vergelyking met die voorbehandelingsgroep gehad. Gedurende die verloop van die studie het die AXOS-aanvullings dieet getoon dat die immunoglobulienvlakke van die vis wat die AXOS 0,6- en AXOS 1.2-behandelings ontvang het, aansienlik verhoog is in vergelyking met die kontrole- en AXOS 0.3-behandelingsgroepe. Aan die einde van die studie het die AXOS 1.2-behandeling 'n aansienlike hoër (P=0.004) immunoglobulienvlak gehad in vergelyking met al die ander behandelings. Die studie het bevind dat die xylanase dieet in staat was om die agterderm mikrobiese diversiteit van *C. gariepinus* aansienlik te verminder, gebaseer op 'n dosisafhanklike wyse. Die afwesigheid van enige negatiewe beduidende effek van die AXOS-bevattende verbinding op die groei- en filetsamestelling het bewys dat AXOS termochemies van 'n afvalproduk geproduseer kan word sonder die teenwoordigheid van semi-antinutrisionele faktore. Daarbenewens het AXOS die immunoglobulienvlakke van *C. gariepinus* aansienlik verhoog op grond van ‘n dosisafhanklike wyse. Oor die algemeen kan die aanvulling van xylanase en AXOS in vismeelvrye diëte van *C. gariepinus* belowende potensiaal hê as funksionele toevoegingstowwe wat die aangebore immuniteit van vis direk kan verbeter deur interaksie met die derm-geassosieerde limfoïedweefsel of indirek deur die modulasie van die agterderm mikrobiota.

Dedication

This thesis is dedicated to Tersia Gericke.

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Preface

This thesis is presented as a compilation of five chapters. Each chapter is an individual entity and is written to the language and style requirements of the *South African Journal of Animal Science*. Chapters three and four are reported in the form of individual potential scientific articles and, therefore, some repetition between these two chapters has been unavoidable.

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Chapter 2	Literature review
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Chapter 5	General conclusion and recommendation

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List of abbreviations

ADC	apparent digestibility coefficient
ADG	average daily growth
AME	apparent metabolizable energy
ANF	antinutritional factors
avDP	average degree of polymerization
avDS	average degree of substitution
AX	arabinoxylans
AXOS	arabinoxylan-oligosaccharides
CP	crude protein
CSM	cottonseed meal
DE	digestible energy
DM	dry matter
DO	dissolved oxygen
DP	digestible protein
FCR	feed conversion ratio
FI	feed intake
FM	fishmeal
FO	fish oil
GE	gross energy
GIT	gastrointestinal tract
HSI	hepatosomatic index
LAB	lactic acid bacteria
MBM	meat and bone meal
ME	metabolizable energy
NDOs	non-digestible oligosaccharides
NSP	non-starch polysaccharides
NSPases	non-starch polysaccharide degrading enzymes
OTU	operational taxonomic unit
PA	phagocytic activity
PBM	poultry by-product meal
PCR	polymerase chain reaction
PCV	packed cell volume
PER	protein efficiency ratio
PRR	pattern recognition receptors
RAS	recirculating aquaculture system
RFI	relative feed intake
RSM	rapeseed meal
SBM	soybean meal
SCFA	short chain fatty acid
SD	standard deviation
SE	standard error
SGR	specific growth rate
VSI	visceral somatic index
WE-AX	water-extractable arabinoxylans
WU-AX	water-unextractable arabinoxylans

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Chapter 1

Introduction

Continual advancements in the efficiency and sustainability of the production of aquaculture feeds are essential for the expansion of the aquaculture industry (Burr *et al*., 2005). Recently, the aquaculture industry has been focusing on the inclusion of functional feeds in prepared diets of aquaculture organisms. Functional feeds contain properties that may promote the health and growth of the host animal to a more extended degree than mere nutrient availability (Gatlin, 2003). These feed additives are, therefore, able to nutritionally complement high plant-containing feedstuffs.

The use of fishmeal (FM) and fish oil (FO) in the production of industrially compounded aquafeeds (especially for carnivorous finfish and marine shrimp species) are deemed unsustainable (Hardy, 2010). In 2006, the average global fish-in fish-out ratio was calculated at 0.7 (Tacon & Metian, 2008). This ratio included the values for herbivorous and omnivorous fish species, such as tilapia, catfish, milkfish and non-filter feeding carp species, which require only small amounts of FM and FO in their diets. However, the use of FM and FO resources by carnivorous aquaculture species, such as salmon, trout, eel and marine shrimp, are considerebaly higher with an average global fish-in fish-out ratio of 3.08 in 2006 (Tacon & Metian, 2008). Therefore, together with the high accompanying costs of these finite resources (Tacon & Metian, 2008), the aquaculture industry has started to incorporate alternative, more sustainable sources of protein (Gatlin *et al*., 2007; NRC, 2011). The incorporation of plant-protein ingredients has shown potential as viable replacements for FM and FO (Gatlin *et al*., 2007; Hardy, 2010) where the latter ingredients are now more regarded as strategic or speciality ingredients (Jackson, 2007). However, FM and FO are still more readily included in the production of aquafeeds than in any other animal feed producing industry (Tacon & Metian, 2008).

The challenge with highly plant-based diets are their high contents of antinutritional factors (ANFs), such as non-starch polysaccharides (NSPs), and their low nutrient efficiency that may be to the detriment of the host's performance (Francis *et al*., 2001; NRC, 2011). The addition of feed additives or functional feeds, e.g. enzymes, prebiotics and probiotics, has shown to curb the negative effects associated with ANFs while simultaneously improving the nutrient efficiency of plant-based diets (Adeola & Cowieson, 2011). These feed additives have also shown to positively affect the immune response and intestinal microbial composition of fish, ultimately benefitting the overall health of the organism (Bedford & Cowieson, 2012; Song *et al*., 2014; Akhter *et al*., 2015; Castillo & Gatlin III, 2015; Hoseinifar *et al*., 2015).

This investigation evaluated two feed additives which can potentially improve the performance of aquaculture animals fed high plant ingredient-containing aquaculture diets: xylanase, which is an NSP-degrading enzyme, and a novel prebiotic product containing arabinoxylan-oligosaccharides (AXOS). This research study consists out of four following chapters that include a literature survey, the effect of xylanase on the parameters investigated on *C. gariepinus*, the effect of AXOS on the parameters investigated on *C. gariepinus*, and a general concluding chapter.

The same parameters were investigated for xylanase and AXOS during a 91-day feeding trial. The parameters investigated include production performance (growth performance and feed efficiency), fillet composition, visceral somatic index, non-specific immunity, hindgut microbial diversity and hindgut short-chain fatty acid production. All the above parameters were measured on African catfish. Based on the experimental design of the study, the xylanase and AXOS trials were run independently and, therefore, each trial will be handled and discussed in separate chapters as the study aimed to evaluate each feed additive exclusively and did not aim to compare the two feed additives with each other.

1.1. Study's aim

The aim of this research study was to contribute to the global trend towards more sustainable aquaculture feeds by investigating the effect of two feed additives to potentially improve the performance of formulated diets containing high levels of plant ingredients fed to African catfish.

1.2. Study objectives

In order to achieve the study's aim, specific objectives were formulated, and are specified inclusively for both of the feed additives investigated. The specific objectives of this study were to:

i. determine a suitable inclusion level for both functional feed additives, as well as their effect on the
ii. selected production performance parameters,
iii. humoral non-specific immunity parameters,
iv. hindgut microbial diversity, and
v. hindgut short-chain fatty acid concentration of *C. gariepinus* fed fishmeal-free diets.

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Chapter 2

Literature review

2.1. Introduction

Aquaculture has recently surpassed the 50% threshold of all fish production for human consumption (FAO, 2016). This is the result of the ever-increasing growth of aquaculture that has contributed to an annual record-high world fish supply of 20 kg per person in 2014, and a concurrent, however slight, improvement in certain natural fish stocks (FAO, 2016). The reason for the aquaculture industry being one of the fastest growing food-producing industries in the world (Ye *et al*., 2017) is thanks to an ever-increasing population, static production of capture-based fisheries and fish still being one of the most-traded food commodities worldwide (FAO, 2018). Currently, the African continent is setting the pace regarding global aquaculture growth and, therefore, contributes significantly to the fact that aquaculture growth is expanding faster than the global annual population growth rate (FAO, 2014). However, natural fish stocks are still under pressure due to unsustainable exploitation of this finite resource. A third of global fish stocks are overfished and almost 60% are being fished at maximum sustainable levels, leaving only 10% of fish stocks still relatively underutilised. Despite international treaties and regulations, unsustainable levels of capture-based fishing are still increasing (FAO, 2016).

The farming of aquatic organisms has previously been heavily reliant on FM as the major feed protein constituent (Naylor *et al*., 2009). The reason for FM being the protein source of choice in aquafeeds is owing to its balanced amino acid profile, high protein content, the absence of antinutrients and good nutrient digestibility (Gatlin *et al*., 2007; Hardy, 2010). Similarly, the aquafeed industry has been heavily reliant on the use of FO thanks to its high digestible energy and essential fatty acid (EFA) content (Naylor *et al*., 2009) which are essential for normal growth development and assists in the absorption of fat-soluble vitamins (NRC, 1993). The aquaculture industry uses 70% and more of global FM and FO production, respectively, far more than the use by industries such as pig and poultry (Tacon & Metian, 2008). This is regardless of the constraints faced with FM and FO usages, such as high market costs and decreasing availability (Tacon & Metian, 2008; Kiron, 2012).

To sustain the growth of the burgeoning aquaculture industry, the need for alternative, more affordable, sustainable and readily available protein sources are imperative (Hardy & Gatlin III, 2002; Gatlin *et al*., 2007; Tacon & Metian, 2008; Naylor *et al*., 2009). Faced by environmental and sustainable issues, the aquaculture industry recently set towards a more sustainable approach by using evermore plant-derived raw materials as alternative ingredients to replace FM in the production of aquafeeds (Hardy, 2010). However, plant ingredients often

contain ANFs that may harm or negatively influence the well-being and growth of fish species (Francis *et al*., 2001; NRC, 2011).

Prior to 2006, antibiotics were used to cure diseases and treat adverse microbiota stimulated by high levels of NSP (Bedford & Cowieson, 2012). However, since the banning of antibiotics, functional feed additives, such as enzymes, prebiotics and probiotics, have proved to be the most cost-effective and commercially viable approach to help improve the nutritive quality of plant ingredients, while eliminating the antinutritive effect of NSP-containing compounds (Burr *et al*., 2005; Adeola & Cowieson, 2011; Bedford & Cowieson, 2012; Castillo & Gatlin III, 2015). Additionally, these feed additives have shown to control enteric disease outbreaks through the manipulation of intestinal microbiota (Bedford & Cowieson, 2012; Kiron, 2012; Ringø *et al*., 2014; Song *et al*., 2014) and, only recently, to possess immunomodulatory properties (Akhter *et al*., 2015; Hoseinifar *et al*., 2015; Mendis *et al*., 2016).

Dietary xylanase and AXOS are regarded as functional feed additives and may have similar microbial and immunomodulatory properties in fish feeds. Endo-xylanase has become well known as the predominant NSP-degrading enzyme (NSP'ase) and is used to alleviate the adverse effects associated with high NSP-containing feedstuffs while enhancing the nutrient availability of plant-based diets (Bedford & Cowieson, 2012; Castillo & Gatlin III, 2015). Due to endogenous xylanase often being unaccounted for in fish (Kuz 'mina, 1996), the exogenous application of xylanase has been described in a number of non-ruminant animal studies, such as poultry (Selle *et al*., 2003) and pig (Nortey *et al*., 2007). On the other hand, AXOS has only recently emerged as a novel prebiotic reported to possess immune- and microbial modulatory activities (Broekaert *et al*., 2011; Mendis *et al*., 2016). Arabinoxylan-oligosaccharides are derived from the enzymatic (endo-xylanase) hydrolysis or thermochemical (steam explosion) processing of arabinoxylans (AX) present in plant materials and will, therefore, likely form when xylanase is added to high plant-ingredient aquaculture diets.

Regardless of their growth and health-enhancing effects, a paucity of information exists regarding the use of xylanase and AXOS in aquaculture studies (Adeola & Cowieson, 2011; Geraylou *et al*., 2012; Jiang *et al*., 2014), and even more so when focusing on African catfish species (Ng *et al*., 1998; Van Weerd *et al*., 1999; Ng & Chen, 2002; Rurangwa *et al*., 2008). The African catfish is a freshwater finfish species that is widely cultured thanks to its robust, omnivorous, high fecundity and air-breathing qualities (Fagbenro & Davies, 2004; Nyina-Wamwiza *et al*., 2010; Tacon *et al*., 2011; Enyidi, 2012). The unique qualities of African catfish promote relatively high stocking densities and fast growth rates compared to other warm water aquaculture species (Verreth *et al*., 1993) and with a total production volume of approximately 246 000 tons in 2015 (FAO, 2018) shows the importance of African catfish as a freshwater aquaculture species.

2.2. Antinutritional factors and non-starch polysaccharides

The majority of plant ingredients are known for their lack of nutrients, poorly balanced essential amino acids and the presence of ANFs when compared to FM (Gatlin *et al*., 2007). Plant ingredients, such as barley, canola, corn, cottonseed, peas, lupins, soybeans and wheat, are often included as alternative sources of energy and protein in aquafeeds (Gatlin *et al*., 2007). Considering its relative affordability, readily availability, high protein content and balanced essential amino acid profile, soybean meal (SBM) is one of the main plant-protein ingredients used to replace FM in aquaculture diets (Rumsey *et al*., 1993; Barros *et al*., 2002; Drew *et al*., 2007). Plant ingredients, including SBM, contain ANFs such as phytic acid, trypsin and protease inhibitors, lectins and antigenic compounds (Choct, 1997; Francis *et al*., 2001). One of the most common ANFs found in plant ingredients is NSP (Sinha *et al*., 2011). Non-starch polysaccharides have attracted much attention in non-ruminant feeds due to their antinutrient and nutrient-shielding effects (Choct, 1997). The indigestibility of NSP in plant ingredients limits the application of plant ingredients in non-ruminant feeds and, therefore, results in low nutrient efficiency and increased environmental pollution (Bedford, 2000; Adeola & Cowieson, 2011).

The endogenous enzymes needed to degrade NSP are absent in most fish species and, therefore, fish are unable to optimally utilise the nutrients stored in plant ingredients (Allan *et al*., 2000). Concurrently, the high inclusion of plant ingredients, such as SBM, may amount to reduced feed utilisation and/or intake, causing a reduced growth of the host. Resultingly, an inclusion of over 50% of SBM in diets of African catfish caused a decrease in nutrient utilisation and growth performance compared to FM diets (Fagbenro & Davies, 2001; Toko *et al*., 2008). However, NSP may also exert beneficial effects upon the host and, therefore, its presence in aquafeeds might be more significant than previously thought.

2.3. The chemical structure of non-starch polysaccharides

Non-starch polysaccharides can be classified as a fraction of the indigestible portion of plant-derived ingredients that cannot be digested by the endogenous enzymes of non-ruminants (Walsh *et al*., 1993; Kuz 'mina, 1996; Masey-O'Neill *et al*., 2014b). The different fractions of dietary fibre have been widely speculated and a fair amount of ambiguity exists around the classification of NSP which makes it no easier to draw a clear conclusion (Choct, 1997). However, most authors do agree on the three main groups of NSP in plant cells (Figure 1), i.e. cellulose, non-cellulosic polymers (pentosans) and pectins (Choct, 1997; Masey-O'Neill *et al*., 2014b). Non-starch polysaccharide-containing crops can be divided into two general categories, namely cereal grains and grain legumes (Sinha *et al*., 2011). Although cellulose is believed to be the most abundant macromolecule in nature (Choct, 1997), pentosans might be considered the most significant regarding cereal grains (Masey-O'Neill *et al*., 2014b).

Pentosans includes arabinoxylan (AX) and β-glucans (Masey-O'Neill *et al*., 2014b), where AX is the main NSP in cereal grains (Grootaert *et al*., 2007) except in barley and oats where β-glucans makes out the majority (Choct, 1997). In cereal grains such as maize and sorghum, very low levels of NSP occur, while high levels of NSP are found in wheat, rye and triticale. Grain legumes such as SBM also contains substantial amounts of NSP usually present as xylan and/or cellulose (Choct, 1997).

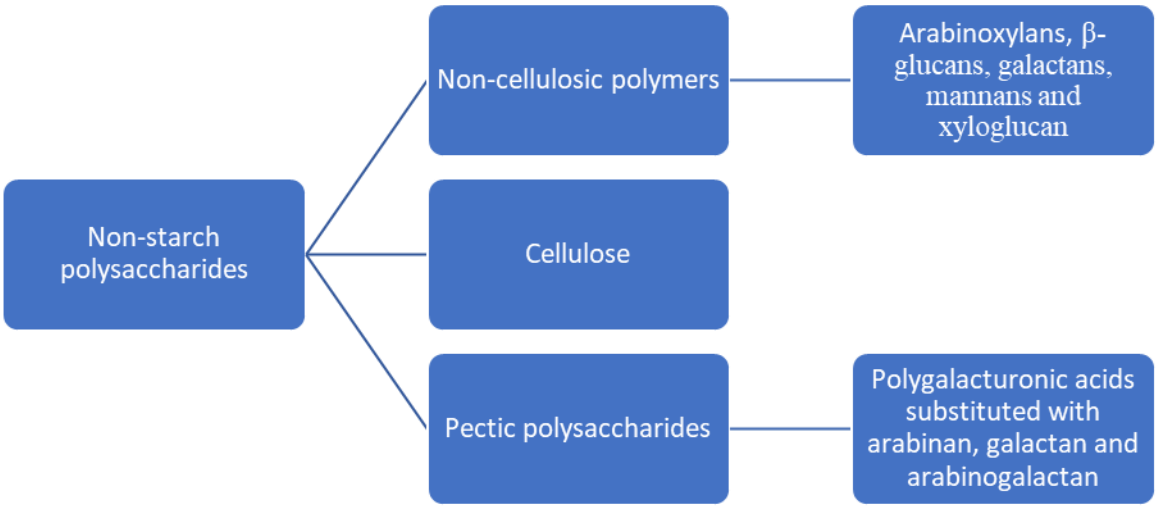

Figure 1 Classification of non-starch polysaccharides (sourced from Choct, 1997)

Depending on their chemical structure, molecular weight and affinity to other cell wall structures, NSP fractions can either be of a water soluble or water insoluble nature (Choct, 1997). Soluble NSP is found in the cell content of plant cells and constitutes a third of the total NSP, while the majority of NSP are found in the cell wall components of plant cells, and apart from its water-holding capability, does not impose great antinutritive effects, unlike soluble NSP (Bach Knudsen, 1997; Choct *et al*., 2004). The indigestibility of these NSP fractions plays an intricate role in the nutrient utilisation of plant ingredients in diets of non-ruminants and is often the main cause of nutrient sequestration (Sinha *et al*., 2011).

2.4. The nutritional significance of non-starch polysaccharides in non-ruminant feeds

Non-starch polysaccharides are indigestible to non-ruminants as their endogenous enzymes can only cleave certain glycosidic bonds between plant polysaccharides, needing microbially derived or special exogenous enzymes to complete the breakdown of NSP to digestible monosaccharide sugars (Smits & Annison, 1996). These special enzymes are particularly low or sometimes even completely absent in most fish species (Krogdahl *et al*., 2005). In plant cell walls, insoluble NSP inhibits the digestion of the cell contents by shielding them from endogenous enzymes, while soluble NSP present in cell contents interfere with nutrient assimilation and nutrient-enzyme interaction (Bach Knudsen, 1997; Adeola & Cowieson, 2011; Castillo & Gatlin III, 2015).

Soluble NSP increases intestinal viscosity by interacting with water molecules and can form cross-links with other parts of its xylan backbone, forming a gel network or junction zones (Smits & Annison, 1996; Choct, 1997). Additionally, soluble NSP can interact with particles through anion and/or cation charged groups or through hydrophobic or hydrophilic association making it possible for them to associate themselves with surfaces of potential nutrients or the epithelial surface of the gut in animals (Smits & Annison, 1996), which may ultimately lead to lower nutrient digestibility (Choct, 1997). Therefore, soluble NSP has been widely associated with the increase in the intestinal viscosity in non-ruminants (Choct, 1997). An increase in intestinal viscosity slows down the rate of digestion and enzyme diffusion, stimulates mucus secretion and reduces the interaction between nutrients and digestive fluids, ultimately causing lowered digestibility and absorption of nutrients and an increase in endogenous losses that may result in decreased animal growth (Johnson & Gee, 1981; Ikegami *et al*., 1990; Smits & Annison, 1996; Masey-O'Neill *et al*., 2014b). Nutrient absorption is also impaired through the thickening of the unstirred water layer of the mucosa, decreasing the rate of absorption through the intestinal wall (Johnson & Gee, 1981). Due to the decreased nutrient assimilation, soluble NSP is also associated with enlargement of the digestive organs which may lead to an increase in digestive secretions (Choct, 1997). These effects have higher energy requirements and can eventually lead to negative growth performance and lower nutrient digestibility (Leenhouwers *et al*., 2006).

Furthermore, the slower rate of digestion, caused by soluble NSP, decreases the availability of oxygen and, therefore, creates a favourable milieu for the development of anaerobic and potentially pathogenic bacteria (Choct *et al*., 1996; Choct, 1997). Increased digesta retention time may also allow for potentially pathogenic bacteria to establish in the proximal part of the intestine (Choct, 1997). MacAuliffe & McGinnis (1971) showed that the addition of antibiotics improved the nutritional value of rye fed to chickens, suggesting that the antinutritive effect of NSP is not exclusively related to high viscosity but also to either a change

in the composition or the shift of the gut microbiota from one part of the intestine to another. High soluble NSP content in diets can also cause lower lipid absorption by binding to bile salts and increasing bile acid secretion (Choct, 1997; Vahjen *et al*., 2007).

Contrary to soluble fibre, insoluble fibre, that constitutes the bulk of dietary fibre, has very little reported effects on nutrient utilisation in non-ruminants (Sinha *et al*., 2011). Due to its water holding capacity, insoluble fibre has shown to decrease the digesta retention time in the GIT, and by increasing the flow rate of digesta, may play a role in the inhibition of anaerobic bacteria in the upper part of the GIT, e.g. 'flushing' of microbiota from the proximal to the distal part of the intestine (Kirwan *et al*., 1974; Choct, 1997).

2.5. The adverse effects of non-starch polysaccharides in aquafeeds

Numerous studies have studied the antinutrient effect of NSP in aquafeeds and resultingly have identified the soluble NSP fraction as one of the main compounds responsible for lowered nutrient efficiency. Leenhouwers *et al*. (2006) found that digesta viscosity was significantly increased by the inclusion of a soluble viscous NSP source (guar gum) in diets of African catfish. The authors suggested that high digesta viscosities may have contributed to the observed reduced nutrient digestibility and increased weight of the intestinal organs. In a similar study on African catfish, a lower protein and lipid digestibility and mineral absorption was reported beyond a certain threshold for digesta viscosity (Leenhouwers *et al*., 2007b). However, the authors could see no direct trend between digesta viscosity levels and nutrient digestibility. The same adverse effects, such as nutrient damping, decrease in digesta dry matter and mineral absorption, were found in other aquaculture species fed diets containing high levels of soluble NSP, such as common carp (Hossain *et al*., 2001), Atlantic salmon (Refstie *et al*., 1999), tilapia (Shiau ' *et al*., 1988; Amirkolaie *et al*., 2005; Leenhouwers *et al*., 2007a), Atlantic cod (Refstie *et al*., 2006) and rainbow trout (Storebakken, 1985). Moreover, a direct association has been reported between intestinal digesta viscosity and animal growth performance in other non-ruminant animals (Dänicke *et al*., 2000; Zhang *et al*., 2000).

The adverse effects of soluble NSP seem to be dependent on the species involved, the maturity of the gut and the animal, and the particular dietary plant ingredients included in the feed (Montagne *et al*., 2003; Sinha *et al*., 2011). African catfish and Nile tilapia had higher intestinal digesta viscosities when fed diets containing rye than in diets containing maize and wheat (Leenhouwers *et al*., 2007b; a). However, Fagbenro & Davies (2004) found that replacement of FM with soy protein concentrate of up to 75% had no adverse effect on African catfish growth, carcass quality and feed utilisation. This may lead to suggest that the lower amount of soluble NSP of soy protein concentrate had no or little effect on digesta viscosity. Refstie *et al*. (1999) found that some soybean products increased the gut viscosity in chickens but not in Atlantic salmon. Older animals may be more resistant to the negative effects of

soluble NSP and may utilise NSP better thanks to a more mature intestinal microbial community compared to younger ones (Choct & Kocher, 2000; Refstie *et al*., 2006).

Cereal grains often constitute large portions of aquafeeds and one of the most important polymers of the cell wall in cereal grains and, therefore, one of the largest constituents of NSP, is arabinoxylan (Saulnier *et al*., 2007; Broekaert *et al*., 2011). Although arabinoxylan (AX) only constitutes a small portion of the grain, their physio-chemical properties play an important part in non-ruminant feeds (Bedford & Schulze, 1998).

2.6. The chemical structure of arabinoxylans

Arabinoxylans are complex polysaccharides that form part of the pentosan components of plant cells and constitutes the majority (up to 60-70%) of NSP in cereal grains, such as wheat, rye and barley (Izydorczyk & Biliaderis, 1995; Choct, 1997; Grootaert *et al*., 2007; Masey-O'Neill *et al*., 2014b; Mccleary, 2017). Arabinoxylans are predominantly composed out of two pentoses, namely xylose and arabinose (Choct, 1997) and consists of a β-(1,4)-linked D-xylopyranosyl (xylose) backbone substituted by side chains of α-L-arabinofuranose (arabinose) that are attached by α-1,2 and α-1,3 glycosidic linkages on the C(O)-2 and/or C(O)-3 position (Ebringerová & Heinze, 2000; Swennen *et al*., 2005; Dornez *et al*., 2009; Sanchez *et al*., 2009; Broekaert *et al*., 2011). Other moieties such as hexoses, hexuronic acids, phenolics (ferulic acid) and proteins may also attach themselves, although less frequently, to the xylan backbone (Geissmann & Neukom, 1973; Fincher, 1975; Neukom, 1976).

Arabinoxylan's structural heterogeneity can be differentiated by their average degree of substitution (avDS), which refers to the average ratio of arabinose to xylose moieties, and their average degree of polymerization (avDP), which is the mean number of xylose residues in their backbone (Grootaert *et al*., 2007; Sanchez *et al*., 2009; Broekaert *et al*., 2011). The degree of substitution and polymerisation plays an important role in their physiochemical properties (Grootaert *et al*., 2007) and may vary according to different plant origin (Broekaert *et al*., 2011) and extraction methods (Mendis *et al*., 2016). Rice and sorghum usually have a higher degree of substitution than wheat, barley and rye (Izydorczyk & Biliaderis, 1995; Ebringerová & Heinze, 2000; Grootaert *et al*., 2007). These physio-chemical properties of AX have been used in the food industry to improve the arts of bread making (Courtin & Delcour, 2002), gluten-starch separation (Frederix *et al*., 2004), refrigerated dough syruping (Courtin *et al*., 2005) and as functional feed additives in animal feeds (Bedford & Schulze, 1998).

In cereal grains, AX consists out of water-unextractable arabinoxylan (WU-AX) and water-extractable arabinoxylan (WE-AX) fractions (Maes & Delcour, 2002). Water-unextractable arabinoxylan, which accounts for two thirds of AX in cereals, is insoluble in water due to their covalent and non-covalent binding to cell walls structures, such as proteins,

cellulose and lignin (Iiyama *et al*., 1994), while the soluble WE-AX, which accounts for the remaining one third of cereal AX, are more loosely bound to cell wall surfaces (Mares & Stone, 1973; Courtin & Delcour, 2001).

In non-ruminant animals, AX is resistant to the host's enzymes and are only hydrolysed by specific bacteria containing AX-degrading enzymes residing in the posterior part of the large intestine (Grootaert *et al*., 2007; Ringø *et al*., 2014). These AX-degrading enzymes mainly consist out of β-D-xylosidase, α-L-arabinofuranosidase and feruloyl esterase with the two most common enzymes being β-glucanase and β-xylanase. The most prominent strain of xylanase used is endo-β-(1,4)-xylanase which randomly cleaves the β-1,4-glycosyl linkage within the backbone of AX, thereby solubilising the WU-AX fraction and fragmenting the WE-AX, thus resulting in shorter fragments of solubilised AX hydrolysis products with a reduced molecular mass, such as arabinoxylan-oligosaccharides (Biely *et al*., 1997; Courtin & Delcour, 2002).

2.7. The microbial- and immunomodulatory properties of arabinoxylans

Dietary AX and its derivatives are known to affect the immune function of fish under certain conditions. Together with other NSP compounds, such as β-glucans, AX has been reported to possess certain immunomodulatory activities and are, therefore, defined as immunosaccharides (Hromádková *et al*., 2013). Immunosaccharides (Figure 2) are chemical compounds that are capable of stimulating the innate immune response of fish (Sinha *et al*., 2011) and have been the subject regarding microbial- and immunomodulating studies on fish, humans and other terrestrial animals (Grootaert *et al*., 2007; Cloetens *et al*., 2008; Courtin *et al*., 2008b; Broekaert *et al*., 2011; Geraylou *et al*., 2012). Immunosaccharides can influence the innate immune system in two ways: Firstly, by directly stimulating the non-specific immune response, and secondly, by altering the growth and composition of intestinal microbiota (Song *et al*., 2014).

The innate immune system in fish is a fast and all-encompassing defensive mechanism comprised out of the epithelial barrier, the humoral and the cellular components (Uribe *et al*., 2011). Fish are considered to be more dependent on their innate/non-specific immune response compared to the adaptive immune response and, therefore, the innate immunity is a reliable way of measuring the immunity of fish (Saurabh & Sahoo, 2008). The non-specific immunity of fish is regarded as the first line of defence (Saurabh & Sahoo, 2008), and includes properties such as phagocytic activity, respiratory burst activity, total serum peroxidase activity, alternative haemolytic complement activity, serum lysozyme activity, total immunoglobulin and total protein. Serum lysozyme hydrolyses the peptidoglycan cell wall of both gram-positive and gram-negative bacteria through its antiviral, antibacterial and anti-inflammatory properties and can be found in various body fluids, tissues and plasma (Saurabh

& Sahoo, 2008; Uribe *et al*., 2011). It is also an effective way of measuring various stresses that may influence the animal, such as water quality, handling, sickness and nutritional stressors (Magnadotir, 2006; Kiron, 2012).

Immunoglobulin or antibodies is an immune parameter in both the innate and the acquired immune response (Magnadotir, 2006). In the innate immune system, these antibodies are found in blood serum and have shown to facilitate a fast and broad defence as part of the immune response in fish species (Vilain *et al*., 1984; Gonzalez *et al*., 1988, 1989). Total serum protein is an indicator between anabolic and catabolic protein metabolism and can, therefore, be used as an indication of the nutritional quality of the diet (Helmy *et al*., 1974).

Immunosaccharides can directly enhance the innate immunity by stimulating the pattern recognition receptors (PRRS) found on non-specific immune cells (Brown *et al*., 2002) and by beneficially influencing the gut-associated lymphoid tissue, GALT, (Akhter *et al*., 2015) as well as by interacting with microbe-associated molecular patterns, MAMPs (Song *et al*., 2014). Arabinoxylans, as part of dietary fibre, have various health benefits and include the suppression of colon cancer (Samuelsen *et al*., 2011), alleviation of type two diabetes (Montonen *et al*., 2003; Lu *et al*., 2004; Rantanen *et al*., 2007; Schulze, 2007; Cao *et al*., 2010; Niewold *et al*., 2012), prevention of cardiovascular disease (Mozaffarian, 2003; Jensen *et al*., 2004), and the enhancement of the immune system (Asp *et al*., 1993; Reddy *et al*., 2000; Gråsten *et al*., 2002; Yu Ã *et al*., 2005).

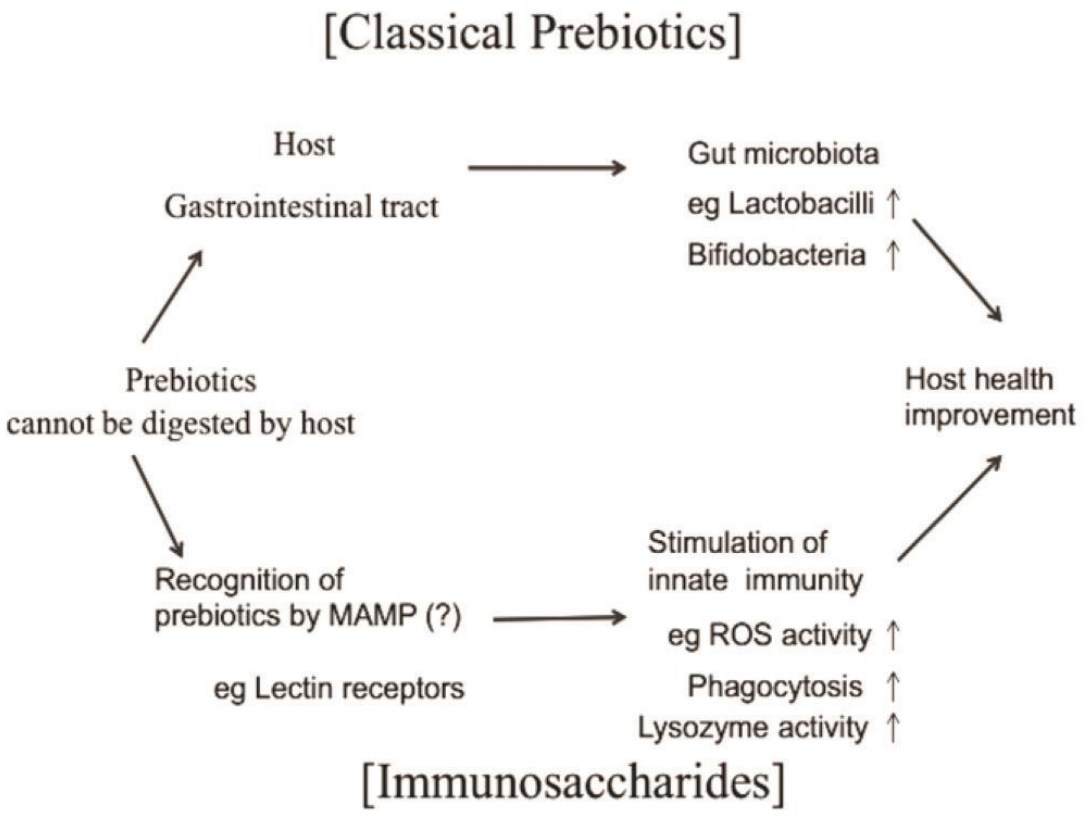

Figure 2 Immunosaccharides vs conventional prebiotics (sourced from Song *et al*. 2014).

Most prebiotic effects on immunity are indirect and modulated through the change in microbiota (Hoseinifar *et al*., 2015). Modulation of the intestinal microbiota plays a crucial role in the health and growth of the host and may assist in the energy homeostasis, the immune system, nutrient digestion, enhancing of the gut morphology, mineral solubility, vitamin synthesis, production of short-chain fatty acids (SCFAs), as well as controlling pathogenic growth (Nayak, 2010; Wardwell *et al*., 2011; Cani *et al*., 2013; Akhter *et al*., 2015). Fermentable AX structures induces a shift in the microbial composition that involves the inhibition of potential pathogenic bacteria, such as *Escherichia coli*, clostridia, veillonellae, enterococci and *Bacteroidaceae* in the proximal part of the intestine (Dänicke *et al*., 1999; Langhout *et al*., 1999; Cloetens, 2009) while stimulating growth of beneficial carbohydrate fermenting bacteria, such as bifidobacteria and lactobacilli (Cloetens, 2009), in the distal part of the large intestine. Given the fact that AX structures are resistant to the endogenous enzymes of non-ruminants, these animals are dependent upon microbes involved in the hydrolysis of AX, such as lactobacilli, bifidobacteria and Bacteroides, to better utilise the NSP fractions of their diet (Grootaert *et al*., 2007; Zhang *et al*., 2014a).

The production of SCFAs is one of the most important benefits accompanying the microbial shift from a proteolytic to a carbohydrate fermenting bacterial community. Short-chain fatty acids may act as immune response enhancers (Pratt *et al*., 1996; Meijer *et al*., 2010; Tremaroli & Backhed, 2012) through improving gut health and development by increasing villi length and crypt depth (Grizard & Barthomeuf, 1999; Choct *et al*., 2004), lowering colonic pH levels that inhibit growth of pathogens (Gibson, 2004) and increases the solubility of minerals, and possesses anti-inflammatory properties (Cloetens, 2009). Additionally, SCFAs may also be used by the immune cells of the GALT, as seen in mammals (Knudsen *et al*., 2003), by activating specific SCFA-receptors.

The main SCFAs are butyrate, propionate and acetate. Butyrate is the preferred energy source for colonocytes (Hamer *et al*., 2008), plays an important role in the prevention of colorectal cancer, maintains the intestinal mucosal integrity (Hamer *et al*., 2008) and stimulates cytokine production of TH cells (Kau *et al*., 2011). Acetate and propionate play a role as an energy source for epithelial cells as it forms part of lipid and glucose metabolism, respectively (Rombeau & Kripke, 1990). Acetate also facilitates the intestinal barrier function (Kau *et al*., 2011) and inhibits the growth of some pathogenic bacteria (Fukuda *et al*., 2011). An increase in SCFAs in the intestine of animals, limits the production of harmful proteolytic metabolites, such as ammonia, and phenolic compounds (Hubener *et al*., 2002; Van Loo, 2004; Nowak & Libudzisz, 2006; Kiarie *et al*., 2007; Sanchez *et al*., 2009) leading to a healthier gut environment. Despite the pivotal role gastrointestinal microorganisms play in the gut

development and regulation of the immune system, these organisms also play an important part in the nutrient digestion of fish, such as through the synthesis of exogenous enzymes (Nayak, 2010; Ray *et al*., 2012).

In the aquaculture industry, the health benefits of AX and its hydrolysis products have been the subject of an increasing number of authors (Burr *et al*., 2005; Ringø *et al*., 2010, 2014; Sinha *et al*., 2011; Bedford & Cowieson, 2012; Song *et al*., 2014; Akhter *et al*., 2015; Castillo & Gatlin III, 2015; Hoseinifar *et al*., 2015), however, to eliminate the ANFs accompanying high NSP-containing feedstuffs and to ensure that AX structures are sufficiently hydrolysed to smaller, fermentable oligosaccharides, plant ingredients need to be processed (biologically or mechanically) to ensure that the NSP fractions of plant materials can be exploited as potential functional feed additives.

2.8. The processing of non-starch polysaccharides in aquafeeds

In order to remove ANFs present and to render NSP as having beneficial rather than negative effects in aquafeeds, plant ingredients need to be processed. Processing methods involve the treatment of feedstuffs by means of biological enhancement and/or mechanical modification (Gatlin *et al*., 2007). Mechanical processing involves hydrothermal treatments, such as extrusion pelleting, as well as the fractionation of crops, such as de-hulling and the production of high-protein concentrates. The mechanical processing of plant ingredients in aquafeeds has shown to increase nutrient digestibility while reducing the presence of ANFs (Allan & Booth, 2004; Gatlin *et al*., 2007; Castillo & Gatlin III, 2015). However, while mechanical processing has shown to increase the nutrient digestibility of various plant ingredients, it does not guarantee the complete removal of ANFs from plant ingredients. Furthermore, the optimal digestibility of the feedstuffs is not always reached due to the absence of enzymes needed to break down the glycosidic bonds of the plant cell wall structure that encapsulates other nutrients (Castillo & Gatlin III, 2015). High temperatures accompanying these processing methods may also damage the protein quality of the feedstuff, causing less than optimal growth in fish (Olsen *et al*., 2001).

Biological enhancement, on the other hand, involves the use of micro-organisms, feed ingredients and/or enzymes to make the ingredient more available to the host (Gatlin *et al*., 2007). Prebiotics, probiotics and exogenous enzymes are the most prevalent feed additives used to biologically enhance animal feedstuffs (Burr *et al*., 2005; Bedford & Cowieson, 2012). Prebiotics are defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (Roberfroid, 2007) whereas probiotics can be defined as live microbial feed additives that beneficially affects the host animal by modulating its microbial composition (Fuller, 1989). However, since the incorporation of probiotics into aquaculture

feeds, it seemed more productive to modulate the intestinal microbiota through the inclusion of prebiotics than to directly incorporate probiotics which have to survive all the accompanied rigours of the digestive tract, viz. digestive secretions and extreme pH fluctuations, as well as the already established intestinal bacteria of the host (Burr *et al*., 2005). Hence, several prebiotics have been studied on aquaculture species and include: inulin, β-glucan, fructooligosaccharides (FOS), mannanoligosaccharides (MOS), trans-galactooligosaccharides (TOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylan-oligosaccharides (AXOS), amylase-resistant gluco-oligosaccharides, lactosucrose, soybean oligosaccharides and isomaltooligosaccharides (IMO) (Macfarlane *et al*., 2006; Ringø *et al*., 2014).

Biologically enhancement of animal feedstuffs also includes the use of NSP-degrading/carbohydrase enzymes (NSP'ase), such as pentosanases, cellulolases, glucanases and xylanases (Adeola & Cowieson, 2011). Carbohydrase enzymes have shown to enhance the nutrient efficiency of plant ingredients through solubilising insoluble NSP in plant cell walls, thereby releasing previously unavailable nutrients, and by hydrolysing soluble NSP in plant cell content, causing reduced intestinal digesta viscosity (Adeola & Cowieson, 2011; Castillo & Gatlin III, 2015). Concurrently, NSP'ases produce non-digestible oligosaccharides (NDOs) through the hydrolysis of NSP compounds which are fermentable by carbohydrate fermenting gastrointestinal bacteria in non-ruminants (Biely *et al*., 1997; Sinha *et al*., 2011). Fermentable NDOs may modulate the intestinal microbiota, which results in a more beneficial microbial community that may ultimately enhance animal health and production performance (Adeola & Cowieson, 2011).

The most significant enzyme involved in the hydrolysis of AX is endo-(1,4)-β-xylanase. The enzymatic hydrolysis of AX results in the formation of AXOS which has shown potential as a prebiotic substance (Grootaert *et al*., 2007; Broekaert *et al*., 2011). Although some of the prebiotic functions of AX have been documented, the effects of AXOS are less studied (Grootaert *et al*., 2007). Both xylanase and AXOS have shown to possess positive growth and health functionalities as feed additives.

2.9. Xylanase as a NSP-degrading enzyme

2.9.1. Origin, types, function and action

Carbohydrase (or NSP-degrading enzymes) are enzymes that hydrolyse the glycosidic bonds between carbohydrate polysaccharides, thereby reducing their molecular weight by forming smaller oligosaccharide fractions (Adeola & Cowieson, 2011). Dietary carbohydrase supplemented in animal feeds is dominated by two enzymes namely, β-xylanase and β-glucanase (Castillo & Gatlin III, 2015), whereas β-xylanase (EC 3.2.1.8) is arguably the most prevalent NSP-degrading enzyme used in animal feeds (Adeola & Cowieson, 2011). Depending on their structure, molecular mass and substrate affinity, xylanases can be classified into the family of glycoside hydrolase (GH), whereas, family groups 10 and 11 are most prominent regarding AX degradation (Henrissat, 1991; Adeola & Cowieson, 2011). Most of the xylanase in the GH 10 family are endo-β-(1,4)-xylanases which have a greater substrate specificity especially for AX structures with a high degree of substitution, while the GH 11 xylanase family prefers unsubstituted AX fractions (Biely *et al*., 1997; Pollet *et al*., 2010; Paës *et al*., 2012). The endo-action does not generate free sugars during hydrolysis (Adeola & Cowieson, 2011), but rather produces NDOs available for microbial fermentation, while the exo-action does produce free sugars as hydrolysis product. The different types of xylanase may affiliate themselves either with the soluble NSP or the insoluble fraction of NSP or both, as has been reported by Choct *et al*. (2004). Xylanases (endo-xylanases) that hydrolyse soluble NSP reduces their viscosity, while xylanases that solubilise insoluble NSP results in soluble NSP fractions being released, which could lead to an increase in intestinal digesta viscosity (Choct *et al*., 2004).

Literature concerning the production of xylanase endogenously (produced by the microorganisms residing in the gut of the animal) is scanty (Ray *et al*., 2012). Fish are unable to produce xylanase by themselves, however, some yeast strains that are capable of producing xylanase may be found in some freshwater species (Gatesoupe, 2007). Other microorganisms known to produce xylanase include fungi (Belancic *et al*., 1995; Sunna &

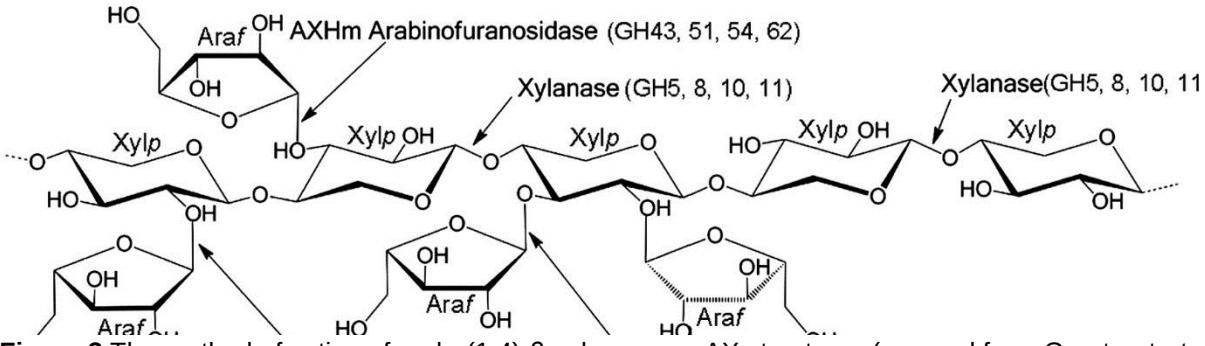

Figure 3 The method of action of endo-(1,4)-β-xylanase on AX structures (sourced from Grootaert *et al*., 2007)

Antranikian, 1997), algae, actinomycetes (Elegir *et al*., 1994) and bacteria (Kulkarni *et al*., 1999).

Furthermore, it is known that numerous fish species are able to synthesise other endogenous enzymes, such as α-amylase, chitinolytic activity, cellulase, lipase, small amounts of phytase and various protease enzymes (Fagbenro *et al*., 2000; Fernandez *et al*., 2001; Ellestad *et al*., 2003; Clements & Raubenheimer, 2006; German *et al*., 2010). These enzymes are however unable to hydrolyse the specific β-1,4-linkages of AX and, therefore, for fish to successfully utilise AX, most species are dependent upon the exogenous supplementation of dietary xylanase or xylanase-producing organisms in their diet.

The activity of β-xylosidase has been found in some catfish species (*Panaque nocturnus*, *Hypostomus pyrineasi*, *Panaque cf. nigrolineatus* and *Pterygoplichthy disjunctivus*) whose diets mainly consisted out of fibrous plant and detritus material (German & Bittong, 2009). The authors speculated that the activity of β-xylosidase originated from the microbes obtained in the diet of the fish rather than produced by resident microorganisms in the GIT of the fish. Moreover, xylanase-producing yeast strains were found in the GIT of a number of carp species and Nile tilapia. Due to the nature of the diet of these omnivorous and herbivorous fish species, it has been suggested that these yeast strains could have been ingested as part of their natural diet (Banerjee & Ghosh, 2014). This is possible since smaller amounts of xylanase are present in cereals (Dornez *et al*., 2009) and produced by microorganisms present on the surface of cereal grains (Dornez *et al*., 2006). Xylanase-producing microbes may thus be present in some herbivorous and omnivorous fish species.

Apart from the pivotal role that microorganisms play in the development of the gut and immunity, they also play an important role in the nutrient digestion of fish and, therefore, further research is needed regarding the ability of resident fish microbiota to produce NSP-degrading enzymes such as xylanase (Ray *et al*., 2012).

2.9.2. The significance of xylanase in non-ruminant feeds

Exogenous feed enzymes are one of the most widely studied areas in animal science whereas xylanase has been one of the most prominent NSP-degrading enzymes (Adeola & Cowieson, 2011). Supplementation of xylanase in high NSP-containing feedstuffs has been extensively studied in poultry (Bedford & Classen, 1992; Vahjen *et al*., 1998; Silversides & Bedford, 1999; Choct *et al*., 1999; Dänicke *et al*., 1999; Hubener *et al*., 2002; Courtin *et al*., 2008b; Cowieson *et al*., 2010; Nian, 2011; Aftab, 2012; Singh *et al*., 2012; Masey O'Neill *et al*., 2012; Zhang *et al*., 2014b; Masey-O'Neill *et al*., 2014a) and pig diets (Inborr *et al*., 1999; Yin *et al*., 2001; Kiarie *et al*., 2007; Vahjen *et al*., 2007; Yáñez *et al*., 2011; Laerke *et al*., 2015). Besides their use in animal feeds, xylanase has also been used in various different industrial applications such as in the paper and pulp (Buchert *et al*., 1994), food processing (Harris & Ramalingam,

2010) as well as in the baking (Courtin & Delcour, 2002) and brewing industries (Kulkarni *et al*., 1999).

However, despite their success shown in terrestrial animal studies, a paucity of information regarding the use of carbohydrase exists in fish nutrition (Ai *et al*., 2007; Adeola & Cowieson, 2011). In most fish species, the presence of carbohydrase enzyme capable of hydrolysing the complex β-glycosidic bonds of plant cell structures are very scarce or even non-existent (Krogdahl *et al*., 2005; NRC, 2011). Non-starch polysaccharides in plant materials can be found either as part of the plant cell structure or as part of the plant cell content. Non-starch polysaccharides in the structures of plant cell walls may entrap nutrients, shielding them from the endogenous enzymes of fish, whereas NSP present in plant cells may increase the digesta viscosity in the intestine and may, therefore, interfere with nutrient assimilation (Bach Knudsen, 1997). Therefore, xylanase supplementation in non-ruminant feeds provides the animal with the ability to digest previously indigestible portions of plant materials (Adeola & Cowieson, 2011) and by hydrolysing the complex NSP structures of plant ingredients, enzymes such as xylanase are able to increase the nutrient efficiency of plant-based diets (Castillo & Gatlin III, 2015).

Xylanase can enhance the nutrient efficiency in non-ruminant feedstuffs in two ways: Firstly, by solubilising the insoluble NSP in plant cell walls causing previously unavailable

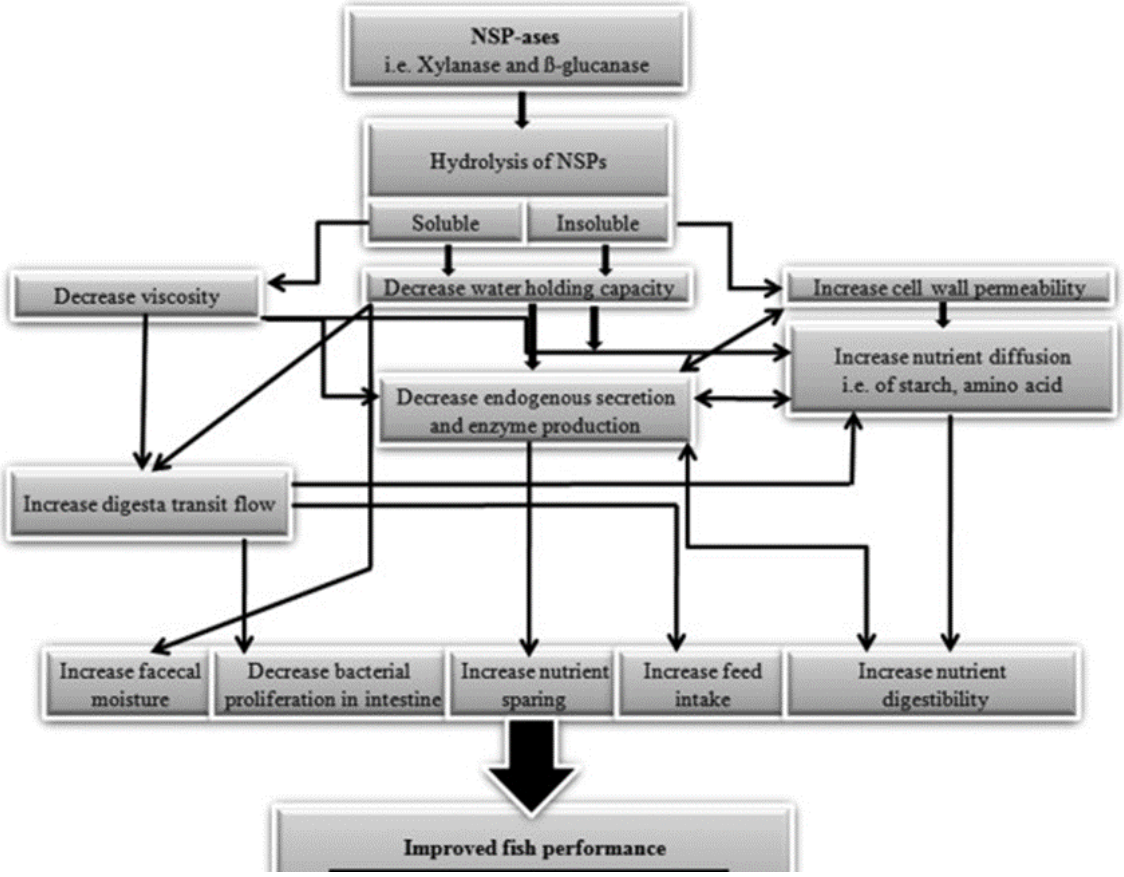

Figure 4 The impact of NSP'ases on the different components of NSP (sourced from Wyatt *et al*., 2008)

nutrients to be released. By disruption of the cell wall, endogenous digestive enzymes, such as pancreatic enzymes, can interact with nutrients previously unavailable for digestion (Bedford & Cowieson, 2012). Secondly, by hydrolysing soluble NSP in the content of plant cells; producing lower molecular weight oligosaccharides (Choct, 1997). Additionally, the hydrolysation of soluble NSP decreases the intestinal digesta viscosity and, resultingly, increases the interaction between nutrients and digestive fluids (Adeola & Bedford, 2004; Vahjen *et al*., 2007). Furthermore, dietary enzymes have also shown to stimulate the host's endogenous enzyme secretion; facilitating the digestion of nutrients (Van Weerd *et al*., 1999; Lin *et al*., 2007; Zhou *et al*., 2009). These activities of xylanase on the different fractions of NSP are shown in Figure 4.

Despite the nutrient-enhancing effect of xylanase in highly plant-based diets, the production of NDOs has shown to have significant effects on intestinal microbial composition (Adeola & Cowieson, 2011). Non-digestible oligosaccharides are indigestible to the host and therefore pass quickly through the GIT towards the distal part of the large intestine where it is fermented by carbohydrate fermenting bacteria (Bedford, 2000; Masey-O'Neill *et al*., 2014b). Resultingly, the gut passage rate of non-ruminants is increased which creates more space in the digestive tract for indigestible feed particles to move more quickly; not allowing potentially pathogenic bacteria to settle in the small intestine (Choct, 1997). The proliferation of bacteria in the upper small intestine may produce enzymes that degrade antimicrobials, such as bile acids, that may impair the lipid and protein digestibility of the animal (Taranto *et al*., 1997; Bedford, 2000). Besides limiting the occurrence of pathogens, NDOs also shifts bacteria from the proximate to the distal intestine, thereby reducing the host-microbe competition for nutrients in the small intestine where the absorption for nutrients is most suited (Adeola & Cowieson, 2011). High concentrations of pancreatic and digestive enzymes and large absorptive surface areas make the upper part of the small intestine the ideal place for nutrient assimilation (Uni *et al*., 1999). Less competition for nutrients may be to the benefit of the animal as there seems to be an inverse relationship between the numbers of bacteria residing in the small intestine and the growth of the animal (Apajalahti & Kettunen, 2006).

Dietary xylanase, through the production of NDOs, stimulates the growth of carbohydrate fermenting bacteria and decreases the occurrence of proteolytic bacteria in the more distal part of the intestine. Resultingly, the increase in carbohydrate fermenting bacteria produces more SCFAs as the products of carbohydrate fermentation (Hubener *et al*., 2002). These SCFAs have various reported health benefits including: reduces proteolytic fermenting bacteria and their harmful metabolites (Gibson, 2004; Kiarie *et al*., 2007), acts as immune enhancers (Meijer *et al*., 2010), improves gut morphology (Choct *et al*., 2004) and increases the solubility of minerals (Cloetens, 2009). The enhancement of fish health and growth through the increase in nutrient efficiency of highly plant-based diets, modulation of the intestinal microbiota, production of SCFAs and enhanced secretion of endogenous digestive fluids are all indicative of the hydrolysis action of NSP (Adeola & Cowieson, 2011). However, the application of xylanase in animal feeds is still a constraint due to the rigours normally associated with the production processes involved in the making of animal feedstuffs as well as the strenuous biological processes involved in the GIT of animals.

2.9.3. The application of xylanase in aquafeeds

The practicality of supplementing exogenous enzymes in aquafeeds is still a major concern (Castillo & Gatlin III, 2015). Most exogenous enzymes are susceptible to high thermal temperatures and pressures during the feed manufacturing process and may therefore be inactivated or degraded during certain processing applications, such as steam extrusion pelleting (Walsh *et al*., 1993). Studies have reported that exogenous enzymes should not be subjected to temperatures exceeding 80°C–95°C to avoid a considerable loss in enzyme activity (Inborr & Bedford, 1994; Spring *et al*., 1996; Samarasinghe *et al*., 2000; Amerah *et al*., 2011).

An approach to avoid the denaturing of enzymes during feed processing is to apply the enzyme after the extrusion pelleting stage, i.e. post-pelleting. Post-pelleting can be applied by spraying the enzyme on the feed pellets and may also be infused with an oil/lipid giving it a protective coating to aid the enzyme against harsh mechanical and biological processes (Adeola & Cowieson, 2011; Castillo & Gatlin III, 2015). An oil coating, however, may lead to an increment in the energy content of the diet and may alter the overall effect of the enzyme. It is believed that enzyme supplementations in aquafeeds should be carried out under less than optimal feeding regimes to ensure that the beneficial effects of the enzymes won't be hidden by a surplus amount of digestible energy in the diets (Cowieson & Ravindran, 2008; Zhou *et al*., 2009). Other means of applying exogenous enzymes to fish feed is to pre-process the plant ingredients prior to extrusion (Stone *et al*., 2003) or through cold feed processing, both of which avoid the heat treatment accompanying the extrusion of fish feed (Ai *et al*., 2007; Dalsgaard *et al*., 2012). These methods, however, do not guarantee the safety of the enzyme

and may not improve the digestibility of the feedstuffs to the same extent as with post-pelleting (Denstadli *et al*., 2011).

2.9.4. The effects of xylanase in aquaculture studies

Compared to other exogenous enzymes, such as phytase, carbohydrase enzymes have been studied to a much lesser extent in aquaculture studies, regardless of their success to enhance nutrient digestibility in the poultry and pig industries (Adeola & Cowieson, 2011). Nevertheless, xylanase has been used in a number of aquaculture studies although it mostly has been supplemented in an enzyme mixture containing other enzymes. Table 1 gives a summary of past research that involved the application of carbohydrase enzymes in mostly plant-based diets of various aquaculture fish species. It is, however, difficult to pinpoint the exact impact of xylanase in these studies due to the variety of species, enzyme mixtures, concentrations and different plant-based ingredients used. The majority of the studies involved the supplementation of enzymes in highly plan-based diets although some involved commercial (high FM content) diets. The following section will describe the findings of previous aquaculture reports relevant to the study objectives mentioned in Chapter 1. In the absence of, or when data on aquaculture species are limited, the author will refer to other relevant non-ruminant animals as to better understand the effects of xylanase.

I) The effect of the feed additive on the production performance parameters (growth, feed conversion, fillet proximate composition and visceral somatic index) of the animal.

The growth promoting effect of carbohydrase enzyme supplementation has been reported by a number of authors. However, only two studies have evaluated the effects of dietary xylanase in diets of African catfish, although both studies involved the supplementation of multi-enzyme complexes. Yildirim & Turan, (2010a) supplemented a multi-enzyme mix (Farmazyme®, containing fungal xylanase, β-glucanase, cellulase and other enzymes) to commercial trout diets (48% protein) fed to African catfish (initial mean weight 46.32 ± 0.29 SD) for a duration of 12-weeks. They found that the inclusion levels (0.25, 0.5 and 0.75 g/kg) of the enzyme complex had a significant effect on the growth performance, feed conversion ratio, protein efficiency ratio and apparent net protein utilisation of African catfish. The fish fed the 0.5 and 0.75 g/kg enzyme treatments had a significantly higher body protein content than the rest of the treatments. This may be attributed to the increased protein utilisation and efficiency caused by the enzyme treatment.

Similar effects were seen when Babalola (2006) investigated the effect of Moina, a micro diet, a micro diet supplemented with xylanase (Nutrex N.V., Belgium at 0.1 g/kg), and a combination of xylanase, micro diet and Moina on the growth and survival of African catfish larvae. Growth performance and survival rates were significantly increased in fish fed diets containing the treatment combination of xylanase, Moina and micro diet. This study, however,

only lasted for a period of 12 days and, as with the study mentioned earlier, also included commercial formulated feed. The only study that involved the sole inclusion of xylanase in fish, studied the effects thereof in diets of Jian carp. The addition of xylanase (220-2470 U/kg diet) significantly increased the growth rate, FCR, protein efficiency ratio and various nutrient and mineral production values with increasing levels of xylanase up to 1480 U/kg diet, and thereafter declined. Diets consisted out of SBM, rice gluten meal, cottonseed meal, rapeseed meal and wheat middling (Jiang *et al*., 2014).

Significant improvements in growth performance have also been reported for other fish species. In plant-based diets of tilapia, the supplementation of a multi-carbohydrase complex showed to increase the growth performance, feed conversion ratio (Goda *et al*., 2012), protein efficiency ratio and body protein content (Yildirim & Turan, 2010b), while stimulating the activity of endogenous digestive enzymes (Lin *et al*., 2007; Li *et al*., 2009; Hlophe-ginindza *et al*., 2016) and increasing the uptake and digestibility of minerals (Wallace *et al*., 2016) and nutrients (Tachibana *et al*., 2010; Maas *et al*., 2018). An increase in the growth performance was reported when a combination of enzymes (phytase, protease and xylanase) and probiotics (*Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus*) were added to diets of Nile tilapia (Adeoye *et al*., 2016b). Carter *et al*. (1994) and Jacobsen *et al*. (2018) studied the effects of a SBM-based diet supplemented with a multi-enzyme complex in Atlantic salmon. Carter *et al*. (1994) found that the enzyme mixture significantly improved the growth rate, feed intake and FCR of fish compared to the fishmeal and SBM treatment without enzyme addition. Jacobsen *et al*. (2018) reported that the addition of the enzyme complex in the dry feed mix, prior to extrusion, significantly increased the growth rate and feed intake compared to the control group.

Studies on rohu carp fed diets containing either gelatinised or non-gelatinised corn-based diets, involved the addition of α-amylase (50, 100 and 150 mg/kg). The treatment containing 50 mg/kg α-amylase significantly improved the growth and protein utilisation while increasing the dry matter digestibility in carp fingerlings. The same treatment also increased the activity of endogenous amylase and protease in non-gelatinised diets (Kumar *et al*., 2006c; a; b). Vajargah *et al*. (2018) studied the effect of a multi-enzyme complex (Kemin) in diets of common carp which were exposed to lethal concentrations of abamectin. The growth rate of fish that received the enzyme treatment was higher compared to the control, however, the opposite was true regarding the survival of the fish. The combined supplementation of xylanase (0.01%) and phytase (0.01%) in non-fermented de-oiled rice bran-based diets significantly increased the growth performance and body protein content of *Labeo rohita* (Ranjan *et al*., 2018). In another study that involved *L. rohita*, phytase and cellulase were administered to either water-soaked or un-soaked plant-based diets and significantly increased the apparent net protein utilisation (Xavier *et al*., 2012). Moreover, exogenous

cellulase (3 g/kg) enhanced the growth and intestinal enzyme activities (cellulase, amylase and protease) of grass carp fed a duckweed-based diet (Zhou *et al*., 2013).

Furthermore, Diler *et al*. (2012) reported that the addition of a multi-enzyme complex (containing xylanase) to a SBM-based diet of rainbow trout increased the protein and lipid digestibility of the plant-based diets, resulting in improved growth performance and feed utilisation. Similar results were observed in highly plant-based diets of Japanese sea bass (Ai *et al*., 2007) and white seabream (Magalhães *et al*., 2016). The addition of NSP'ase in diets of large yellow croaker, significantly improved the growth performance as well as the endogenous amylase activity in the stomach and intestine of the fish. However, no significant effect of the exogenous enzyme supplementation was seen on the body moisture, protein, lipid or gross energy values (Zhang *et al*., 2006). Similarly, in another study by the same author, Japanese sea bass significantly benefited in growth performance and endogenous amylase levels through the supplementation of a multi-enzyme complex although no differences existed in the body proximate analysis (Zhang *et al*., 2009). Although the studies of Ghomi *et al*. (2012) and Zamini *et al*. (2014) involved commercial, highly digestible diets they also observed positive growth and FCR effects due to multi-enzyme complex supplementation in great sturgeon and Caspian salmon, respectively.

Contrary to the above findings, the lack of significant growth performance in fish fed carbohydrase enzymes also exists. Carbohydrase supplementation had no significant effect on growth performance of rainbow trout fed dehulled lupin meal (Farhangi & Carter, 2007) and SBM-based diets (Ogunkoya *et al*., 2006). They did, however, see an increase in nutrient digestibility and reduced nutrient excretion, respectively. In a study that lasted only 17-19 days, Dalsgaard *et al*. (2012) found that xylanase addition to plant-based diets had no effect on the growth performance of juvenile rainbow trout, while β-glucanase and protease induced an increase in the apparent lipid digestibility of a SBM-based diet.

Similarly, no significant increase in the production performance of *Mugil liza* fed SBM-based diets supplemented with an enzyme complex was observed although the calcium bone retention of the enzyme treatments was significantly higher than the fish fed the control treatment (Ramos *et al*., 2017). Furthermore, the addition of an enzyme mixture (containing xylanase) had no significant effect on the growth performance or the FCR of gilthead sea bream fed a SBM-based diet (Ayhan *et al*., 2008) while tilapia fed canola meal-based diets supplemented with cellulase experienced no significant improvements in growth (Yigit & Olmez, 2011). The supplementation of an enzyme blend (Natugrain-blend®), that contained xylanase and β-glucanase, at three inclusion levels (75, 150 & 300 µL/kg) in wheat or dehulled lupin diets of silver perch had no effect on nutrient digestibility and growth of the fish fed the enzyme treatments (Stone *et al*., 2003). Exogenous phytase (300 mg/kg), protease (200 mg/kg) and carbohydrase (300 mg/kg) were supplemented to plant-based diets of Nile tilapia,

however, when compared to the control, only the phytase treatment significantly improved the SGR, FCR and protein efficiency ratio of the fish (Adeoye *et al*., 2016a). In the same study, no significant difference was seen between treatments regarding whole body composition.

Some adverse effects of exogenous enzyme supplementation have also been reported. In highly plant-based diets of common carp, the addition of a multi-enzyme complex (Endofeed W) resulted in decreased growth performance of the fish fed the enzyme treatments (Kazerani & Shahsavani, 2011). Denstadli *et al*. (2011) reported that the in vitro pre-treatment of various plant ingredients (SBM, rapeseed meal and sunflower cake) with a multi-enzyme mix (RONOZYME® VP) resulted in a significant reduction in the NSP content of the plant feedstuffs, however, when the same experiment was done on rainbow trout, no significant effect was seen on nutrient digestibility while the FCR was negatively influenced by the enzyme treatment. Moreover, the complete replacement of FM with SBM led to the retardation of growth and feed conversion despite the addition of carbohydrase and phytase enzymes in Nile tilapia (Mahmoud *et al*., 2014).

Soluble NSP may increase the digesta viscosity in the intestine and thereby slow down the rate of passage in the GIT of fish (Choct, 1997). This negatively affects the interaction between endogenous enzymes and their potential nutrient substrates (Ikegami *et al*., 1990). In order to increase nutrient assimilation, more digestive secretions are produced which may cause the digestive organs to enlarge (Ikegami *et al*., 1990). Leenhouwers *et al*. (2006) investigated the effects of high NSP-containing guar gum-based feedstuffs on African catfish. They reported that the NSP-containing feedstuffs stimulated the weights of digestive organs, such as the stomach and intestines. Positive effects related to the weight of intestinal organs were seen after the addition of a multi-enzyme complex to diets of hybrid tilapia. The multi-enzyme complex significantly deceased the viscera ratio, liver lipid and the hepatosomatic index with increasing levels of the enzyme complex (Lin *et al*., 2007). However, results regarding the effect of exogenous enzymes on the weight of digestive organs seems to be equivocal. Kumar *et al*. (2006c) tested the effects of α-amylase on rohu carp and found that the enzyme addition significantly increased the hepatosomatic index and liver glycogen. Similar findings were reported in carp where the addition of xylanase significantly increased the hepatosomatic index and hepatopancreas weight and protein (Jiang *et al*., 2014). On the other hand, no significant effects were found in the hepatosomatic index and intestinal somatic index of Nile tilapia (Adeoye *et al*., 2016b; a), white seabream (Magalhães *et al*., 2016) and *L. rohita* (Ranjan *et al*., 2018) fed plant-based diets supplemented with carbohydrase enzymes.

II) The effect of the feed additive on selected humoral non-specific immunity parameters, as an indication of any impacts on the health status of the animal.

Only a few studies exist that focused on the immunomodulatory properties of dietary carbohydrase supplementation in fish. Saputra *et al*. (2016) studied the effect of a xylanase-expressing probiotic in diets of Nile tilapia and found that the phagocytic activity, respiratory burst activity and the serum lysozyme activity significantly increased upon the addition of the probiotic. Another study on tilapia showed that the addition of a combination of probiotics had a significantly higher serum lysozyme activity compared to that of an enzyme mixture treatment (Adeoye *et al*., 2016b). The increase in the non-specific immune system and haematological indices was also seen in rohu carp fed diets containing α-amylase (Kumar *et al*., 2005, 2006c; b, 2009) and tilapia fed carbohydrase (Adeoye *et al*., 2016a) as well as in tilapia fed exogenous digestive enzymes (Goda *et al*., 2012), although these significant effects were not seen in Caspian salmon fed diets supplemented with a multi-enzyme complex (Zamini *et al*., 2014).

III) The effect of the feed additive on the hindgut microbial diversity of the animal.

It is known that the microbiota residing in the GIT play a pivotal role in the nutrient digestion and health status of fish (Nayak, 2010; Ray *et al*., 2012). However, the effect of dietary xylanase on the intestinal microbiota of fish remains largely unknown. Only one study evaluated the sole effect of xylanase on the hindgut microbial community of fish. Jiang *et al*. (2014) studied the effect of dietary xylanase on the intestinal microbiota of juvenile Jian carp. They found that xylanase inclusion in plant-based diets significantly increased the growth of lactobacilli, which is regarded as a beneficial bacterium (Aguirre & Collins, 1993), while significantly decreasing the numbers of *Escherichia coli* and *Aeromonas*, which are regarded as pathogenic bacteria in the intestines of fish (Merino *et al*., 1995; Del Rio-Rodriguez *et al*., 1997). The authors speculated that the intestinal microbiota may have been affected by the production of AX hydrolysis products caused by the addition of xylanase. Other studies also reported the modulating effect of carbohydrase enzyme mixtures on the intestinal microbiota of fish. In diets of Nile tilapia, the treatment containing a carbohydrate enzyme mixture significantly modulated the microbial community compared to the control treatment (Adeoye *et al*., 2016a). Similar effects were reported when the dietary addition of cellulase showed to significantly modulate the gut microflora of grass carp fed duckweed and wheat flour-based diets (Zhou *et al*., 2013). However, no significant effects were seen regarding the intestinal microbial diversity when a combination of enzymes (including xylanase) and probiotics was supplemented to commercial diets of Nile tilapia (Adeoye *et al*., 2016b).

IV) The effect of the feed additive on the hindgut short-chain fatty acid production of the animal.

Literature regarding the effect of dietary carbohydrase on SCFA production in fish warrants more study. Carbohydrase enzymes, such as xylanase, produces NDOs from dietary fibre that is fermented by specific facultative anaerobic bacteria in the distal part of the large intestine (Grootaert *et al*., 2007; Broekaert *et al*., 2011). Therefore, by increasing the available fermentable substrate, the amount of beneficial carbohydrate fermenting bacteria will increase. These bacteria produce organic acids, such as SCFAs, which have several benefits including the inhibition of gut pathogens (Gibson *et al*., 2004) and acts as an additional source of energy (Mcneil, 1984) for gut microbes and the host alike (Masey-O'Neill *et al*., 2014b). Short-chain fatty acids, mainly acetic acid, butyric acid and propionic acid directly stimulates the immune response (Akhter *et al*., 2015) and may subsequently promote the health of the host (Tremaroli & Backhed, 2012). Therefore, it seemed viable to test for such organic acids in the hindgut of fish as it would have given a good indication of the formation of oligosaccharides that are produced through the hydrolysis of dietary NSPs.

In broiler chickens, the addition of an enzyme (β-glycanase) in diets containing a high amount of NSP caused a decreased in the intestinal digesta viscosity as well as in the fermentation rate (SCFA production) in the ileum of the broilers. The fermentation was rather shifted to the caeca of the chickens. This decreased the competition between the host and microbes for nutrients in the small intestine. Resultingly, the supplementation of the enzyme caused an increased in the growth and feed utilisation performance of the chickens (Choct *et al*., 1996). In low-metabolisable energy wheat-based diets of broiler chickens, dietary xylanase addition significantly decreased the ileum fermentation rate while significantly increasing the rate of fermentation in the caeca of the chickens fed the enzyme-treated diets (Choct *et al*., 1999). Similarly, dietary xylanase significantly increased caeca SCFA concentrations of broiler chickens fed maize and wheat-based diets (Masey-O'Neill *et al*., 2014a). Contradictory, however, dietary xylanase supplemented in maize-soybean-based diets of broiler chickens, significantly decreased the SCFA production in the caeca of the chickens (Singh *et al*., 2012).

Table 1 Summary of carbohydrase enzyme studies on aquaculture species

Species	Carnivorous, omnivorous or herbivorous	Exogenous enzyme(s)	Enzyme administration	Feedstuffs	Effects & results	Reference(s)
Clarias gariepinus	Omnivorous	Farmazyme® multi-enzyme complex (containing fungal xylanase)	0.25, 0.5 & 0.75 g/kg for 12 weeks	Commercial trout diet	↑growth, FCR, PER & body protein content for all enzyme treatments (inclusion level dependency)	Yildirim & Turan, (2010a)
C. gariepinus	Omnivorous	Xylanase, Moina & microdiet	0.1 g/kg for 12 days	-	↑growth & survival (combination of xylanase, Moina & microdiet)	Babalola (2006)
Oreochromis aureus	Omnivorous	Farmazyme® multi-enzyme complex (containing fungal xylanase)	0.25, 0.5 & 0.75 g/kg for 90 days	Commercial carp diet	↑growth, FCR, PER, apparent net protein utilisation and body protein content for all enzyme treatments (inclusion level dependency)	Yildirim & Turan, (2010b)
O. mossambicus	Omnivorous	Natuzyme 50® multi-enzyme complex (containing xylanase, phytase & cellulase)	0.25, 0.5, 0.75 & 1.0 g/kg for 60 days	Kikuyu-based diet	↑growth & digestive enzyme activity for all enzyme treatments (0.5 g/kg enzyme treatment performed the best)	Hlophe-ginindza *et al.* (2016)

O. niloticus	Omnivorous	Phytase, protease & carbohydrase	0.3, 0.2 & 0.3 g/kg, respectively, for six weeks	Soybean protein, lupin meal, maize starch & FM	↑RBC, microvilli density & modulated microbial community (carbohydrase treatment); ↑growth (phytase treatment); →somatic indices (all treatments)	Adeoye *et al.* (2016a)
O. niloticus	Omnivorous	Xylanase-expressing *Bacillus amyloliquefaciens* R8	Administered for two months	FM, SBM, wheat middling & rice bran	↑growth, FCR, condition factor, survival, phagocytic activity, respiratory burst activity and serum lysozyme activity (*B. amyloliquefaciens* R8)	Saputra *et al*. (2016)
O. niloticus x O. mossambicus	Omnivorous	Ronozyme® (xylanase & phytase)	0.385 & 0.075 g/kg, respectively, for 80 days	SBM and maize meal	↑PER, phosphorous digestibility & trace mineral uptake (enzyme treatment justifies a 2% FM reduction)	Wallace *et al*. (2016)
O. niloticus	Omnivorous	Phytase & xylanase	Phytase (0 & 1000 FTU/kg), xylanase (0 & 4000 U/kg) for 38 days	SBM, wheat DDGS, RSM & sunflower meal	↑growth (combination of phytase & xylanase); ↑nutrient digestibility (phytase & xylanase separately); →body composition (xylanase)	Maas *et al*. (2018)
O. niloticus	Omnivorous	Combination of phytase, protease & xylanase with or without	0.75, 3.0 & 2.5 g/kg, respectively, for seven weeks	Commercial feed (35% protein & 5% lipid)	↑growth & intestinal morphology (combination of enzymes & probiotics); ↑serum lysozyme activity	Adeoye *et al.* (2016b)

		probiotics (*Bacillus subtilis, B. licheniformis* & *B. pumilus*)			(probiotic treatment); →intestinal microbial diversity (all treatments); →somatic indices (all treatments)	
O. niloticus	Omnivorous	Natugrain Blend L® (glucanase & xylanase)	0, 150, 300, 450 & 600 g/kg	Triticale	↑dry matter apparent digestibility & energy (300 g/kg enzyme treatment)	Tachibana *et al*. (2010)
O. niloticus x O. aureus	Omnivorous	NSP'ase, phytase & citric acid	1.0, 1.0 & 10.0 g/kg, respectively, for three weeks	SBM, wheat middling & FM	↑intestinal amylase activity (NSP'ase)	Li *et al*. (2009)
O. niloticus	Omnivorous	Cellulase	1-5 g/kg for 90 days	Canola meal	→growth & nutrient digestibility	Yigit & Olmez (2011)
O. niloticus	Omnivorous	Exogenous digestive enzymes (pepsin, papain & α-amylase)	Pepsin (0.64 & 1.28%), papain (1.28 & 2.56%), α-amylase (0.16 & 0.32%) for 119 days	Maize gluten & SBM	↑growth, FCR & haematological indices	Goda *et al*. (2012)
O. niloticus	Omnivorous	Pan Zyme multi-enzyme complex & Phytase-plus broiler 500	Administered for 83 days	SBM	↓feed intake & growth; →endogenous enzyme activity	Mahmoud *et al*. (2014)
O. niloticus x O. aureus	Omnivorous	Multi-enzyme complex (protease, glucanase & xylanase)	0, 1 & 1.5 g/kg for 12 weeks	SBM, CSM & RSM	↑growth, FCR, dry matter digestibility, apparent protein retention & endogenous digestive enzyme activity; ↓feed intake, liver lipid, viscera	Lin *et al*. (2007)

					ratio & HSI; →body composition (all treatments)	
Oncorhynchus mykiss	Carnivorous	Ronozyme® (xylanase, β-glucanase & protease)	0.208, 0.67 & 0.288 g/kg, respectively, for 17-19 days	De-hulled, solvent extracted SBM, sunflower meal & RSM	↑protein digestibility (xylanase & SBM); ↑lipid digestibility (xylanase & RSM); →growth (all treatments)	Dalsgaard *et al*. (2012)
O. mykiss	Carnivorous	Ronozyme® VP (β-glucanase, hemicellulase & pectinase)	2.1 mL/kg	SBM, RSM & sunflower cake	→nutrient digestibility; ↓FCR	Denstadli *et al*. (2011)
O. mykiss	Carnivorous	Superzyme CS multi-enzyme complex	0, 1.0 & 2.5 g/kg for 16 weeks	SBM	↓faeces cohesiveness & sinking speed; →growth & nutrient digestibility	Ogunkoya *et al*. (2006)
O. mykiss	Carnivorous	Multi-enzyme complex (protease, cellulase, xylanase, β-glucanase & phytase)	0.02%	SBM	↑growth & FCR (phytase & protease separately)	Diler *et al*. (2012)
O. mykiss	Carnivorous	Energex™ (hemicellulase), Bio-feed™ (protease) & Alpha	1800, 300 & 3000 PPM, respectively, for six weeks	De-hulled lupin-based diets	↑apparent digestibility of dry matter, crude protein & gross energy (Energex™); ↑PER (combination of	Farhangi & Carter (2007)

		galactosidase™ (α-galactosidase)			enzymes); →growth (all enzyme treatments)	
Cyprinus carpio	Omnivorous	Xylanase	220, 650, 1070, 1480, 1810 & 2470 U/kg for 10 weeks	FM, SBM, RSM, CSM & rice gluten meal	↑growth, FCR, PER, nutrient efficiency, HSI, intestinal enzyme activity & intestinal modulation of microbial communities (optimal enzyme concentration = 1259 U/kg)	Jiang *et al*. (2014)
C. carpio	Omnivorous	Kemin® multi-enzyme complex	0 & 1000 mg/kg for six weeks	-	↑growth	Vajargah *et al*. (2018)
C. carpio	Omnivorous	Endofeed® W multi-enzyme complex (xylanase, β-glucanase, celullase & hemicellulase)	0.25, 0.5, 1, 2, & 3 g/kg for 60 days	Commercial feed & cracked wheat	↓growth (inclusion level dependency)	Kazerani & Shahsavani (2011)
Labeo rohita	Omnivorous	Xylanase & phytase	0.01% for 60 days	Fermented & non-fermented de-oiled rice bran-based diet	↑weight gain, SGR, FCR & PER (non-fermented supplemented with enzymes); ↑body protein content; →somatic indices (all treatments)	Ranjan *et al*. (2018)
L. rohita	Omnivorous	Phytase & cellulase	Phytase (0 & 500 U/kg), cellulase (0, 0.2%) for 60 days	Soaked & un-soaked plant-based ingredients	Soaking reduced tannin content of feed; ↑protein utilisation, ash & tissue lipid content (phytase & cellulase); ↑body crude	Xavier *et al*. (2012)

					protein content & protease activity (cellulase)	
L. rohita	Omnivorous	α-amylase	0, 50, 100 & 150 mg/kg for 60 days	Gelatinised (G) & non-gelatinised (NG) maize flour	↑ non-specific immune response, growth, dry matter digestibility, HSI, liver glycogen, intestinal amylase & protease activity, blood glucose & PER (NG & 50 mg/kg enzyme treatment)	Kumar *et al.* (2005, 2006c; a; b, 2009)
Ctenopharyngodon idella	Herbivorous	Cellulase	3 g/kg for two months	Duckweed & wheat flour	↑growth, endogenous enzyme activities & modulation of gut microflora	Zhou *et al*. (2013)
Salmo salar	Carnivorous	Multi-enzyme complex (phytase, protease, xylanase & cellulase)	Administered for 93 days	De-hulled solvent extracted SBM	↑Iron apparent digestibility (enzyme coating post extrusion); ↑growth & feed intake (enzyme addition in dry mix prior to extrusion); ↓protein apparent digestibility (enzyme pre-processed SBM); →whole body analysis (all treatments)	Jacobsen *et al*. (2018)
S. salar	Carnivorous	Multi-enzyme complex (proteolytic	1.0% for 12 weeks	SBM	↑feed intake, final body weight, growth, FCR & maintenance ratio	Carter *et al*. (1994)

		enzymes & carbohydrases)				
Salmo trutta caspius	Carnivorous	Natuzyme® & Hemicell® multi-enzyme complexes	0, 0.25 & 0.5 g/kg for eight weeks	Commercial trout diet	↑survival, growth, WBC & FCR (0.5 g/kg Natuzyme® & 0.5 g/kg Hemicell®); →haematological indices	Zamini *et al*. (2014)
Bidyanus bidyanus	Omnivorous	Natugrain-blend® (β-glucanase & β-xylanase)	0, 75, 150 & 300 µL/kg for 26 days	Wheat & de-hulled lupin-based diet	→dry matter, energy & protein digestibility	Stone *et al*. (2003)
Sparus aurata	Omnivorous	Multi-enzyme mix (protease, cellulase, xylanase, β-glucanase & phytase)	2 g/kg for 12 weeks	FM & de-hulled SBM	→weight gain & SGR	Ayhan *et al*. (2008)
Siganus canaliculatus	Herbivorous	Cellulase, xylanase & β-glucanase	0.2% for eight weeks	Basal diet supplemented with or without 10% *Ulva pertusa*	Proteobacteria & Firmicutes = dominant bacterial groups; *Ruminococcus, Clostridium* & *Lachnospiraceae* = potential NSP-degrading bacteria	Zhang *et al*. (2018)
Diplodus sargus	Omnivorous	Natugrain® TS enzyme complex (carbohydrase)	0.04% for 10 weeks	SBM	↑FCR, PER, nutrient & energy efficiency, whole body protein & lipid content, lipase & amylase	Magalhães *et al*. (2016)

					activity; ↓nitrogen & energy faecal losses	
Lateolabrax japonicus	Carnivorous	Multi-enzyme complex (phytase, glucanase, pentosanase, cellulase & xylanase)	200 – 800 mg/kg for eight weeks	SBM, RSM & peanut meal	↑ growth, FCR (NSP'ase), phosphorous (phytase) & nitrogen retention (NSP'ase)	Ai *et al*. (2007)
Lateolabrax japonicus	Carnivorous	Phytase & NSP'ase	Phytase (200 mg/kg), NSP'ase (800 & 400 mg/kg)	FM and various other plant ingredients	↑ growth performance; ↑ endogenous amylase activity (NSP'ase treatment); → body proximate analysis	Zhang *et al*. (2009)
Huso huso	Carnivorous	Multi-enzyme complex	0, 250, 500, 750 & 1000 mg/kg for 46 days	Commercial diet	↑ weight gain, growth rate & FCR (250 mg/kg enzyme treatment)	Ghomi *et al*. (2012)
Pseudosciaena crocea	Carnivorous	Phytase & NSP'ase	-	-	↑ growth performance; ↑ endogenous amylase activity (NSP'ase treatment); → body proximate analysis	Zhang *et al*. (2006)
Mugil liza	Omnivorous	Multi-enzyme complex (NSP'ase)	0, 50, 100, 150 & 200 g/ton for 75 days	SBM	↑ Calcium bone retention & lipid deposition; → performance parameters	Ramos *et al*. 2017)

↑: statistically significant improvement, ↓: statistically significant deterioration, →: no statistically significant effect, CSM: cottonseed meal, DDGS: distiller's dried grains with solubles, HSI: hepatosomatic index, PER: protein efficiency ratio, RBC: red blood cells, RSM: rapeseed meal, WBC: white blood cells.

2.10. Arabinoxylan-oligosaccharides as a prebiotic substance

2.10.1. Structure, function and type

Arabinoxylan-oligosaccharides is produced through the hydrolysis of AX using AX-degrading enzymes and are regarded as NDOs that has shown to possess potential prebiotic properties (Van Laere *et al*., 2000; Geraylou *et al*., 2013a). These NDOs are low molecular weight glycosides that contain between two and ten sugar moieties (Zhang *et al*., 2003) and acts as intermediates between simple sugars and polysaccharides (Geraylou *et al*., 2014). Existing commercially produced oligosaccharides are produced from natural plant sugars such as sucrose (Hang & Woodams, 1996), lactose (Zarate & Lopez-Leiva, 1990), maltose (Hayashi *et al*., 1994) and starch (Monsan *et al*., 1989), and are structurally different from plant cell wall oligosaccharides, such as AXOS (Playne & Crittenden, 1996). Arabinoxylan-oligosaccharides has only recently been recognised as having potential prebiotic properties (Hughes *et al*., 2007; Vardakou *et al*., 2007; Grootaert *et al*., 2007), although it has been reported that these types of oligosaccharides are naturally being formed in the colon by NSP-fermenting bacteria (Van Laere *et al*., 2000; Broekaert *et al*., 2011). Despite its use as a prebiotic substance in animal feeds, AXOS has also been used in the food processing industry to help improve processes such as bread-making, dough syruping and beer brewing (Courtin & Delcour, 2002; Courtin *et al*., 2005).

Depending on the AX fragmentation process, different AXOS compounds with varying degrees of avDP (x) and avDS (y) are produced and can be denominated as AXOS-x-y (Geraylou *et al*., 2013a). The average degree of substitution (avDS) is the average ratio of arabinose to xylose moieties, whereas the average degree of polymerization (avDP) is the mean number of xylose residues in their backbone (Grootaert *et al*., 2007; Sanchez *et al*., 2009; Broekaert *et al*., 2011). This distinguishes AXOS from conventional prebiotics that only differ in their degree of polymerization (Grootaert *et al*., 2007). It has been reported that the biodegradability of AXOS is indirectly proportional to the degree of substitution and polymerization (Hughes & Rowland, 2001; Amrein *et al*., 2003; Swennen *et al*., 2006; Sanchez *et al*., 2009; Ringø *et al*., 2010; Hoseinifar *et al*., 2015), and are fermented in different parts of the GIT depending on their chemical structure (Sanchez *et al*., 2009). Conversely, Sanchez *et al*. (2009) found that AXOS with a higher avDP allowed more oligosaccharides to reach the distal colon than lower avDP AXOS, the latter being more completely fermented in the proximal colon. The author also reported that AXOS supplementation can mediate the protein-rich environment of the distal colon to a more saccharolytic (carbohydrate-fermenting) environment, decreasing the production of harmful protein fermentation metabolites such as phenol and p-cresol.

Potential prebiotics need to pass a certain criterion in order for them to be classified as prebiotic substances. Such a criterion has been established by Gibson *et al*. (2004) and consists out of the following three requirements: 1) potential prebiotics must be resistant to gastric acidity, to gastrointestinal absorption and to the hydrolysis by mammalian enzymes; 2) it must be fermentable by gut microbes; and 3) it must selectively stimulate the growth and/or activity of health-promoting bacteria. According to Broekaert *et al*. (2011) and Cloetens (2009), AXOS has fulfilled the above-mentioned requirements and may, therefore, be classified as a prebiotic substance.

2.10.2. The significance of arabinoxylan-oligosaccharides in animal feeds

Arabinoxylan-oligosaccharides has shown to possess certain prebiotic properties (Kabel *et al*., 2002) and may directly interact with the gut-associated lymphoid tissue (GALT). However, perhaps the most significant effect of AXOS is to stimulate health-promoting bacteria while limiting the growth of deleterious microbiota. A beneficial microbial community may also exert immunostimulatory effects and may, therefore, assist in promoting the health of the host (Sang *et al*., 2011; Zhang *et al*., 2011). Together, these two properties of AXOS may have a significant effect on the growth and health of the host. The microbial modulating effect of AXOS has been studied in humans (Gråsten *et al*., 2003; Cloetens, 2009; Sanchez *et al*., 2009; Grootaert *et al*., 2009; Cloetens *et al*., 2010; Broekaert *et al*., 2011), rats (Van Craeyveld *et al*., 2008; Damen *et al*., 2011), mice (Neyrinck *et al*., 2012), pigs (Niewold *et al*., 2012), broilers (Courtin *et al*., 2008b; a; Eeckhaut *et al*., 2008) and fish (Delaedt *et al*., 2008; Rurangwa *et al*., 2008; Geraylou *et al*., 2012, 2013a; b). These studies reported that AXOS may stimulate a select few health-promoting bacteria while reducing potentially pathogenic bacteria in the lower part of the large intestine (Courtin *et al*., 2008a). The microbial species mostly involved in the hydrolysis of dietary fibre include groups from the genera of *Bacteroides*, *Bifidobacterium*, *Ruminococcus*, *Eubacterium*, *Lactobacillus* and *Clostridium* (Van Laere *et al*., 2000). However, AXOS can only be utilised by a certain number of bacteria, such as *Bifidobacterium*, *Lactobacillus* and *Bacteroides* species, making it a relatively specific fermentable substrate (Van Laere *et al*., 2000; Moura *et al*., 2007).

Arabinoxylan-oligosaccharides has especially strong bifidogenic properties in animals and humans (Courtin *et al*., 2008a; Broekaert *et al*., 2011) which might contribute to the fact that, in previous studies, AXOS showed to have a more potent ability to modulate the intestinal microbiota in animals compared to more conventional prebiotics such as inulin and fructooligosaccharides (Courtin *et al*., 2008a; Van Craeyveld *et al*., 2008). Conversely, the supplementation of AXOS increased the levels of bifidobacteria in the caeca as well as improved the FCR of broilers chickens fed either maize or wheat-based diets (Courtin *et al*., 2008a). Although the modulation of the intestinal microbiota in itself cannot be seen as a health

benefitting effect (Broekaert *et al*., 2011), AXOS may also possess immunological properties (Mendis *et al*., 2016). These properties include, increasing the solubility of minerals, antitumor activities, facilitation of the intestinal integrity, production of SCFAs, reducing proteolytic metabolites, stimulating lipid and glucose metabolism, preventing metabolic disorders, enhancing the immune system and exhibiting antioxidant effects (Teitelbaum & Walker, 2002; Gråsten *et al*., 2003; Macfarlane *et al*., 2006; Wong *et al*., 2006; Ou *et al*., 2007; Eeckhaut *et al*., 2008; Van Craeyveld *et al*., 2008; Cloetens, 2009).

2.10.3. The effect of arabinoxylan-oligosaccharides in aquafeeds

Literature regarding the effects of AXOS in aquaculture studies is putative compared to other animals and humans (Geraylou *et al*., 2013b). To the author's knowledge, AXOS has only been studied on two aquaculture species namely, African catfish and Siberian sturgeon. Table 2 contains the most relevant information reviewed by the author regarding prebiotic supplementation in studies of catfish species. The following section will discuss the findings of these studies according to the study objectives mentioned in Chapter 1.

I) The effect of the feed additive on the production performance parameters (growth and feed conversion efficiency) of the animal.

In previous aquaculture studies, AXOS did not have a prominent effect on the growth performance of fish. Geraylou *et al*. (2012) studied the effects of two different structures of AXOS, viz. AXOS-32-0.30 with an avDP of 32 and avDS of 0.30 and AXOS-3-0.25 with an avDP of 3.0 and avDS of 0.25, on the growth performance of juvenile Siberian sturgeon. Both AXOS structures were administered to the diets at 2% inclusion level and were fed for 12 weeks. The growth performance and feed utilisation were statistically similar for all treatments. The addition of AXOS-32-0.30 tended to slightly improve the growth and feed conversion ratio of the fish although only numerically. In a similar study by the same author, two separate feeding trials were used to evaluate the effect of AXOS on Siberian sturgeon. In the first trial, AXOS-32-0.30 was administered at both 2% and 4% to diets of juvenile Siberian sturgeon while in the second trial 2% of AXOS-32-0.30 and AXOS-3-0.25 were administered seperately. Similar non-significant results were seen where 2% of AXOS-32-0.30 numerically improved the growth performance of the fish in both trials (Geraylou *et al*., 2013a).

Moreover, Rurangwa *et al*. (2008) studied the effect of AXOS inclusion in diets of Siberian sturgeon and African catfish. Dietary AXOS, administered at 10 and 20 g/kg for a duration of 10 weeks, resulted in no significant difference in growth performance parameters between the treatments. However, contrary to the above-mentioned studies, Geraylou *et al*. (2013b) investigated the separate and combined supplementation of 2% AXOS-32-0.30 and endogenous probiotics (*Lactococcus lactis spp. lactis* and *Bacillus circulans*) on the growth performance of Siberian sturgeon. The authors reported significant improvements in the

growth and FCR of fish fed the treatment containing 2% AXOS and *L. lactis* ST G45, compared to the other treatments.

II) The effect of the feed additive on selected humoral non-specific immunity parameters, as an indication of any impacts on the health status of the animal.

The immune-enhancing effect of AXOS in aquaculture studies has been reported by some authors, although, when compared to other contemporary prebiotics, literature concerning AXOS is severely limited. Geraylou *et al*. (2012) investigated the reputed immunomodulatory properties of AXOS (AXOS-32-0.30 and AXOS-3-0.25) on Siberian sturgeon and found that neither AXOS structures had any significant effect on the serum lysozyme activity of Siberian sturgeon. However, both AXOS preparations significantly enhanced the phagocytic activity (PA) of fish macrophages compared to the control treatment. Furthermore, the AXOS-32-0.30 treatment proved to significantly increase the alternative haemolytic complement activity (ACH_{50}) and total serum peroxidase activity (PO) of the fish. In another study, AXOS-32-0.30 supplemented at 2% were added separately and in combination with endogenous probiotics (*L. lactis spp. lactis* and *B. circulans*) to diets of Siberian sturgeon. The results indicated that the combined effect of AXOS and *L. lactis spp. lactis* G45 had a significant effect on the ACH_{50} of the fish. Moreover, the PA and PO activity were significantly stimulated by the separate inclusion of AXOS and probiotic treatments while the respiratory burst activity (RBA) of the macrophages was also significantly enhanced through AXOS treatment. However, no significant difference was found for the immunoglobulin levels between dietary treatments (Geraylou *et al*., 2013b).

III) The effect of the feed additive on the intestinal microbial diversity of the animal.

The ability of AXOS to modulate the microbial community of aquaculture species has been previously reported. The structure-function relationship of AXOS seems to be the biggest factor influencing the diversity of hindgut microbial populations of fish. The microbial modulating effect of two AXOS structures (AXOS-32-0.30 & AXOS-3-0.25) were studied on the hindgut microbiota of juvenile Siberian sturgeon. The microbial community of the fish fed different dietary treatments were analysed by means of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and it was shown that both AXOS structures significantly influenced the bacterial community of the fish by stimulating the growth of lactic acid bacteria (LAB) and *Clostridium sp*. It was also proven, through the means of a redundancy analysis, that AXOS-32-0.30 had a more pronounced effect than AXOS-3-0.25 on the hindgut microbial community (Geraylou *et al*., 2012). The microbial modulating effect of AXOS was again proven in another study involving the hindgut microbiota of Siberian sturgeon (Delaedt *et al*., 2008).

It has been reported that dietary AXOS is fermented by carbohydrate fermenting bacteria in the hindgut of non-ruminant animals and, therefore, stimulates the growth of lactic acid bacteria. Geraylou *et al*. (2013b) reported that dietary AXOS stimulated the growth and colonisation of *Lactococcus lactis spp. lactis* when supplemented together with *L. lactis spp. lactis* in diets of juvenile Siberian sturgeon. In the same study, AXOS, supplemented at 2% inclusion level, together with *L. lactis* ST G45 significantly decreased the hindgut microbial diversity of fish by means of a significantly decreased Shannon diversity index and a specific species richness index. Additionally, the combination of AXOS and *Bacillus circulans* ST M53 caused a significant decrease in the microbial species richness of juvenile Siberian sturgeon. The authors suggested that the reduced microbial diversity could have been due to the strong antimicrobial properties of these two probiotics, although similar decreases in bacterial diversity have been seen for Atlantic salmon fed a supplementation of inulin (Bakke-McKellep *et al*., 2007) and Arctic char fed dietary carbohydrates (Ringø & Olsen, 1999).

The supplementation of dietary AXOS has shown to affect the microbial community without significantly affecting the hindgut species diversity of Siberian sturgeon. The supplementation of 2% AXOS-32-0.30 induced a significant effect on the relative abundance of hindgut microbiota, although it did not significantly affect the bacterial diversity. However, the same AXOS treatment did enhance the growth of *Eubacteriaceae*, *Clostridiaceae*, *Streptococcaceae*, *Lactobacillaceae*, *Lactobacillus spp*. and *Lactococcus lactis*. In the same study, the addition of 2% AXOS-3-0.25 increased the bacterial diversity and species richness of the hindgut microbiota of Siberian sturgeon. This is in contrast to some of the above-mentioned studies that reported the significant decrease of species richness accompanying the addition of dietary AXOS (Geraylou *et al*., 2013a). Results from an in vitro study on Siberian sturgeon and African catfish hindgut microbiota showed that AXOS, oligofructose, xylose and fructose had a significant impact on the microbial fermentation activity of both species in a substrate and species dependent manner (Geraylou *et al*., 2014). However, in a different in vitro study, dietary AXOS had the lowest effect on the growth of bacteria (*B. subtilis*, *Carnobacterium piscicola*, *L. plantarum* and *L. delbrueckii*) compared to other prebiotics and monosaccharides, such as inulin, xylo-oligosaccharides and β-glucan (Rurangwa *et al*., 2009).

IV) The effect of the feed additive on the hindgut short-chain fatty acid production of the animal.

Studies have shown that dietary AXOS supplementation in fish feeds are capable of significantly influencing hindgut SCFA production in Siberian sturgeon and African catfish. In diets of Siberian sturgeon, the addition of an AXOS preparation (high avDP and avDS) significantly increased the production of acetate, butyrate and total SCFAs compared to an AXOS structure with a lower avDP and avDS and a control treatment (Geraylou *et al*., 2012, 2013a). In diets of Siberian sturgeon and African catfish, AXOS supplementation significantly enhanced the production of acetate, propionate and total SCFAs compared to a control treatment. However, in the same study, the levels of butyrate were unaffected by AXOS supplementation (Rurangwa *et al*., 2008). An in vitro study involving the fermentation rate of AXOS, oligofructose and their monomeric sugars (xylose and fructose, respectively) by hindgut bacteria derived from Siberian sturgeon and African catfish, found that the incubation of xylose and oligofructose resulted in a higher production of SCFAs compared to the incubation of AXOS and fructose. The same study revealed that the hindgut microbiota of Siberian sturgeon had a higher fermentation capacity compared to those of African catfish (Geraylou *et al*., 2014).

Table 2 Summary of prebiotic studies on catfish species

Species	AXOS structure	AXOS administration	Effects and results	Reference(s)
Acipenser baerii	AXOS-3-0.25 & AXOS-32-0.30	2% for 12 weeks	↑ACH_{50}, PO, LAB & *Clostridium sp*. (AXOS-32-0.30); ↑PA (both AXOS treatments); ↑acetate, butyrate & total SCFA production (AXOS-32-0.30); →weight gain, SGR, FCR & lysozyme activity (all AXOS treatments)	Geraylou *et al.* (2012)
A. baerii & C. gariepinus	-	10 & 20 g/kg for 10 weeks	↑acetate, propionate & total SCFA production; →growth & butyrate	Rurangwa *et al.* (2008)
A. baerii	-	10 g/kg for 18 weeks	Modulation of hindgut microbiota (AXOS treatment's intestinal microbiota more diverse than control treatment's)	Delaedt *et al.* (2008)
A. baerii	*L. lactis spp. lactis, B. circulans* & AXOS-32-0.30 (separately and in combination)	2% for 4 weeks	↑growth & FCR (*L. lactis* + AXOS); ↑PA, RBA, PO & ACH_{50} (separate action of probiotics & AXOS); ↑RBA (AXOS); ↑ACH_{50} (combination of AXOS & probiotics); →total immunoglobulin (all treatments); ↓bacterial diversity (*L. lactis* + AXOS)	Geraylou *et al.* (2013b)
In vitro tested bacterial growth on dietary AXOS	AXOS (avDP = 30)	-	Acetate dominated the amount of SCFAs produced; Compared to other prebiotics (e.g. inulin, XOS & β-glucan); AXOS had lowest effect on growth of bacteria tested in vitro	Rurangwa *et al.* (2009)
A. baerii	AXOS-32-0.30 +	2 & 4% for 10 weeks	↑acetate, butyrate and total SCFA production (2% AXOS-32-0.30); ↑hindgut bacterial diversity (2% AXOS-3-0.25); ↓survival (4% AXOS-32-0.30); →growth performance (all	Geraylou *et al.* (2013a)

	AXOS-32-0.30 & AXOS-3-0.25	2% for 12 weeks	AXOS treatments); →hindgut bacterial diversity (2 & 4% AXOS-32-0.30) AXOS had a significant effect on the abundance & clustering of microbiota	
In vitro tested fermentation rate by hindgut bacteria derived from *A. baerii* and *C. gariepinus*	AXOS, OF, xylose & fructose	1.0% incubated for 48 hours	Acetic acid dominated the SCFAs produced Hindgut microbiota from Siberian sturgeon had higher fermentation capacity that those from African catfish Microbiota of Siberian sturgeon fermented xylose quicker than AXOS while microbiota from African catfish fermented OF quicker than fructose AXOS and its monomer xylose had an impact on microbial fermentation of hindgut bacteria in both species	Geraylou *et al.* (2014)
Ictalurus punctatus	Bio-MOS® (sink & float)	2 g/kg for 6 weeks + 3 weeks *E. ictaluri* challenge	↑resistance against *E. ictaluri* (MOS-sink); →weight gain, SGR & FCR (all treatments)	Peterson *et al.* (2010)
I. punctatus	Bio-MOS® Aqua Grade	2 g/kg for 4 weeks	→growth performance, haematology, immune response, resistance against *E. ictaluri*; ↓plasma cortisol	Welker *et al.* (2007)
Rhamdia quelen	Bio-MOS®	2 g/kg for 60 days	↑survival; →SGR	Hernández *et al.* (2012)

↑: statistically significant improvement, ↓: statistically significant deterioration, →: no statistically significant effect, ACH_50: alternative haemolytic complement activity, LAB: lactic acid bacteria, OF: oligofructose, PA: phagocytic activity, PO: total serum peroxidase activity, RBA: respiratory burst activity, XOS: xylo-oligosaccharide.

2.11. African catfish, *Clarias gariepinus*, as an experimental species

The African 'sharptooth' catfish is a hardy and robust species known for its tolerance of severe environmental conditions, high-density culture and fast growth rate (Quick & Bruton, 1984; Huisman *et al*., 1987; Haylor, 1993; Ali & Jauncey, 2005). Its high fecundity (Bruton, 1979) and airbreathing capabilities (Moussa, 1957) contribute to its wide distribution within Africa and even some parts of Eastern Europe (Teugels, 1986). Conversely, the African catfish is the freshwater species with the widest latitudinal range in the world (Bruton, 1988). *Clarias gariepinus* can utilise atmospheric oxygen through the aid of their suprabranchial organs. Their ability to utilise atmospheric oxygen, and to withstand poor water quality, may explain why these fish can be stocked at very high densities per unit of water volume (Huisman *et al*., 1987). The intensive farming of African catfish has long been proposed by several authors (De Kimpe & Micha, 1974; Richter, 1976; Hogendoorn, 1979). In South Africa and in the Netherlands, the commercialisation of this species were realised by pioneers in the field and involved extensive studies on propagation (Hogendoorn & Vismans, 1980; Hecht, 1982; Van Oordt & Goos, 1987), intensive rearing (Hecht & Appelbaum, 1987; Hogendoorn, 1980, 1981), optimal dietary requirements (Uys & Hecht, 1985; Appelbaum & Van Damme, 1988), as well as biological and environmental requirements (Britz & Hecht, 1987).

Table 3 Nutritional requirements (protein, lipid & energy) of African catfish

Reference(s)	Protein (%)	Lipid (%)	Energy (kJ/g)	Protein-to-energy ratio (mg/kJ) GE
Uys (1989)	40-42	10-12	14-16 (DE)	26-29
Lovell (1998)[a]	27 (DP)	-	13 (DE)	21
Machiels & Henken (1985)	>40	-	13 (DE)	31
Ali & Jauncey (2005)	43	8	21.2 (GE)	20.5
Henken *et al.* (1986)	-	-	-	25-35
Van Weerd (1995)	40	-	13-17	-
Degani *et al.* (1989)	40	-	11-13 (GE)	31-36
Giri *et al.* (2003)	35-40	-	-	-
Ahmad (2008)	30	12	-	-
Hecht *et al.* (1988)	38-42	10-11	12	-

[a]Nutrient values based on channel catfish. DE: digestible energy, DP: digestible protein, GE: gross energy.

The nutritional requirements of African catfish are not yet fully established (Pantazis, 1999). However, due to the similarity between African catfish and channel catfish, the nutrient requirements of these two species are comparable (Goda *et al*., 2007; Ayoola, 2016). The crude protein, lipid and energy requirements of African catfish are provided in Table 3. The building blocks of protein are defined as amino acids and can be distinguished into two groups namely, essential and non-essential amino acids. Most animals can produce non-essential amino acids *de novo*, however, essential amino acids can only be obtained from their diet. Together, essential and non-essential amino acids comprise the quality and composition of all protein materials. Table 4 contains the essential amino acid requirements of channel catfish, as reported by previous studies. The values of Table 3 and 4 are mainly based on and/or supplemented with data based on the nutritional requirements of channel catfish due to the lack of information regarding *C. gariepinus*.

Table 4 Essential amino acid requirements of channel catfish (Lovell 1991; NRC 1993)

Essential amino acid	Requirement (% as-fed basis)	Requirement (% dry diet)
Histidine	0.48	0.40
Arginine	1.38	1.00
Threonine	0.64	0.50
Lysine	1.63	1.20 – 2.30[c]
Methionine	0.74[a]	0.60 – 1.30[c]
Valine	0.96	0.71
Isoleucine	0.83	0.60
Leucine	1.12	0.80
Phenylalanine	1.60[b]	1.20
Tryptophan	0.16	0.12 – 0.43[c]

[a]Methionine + cystine. [b]Phenylalanine + tyrosine. [c]Based on African catfish data (Fagbenro *et al*., 1998, 1999; Fagbenro & Nwanna, 1999).

Warm-water omnivorous fish species are known to have a high tendency to utilise carbohydrates (Wilson, 1994; Lovell, 1998). Conversely, studies on the digestive enzymes of *C. gariepinus* revealed proteolytic enzyme activities similar to those of carnivorous fish species and carbohydrase enzyme activities corresponding to those of specialised herbivorous fish species (Uys & Hecht, 1987; Uys, 1989). The natural diet of African catfish often includes a variety of plant-based products and may, therefore, support the fact that they can utilise plant ingredients relatively well (Clay, 1981). High lysozyme levels have been reported in *C. gariepinus*, suggesting it plays a role in the omnivorous nature of this opportunistic predator

(Uys, 1989). Compared to other warm-water fish species, such as eels (Degani *et al*., 1985) or tilapia (Degani *et al*., 1982), African catfish exhibited a higher growth rate when fed a low animal-protein diet (Degani *et al*., 1989).

The inherent ability of omnivorous fish species, such as African catfish, to better equip themselves with endogenous digestive enzymes and metabolites, facilitating the digestibility of energy-rich plant materials (Stone *et al*., 2003; Sá *et al*., 2007), might explain why these fish species seem to have more tolerance against the anti-nutritive factors present in some plant ingredients compared to carnivorous species (NRC, 2012). Due to their omnivorous nature, the African catfish seem to be a good candidate regarding fishmeal replacement studies. Table 5 contains some important studies that involve the replacement of fishmeal with alternative plant ingredients in diets of catfish species. Some of these studies have shown that African catfish are capable of utilising FM-free diets supplemented with SBM and poultry by-product meal without any negative growth effects (Goda *et al*., 2007). Other studies reported that soybean flour and SBM can replace FM up to 50% in diets of African catfish without compromising the growth of the species (Fagbenro & Davies, 2001; Enyidi, 2012).

Table 5 Important aquaculture studies regarding the replacement of fishmeal in diets of catfish species

Ingredient(s)	Effects and results	Reference(s)
PBM, MBM & SBM	PBM (75 & 100%), SBM (75 & 100%) & MBM (75%) replacement had no significant effects on FBW & SGR compared to control (FM 25%). SBM (100%) had significantly lower whole-body moisture although higher fat & energy contents compared to control.	Goda et al. (2007)
De-hulled, solvent-extracted soybean flour	Soybean flour can replace 50% of FM and, if supplemented with methionine, up to 75%.	Fagbenro & Davies (2001)
Raw & Cooked SBM	Cooked SBM performed significantly better than raw SBM in all aspects. No significant differences were found between control and 100% cooked SBM treatments.	Balogun & Ologhobo (1989)
SBM & phytase	Treated (oven-dried, cooked & toasted) SBM with phytase had significantly higher growth rates than untreated SBM with phytase. Treated SBM with phytase had significantly less phosphorous discharge than the other treatments.	Nwanna et al. (2005)

SBM & CSM	600 g/kg SBM & CSM treatments significantly decreased growth performance. SBM & CSM induced significant decreases in body mineral composition mainly due to phytic acid concentrations. SBM & CSM can replace FM up to 300 g/kg without negatively affecting growth performance.	Toko et al. (2008)
Maize meal & SBM	Maize meal induced significantly poor growth performance while SBM can replace FM up to 50% or 300 g/kg without any adverse effects on growth performance.	Enyidi (2012)
SBM, tomato waste & brewer's yeast	Soybean products induced a significant decrease in growth possibly due to ANFs present. Tomato waste had a better effect on growth but may have been due to higher amount of FM present in the diet.	Hoffman et al. (1997)
Cereal grains differing in viscosity (maize, wheat, barley & rye)	Proximal intestine digesta viscosity differed significantly between treatments and increased with higher dietary viscosity. The highest dietary viscosity diet (400 g/kg rye) had the lowest ($P<0.05$) protein and fat digestibility and plasma cholesterol levels. The wheat-based diet (400 g/kg) had the lowest NSP digestibility, while the maize-based diet had the highest ($P<0.05$) digestibility, indicating that C. gariepinus could better utilise maize NSP than NSP from wheat and rye.	Leenhouwers et al. (2007b)
Guar gum (soluble NSP)	Guar gum supplementation significantly increased digesta viscosity in the proximal & distal intestine. ADC & somatic stomach index differed significantly between control & highest guar gum inclusion treatments. Intestinal somatic index directly increased with increasing levels of guar gum supplementation.	Leenhouwers et al. (2006)
Various oilseed cakes/meals	Soybean cake had the highest protein digestibility by African catfish with an ADC-protein of 86.9% and an ADC-energy of 77.4%.	Fagbenro (1998)

Soy protein concentrate	Soy protein concentrate can replace 75% of FM and, with supplemental methionine, up to 100%.	Fagbenro & Davies (2004)
Phytase-treated SBM	SBM induced a significant reduction in growth rate & feed utilisation. Phytase improved phosphorous digestibility & retention, concurrently reducing phosphorous leaching.	Van Weerd et al. (1999)
ADC of plant and animal feedstuffs	ADC-dry matter values of plant feedstuffs were significantly lower than those of animal origin, while the ADC-protein values were statistically similar between plant and animal feedstuffs. SBM & yellow maize meal had the highest digestibility by C. isheriensis species. FM, PBM & SBM had the highest ADC-energy values. Protein & energy were equally digestible between plant & animal ingredients.	Fagbenro (1996)
PBM	PBM may replace up to 40% of FM in diets of African catfish.	Abdel-Warith et al. (2001)

ADC: apparent digestibility coefficients, CSM: cottonseed meal, FBW: final body weight, MBM: meat and bone meal, PBM: poultry by-product meal, SBM: soybean meal, SGR: specific growth rate.

2.12. Conclusion

Fishmeal and fish oil are becoming scarcer and more expensive due to the stagnant production growth of capture-based fisheries and the increasing demand for FM and FO resources. Furthermore, it can be argued that the use of FM and FO in industrially compounded aquafeeds are unsustainable especially for carnivorous marine finfish species based on the global average fish-in fish-out ratios which are generally greater than one for these high-value aquaculture species. Therefore, more affordable and available alternatives, such as plant-protein ingredients, are being readily incorporated into diets of fish. However, despite the presence of ANFs, such as NSP, most fish species do not possess the endogenous enzymes to optimally utilise plant ingredients.

The indigestible portion of aquafeeds, viz. NDOs, are regarded as functional feed additives and may exert positive effects on the growth and health of aquatic animals. Non-digestible oligosaccharides or immunosaccharies possess beneficial, immune stimulating properties and may enhance the innate immune response of fish in two ways: Firstly, immunosaccharides have shown to enhance the innate immunity through directly stimulating pattern recognition receptors and interacting with carbohydrate receptors on leucocytes in the GALT of fish. Secondly, immunosaccharides indirectly modulate the intestinal microbial community by stimulating the growth of specific, beneficial bacteria, such as lactic acid

bacteria, while also inhibiting the growth of harmful, pathogenic microbiota in the intestines of fish. Accompanying the enhanced growth of LAB is an increase in the production of beneficial metabolites, such as SCFAs, while harmful proteolytic metabolites are inhibited. Short-chain fatty acids are beneficial in that they may act as additional energy sources as well as having health-benefitting properties. Non-digestible oligosaccharides may be supplemented to fish feeds directly (via prebiotics) or indirectly (via NSP-degrading enzymes). The current study aimed to evaluate the effect of a prebiotic (AXOS) and an NSP-degrading enzyme (xylanase) on the production performance, innate immunity, hindgut SCFA production and hindgut microbial diversity of African catfish.

Dietary xylanase has shown to increase the nutrient digestibility and availability of aquafeeds through i) hydrolysing soluble NSP in the cell contents of plant cells, as well as ii) solubilising insoluble NSP in the cell wall fraction of plant cells. Soluble NSP in aquafeeds interferes with the interaction between nutrients and endogenous enzymes through increasing the intestinal digesta viscosity. On the other hand, insoluble NSP encapsulates potential nutrients inside the plant cell wall. Non-ruminant animals lack the endogenous enzymes that are capable of hydrolysing the glycosidic bonds of plant ingredients and are, therefore, unable to utilise plant feedstuffs optimally. Through hydrolysing the different NSP fractions of plant ingredients, xylanase increases the nutrient efficiency and digestibility of highly plant-based diets. Additionally, xylanase also produces NDOs that are fermented by carbohydrate fermenting bacteria in the large intestine of fish.

Information regarding the supplementation of dietary xylanase in aquaculture studies is lacking despite the positive results shown in the poultry and pig industries. From the literature survey, it is concluded that the effects of dietary xylanase in aquaculture studies vary according to the species, enzyme mixture, enzyme concentrations, and the diet constituents. It is, however, difficult to distinguish the effects of xylanase on fish performance as most studies involved the addition of xylanase in a multi-enzyme complex containing various other enzymes. Nevertheless, xylanase has shown to increase the nutrient efficiency of plant-based diets, especially wheat, but also maize and SBM-based diets. An increase in the growth performance, survival, body protein content, protein efficiency ratio and an improved feed conversion ratio, are among the most reported benefits regarding the supplementation of dietary xylanase in plant-based diets of fish.

Significant improvements have been reported regarding innate immunity parameters, endogenous enzyme activities and intestinal morphology. Additionally, studies have also shown that xylanase supplementation modulated the intestinal microbial communities of fish. The increase in growth performance caused by xylanase and other carbohydrase supplementation is most likely because of the increased nutrient utilisation efficiency caused by the increase in nutrient digestibility, intestinal morphology, physiological factors and a more

beneficial microbial community. These beneficial effects of xylanase seem to be more profound, but not limited to, omnivorous and herbivorous, freshwater species. This might be due to the greater inherent capability of these species to utilise plant ingredients compared to carnivorous, saltwater species.

Arabinoxylan-oligosaccharides are the hydrolysis products of arabinoxylan and have shown to possess potential prebiotic properties. Arabinoxylan-oligosaccharides can be produced through enzymatic (xylanase) hydrolysis or through thermochemical methods, such as steam explosion, of AX-containing raw materials, such as brewer's spent grains. Depending on the method and raw material used, different AXOS preparations are produced. These different preparations of AXOS have different chemical structures and, therefore, different rates of fermentability. Dietary AXOS are indigestible to fish and thus passes through the GIT to the hindgut where it is fermented by facultative anaerobic bacteria. Positive microbial and immunomodulating effects seen in fish may be caused by the direct or indirect action of AXOS supplementation. Dietary AXOS may directly enhance the innate immune response by interacting with microbial-associated molecular patterns and pattern recognition receptors in the GALT of fish. However, most effects of AXOS on the immune system are indirect which involves the modulation of the intestinal microbiota.

Despite the reported benefits of AXOS supplementation in other animals and human studies, literature concerning the supplementation of dietary AXOS in aquafeeds are limited. From this literature study, it can be gathered that AXOS possesses prebiotic properties, including microbial and immunomodulating activities, that may enhance the growth and health of fish. The intricate structure of AXOS, compared to conventional prebiotics, may convolute its functional properties and, therefore, some discrepancies exist between studies. Dietary AXOS have shown to significantly increase innate immune response parameters and hindgut SCFA production of Siberian sturgeon and African catfish. Supplementation of AXOS has also shown to significantly modulate the hindgut microbial community of both these species. A significant decrease in the hindgut microbial diversity has been reported in Siberian sturgeon fed diets supplemented with dietary AXOS. Although the effects of AXOS are more profound on the intestinal microbial community and non-specific immunity parameters than on growth performance, one study did reveal that a combination of AXOS and a probiotic significantly increased the growth rate of juvenile Siberian sturgeon. These effects of AXOS on fish performance may be depended upon the AXOS preparation, fish species, supplementation dosage and the mixture of other prebiotics or probiotics used in combination with AXOS.

The African catfish species is a hardy, omnivorous, highly fecund, fast growing and airbreathing freshwater fish that is not only cultured in Africa but also in parts of Eastern Europe. The inherent capability of *C. gariepinus* to utilise plant ingredients relatively well may be attributed to the fact that its endogenous enzyme activities are similar to both herbivorous

and carnivorous fish species. However, literature has shown that in order to successfully rear African catfish the nutrient efficiency of highly plant-based diets needs to be improved while the presence of ANFs needs to be removed. Functional feed additives, such as xylanase and AXOS, have proved to be able to alleviate ANFs, such as NSP, as well as enhance the nutrient digestibility of plant-based diets whether by degradation of the NSP contents of plant products or by beneficially altering the microbial community of the host, ultimately leading to an improved health and growth of the host animal. However, information regarding the use of these feed additives in aquatic animals are scarce and, therefore, their effects on African catfish performance warrants more study.

2.13. References

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Chapter 3

The effect of dietary endo-(1,4)-β-xylanase on the production performance, non-specific immunity, hindgut microbial diversity and hindgut short-chain fatty acid production of African catfish, *Clarias gariepinus*, fed fishmeal-free diets.

Abstract

A 91-day feeding trial was conducted to investigate the effects of Nutrase Xyla (an endo-(1,4)-β-xylanase blend) on the production performance parameters (growth performance and feed efficiency), fillet proximate analysis, visceral somatic index, non-specific immunity, hindgut microbial diversity and the production of short-chain fatty acids in the hindgut of African catfish fed fishmeal-free diets. The trial consisted of four dietary treatments, each containing the same basal diet. Treatments included one control group and three test diets, each supplemented with an increasing level (100, 150 and 200 PPM) of liquid xylanase supplied by Nutrex, Belgium. All treatments were replicated six times and received six randomly allocated, mixed sexed African catfish, with an initial average body mass of 513.0 ± 94.2 g (mean ± SD) at the start of the trial. No statistically significant differences existed between treatments for the growth performance, feed efficiency, fillet proximate analysis, visceral somatic index and SCFA production during the course of the trial. However, between Day 0 and Day 91, serum lysozyme activity and total immunoglobulin levels significantly increased, although no significant differences were found between treatments. Through the means of automated ribosomal intergenic spacer analysis (ARISA), statistically significant differences were found for the alpha and beta microbial diversity indices. Regarding the alpha diversity, significant differences existed for the Shannon diversity (P=0.041) and the Simpson diversity (P=0.050) indices between dietary treatments. The hindgut microbial diversity seemed to be dependent on xylanase inclusion level. The xylanase 200 treatment had a significantly lower Shannon diversity score, indicating a lowered species diversity, compared to the control treatment. Furthermore, the Bray-Curtis dissimilarity index showed statistically significant differences regarding the beta diversity between the start and termination of the trial. The results indicated that African catfish can be reared on diets completely devoid of fishmeal and fish oil and that dietary xylanase may have potential microbial modulating properties. The statistically similar results obtained in the trial regarding the production performance of the fish may be attributed to the large initial size of the fish at the start of the trial. It is therefore recommended that future studies should aim to use smaller fish with a faster growth rate and less adaptability towards highly plant-based diets. It is also recommended that future studies should aim to use

gnotobiotic environments when studying the microbial modulating effect of xylanase as to better understand the specific microbial species that might be involved in the upregulation of the innate immune system.

3.1. Introduction

There is a scarcity of information on the potential benefits of adding xylanase to aquaculture diets, despite widespread use in terrestrial monogastric diets (Ai *et al*., 2007; Jiang *et al*., 2014). The commercial use of xylanase in animal feed is largely based on the success found in the pig (Bartelt *et al*., 2002; Nortey *et al*., 2007a; b; Kim *et al*., 2008) and poultry (Choct *et al*., 1999; Selle *et al*., 2003a; b; Engberg *et al*., 2004; Wu *et al*., 2004; Selle, 2009; Singh *et al*., 2012; Masey-O'Neill *et al*., 2014) industries. The global trend towards more sustainable and affordable fish feeds involves the replacement of fishmeal with alternative plant protein ingredients (Gatlin *et al*., 2007). Plant ingredients, however, contain various antinutritional factors (ANFs), such as non-starch polysaccharides (NSP), and are largely indigestible to monogastric animals (Choct, 1997; Sinha *et al*., 2011). Non-ruminant animals, such as fish, lack the endogenous enzymes needed for the degradation of NSP (Kuz 'mina, 1996; Sinha *et al*., 2011; Glencross *et al*., 2012).

Dietary xylanase hydrolyses the glycosidic bonds of NSP and thereby reduces the associated negative effects, such as increased digesta viscosity and interference with nutrient absorption (Smits & Annison, 1996; Choct, 1997; Bedford & Schulze, 1998; Austin *et al*., 1999). Additionally, xylanase hydrolyses complex carbohydrates into lower molecular weight oligosaccharides, thereby releasing trapped nutrients inside plant cells which increases the nutritional value of otherwise less digestible feedstuffs (Bedford & Schulze, 1998). Therefore, with the supplementation of xylanase, animal feedstuffs can be formulated to contain a lower nutrient (protein) and energy content due to increased nutrient efficiency. Despite the increase in nutrient efficiency, the enzymatic hydrolysis of arabinoxylans (AX) releases non-digestible oligosaccharides (NDOs), such as arabinoxylan-oligosaccharides (AXOS), which have shown to exert health and microbial promoting effects in fish, humans and chickens (Grootaert *et al*., 2007; Courtin *et al*., 2008; Broekaert *et al*., 2011; Geraylou *et al*., 2012, 2013a; b). Therefore, dietary xylanase addition can be seen as a potential method of producing prebiotic oligosaccharides from plant-based dietary ingredients.

Xylanase addition to aquafeeds could be a strategy which allows higher rates of fishmeal substitution using plant ingredients. Exogenous xylanase addition to monogastric diets may alleviate the ANFs associated with plant ingredients while also increasing the nutrient efficiency of the feedstuff. Previous aquaculture studies involving the addition of xylanase have shown significant increases in the nutrient efficiency of plant-based diets (Babalola, 2006; Ai *et al*., 2007; Lin *et al*., 2007; Yildirim & Turan, 2010a; Jiang *et al*., 2014;

Adeoye *et al*., 2016a; Wallace *et al*., 2016). In most of these studies, the increased nutrient efficiency of the diet resulted in significant improvements in growth performance. However, the majority of studies that involved the supplementation of xylanase in aquafeeds used it as part of a multi-enzyme complex and, therefore, more information regarding the sole effects of xylanase in aquafeeds is warranted. Due to the use of different multi-enzyme complexes, species and diet constituents, ambiguity exists around the inclusion level of dietary xylanase in fish studies. One of the few studies that involved the sole inclusion of xylanase studied its effect on juvenile Jian carp and found that the best performance was achieved by supplementing xylanase at levels between 0.175 - 0.250 g/kg (Jiang *et al*., 2014).

Studies involving the xylanase supplementation in diets of African catfish, studied their effect as part of multi-enzyme complexes at inclusion levels ranging from 0.1 - 0.75 g/kg (Babalola, 2006; Yildirim & Turan, 2010a), whereas the best growth results were seen for fish fed a 0.75 g/kg enzyme mixture treatment (Yildirim & Turan, 2010a). Thus, due to the paucity of information regarding xylanase addition in aquaculture diets, the optimal inclusion level of xylanase seems to be poorly defined and, therefore, one of the objectives of the current study was to determine a suitable inclusion level for dietary xylanase in fishmeal-free diets of African catfish.

The addition of exogenous enzymes in diets of African catfish is poorly studied (Ng *et al*., 1998; Van Weerd *et al*., 1999; Ng & Chen, 2002). Furthermore, despite evidence of having microbial- and immunomodulatory properties (Adeola & Cowieson, 2011; Bedford & Cowieson, 2012), dietary xylanase studies on African catfish are limited to the evaluation of growth performance and feed utilisation (Babalola, 2006; Yildirim & Turan, 2010a). These studies often incorporated xylanase as part of a multi-enzyme complex and, thus the sole effect of xylanase in diets of African catfish is unknown. The African catfish is a major freshwater aquaculture species indigenous to Africa, and due to its air-breathing, robust, fast-growing and omnivorous qualities (Huisman *et al*., 1987; Fagbenro & Davies, 2001; Turan & Akyurt, 2005), have since been cultured in other countries in Eastern Europe and Southern Asia, which led to the total amount of African catfish production of 246 000 tons in 2015 (FAO, 2018).

The aim of this trial was to determine if dietary xylanase could improve the nutritive quality of fishmeal-free diets of African catfish. In order to achieve the study's aim, the following specific objectives were formulated: To determine the effects of xylanase at i) different dietary inclusion levels on the ii) production performance, iii) non-specific immunity, iv) hindgut microbial diversity and v) hindgut short-chain fatty acid production of African catfish fed fishmeal-free diets.

3.2. Materials and methods

This section includes the experimental design, production of the dietary treatments, equipment used, experimental procedures followed, and the analytical methods used to determine the various parameters studied on African catfish. Parameters tested on African catfish include production performance parameters (growth and feed efficiency), fillet proximate analysis, visceral somatic index, non-specific immunity assays, haematocrit values, hindgut microbial diversity and hindgut short-chain fatty acid (SCFA) concentrations. All dietary treatments were subjected to a feed proximate analysis (based on the Weende nutrient system) where moisture, ash, crude protein, crude fibre and crude fat values were obtained. African catfish fillet samples were subjected to a meat proximate analysis where moisture, ash, total fat and crude protein values were obtained. All diets and major feed ingredients were also subjected to an amino acid analysis. The proximate composition of feed and fish fillet samples were analysed according to the methods described by AOAC (2002).

3.2.1. Experimental design, animals and system

This research study applied and obtained ethical clearance from the Stellenbosch University Research Ethics Committee: Animal Care and Use (Protocol # 2820 ACU-2018-2820, attached in Appendix A) before the commencement of this trial and, therefore, all experimental procedures have been approved by the above-mentioned committee.

A 91-day feeding trial was established to evaluate the effects of dietary endo-(1,4)-β-xylanase (Nutrase Xyla) on the production performance, non-specific immunity, hindgut microbial species diversity and hindgut SCFA production of African catfish. The trial commenced on the 23rd of November 2017 and carried through until the 22nd of February 2018. The experimental system consisted out of an indoor, temperature controlled, recirculating aquaculture system (RAS) situated at Welgevallen Experimental Farm, Stellenbosch University. The system consisted out of 88 x 75-litre plastic tanks housed in a closed building equipped with an air-conditioning system and extractor fan to regulate atmospheric and water temperature. Each tank received continuous aeration supply through a 1.1 kW air blower (Model SCL V4, Incorezzo, Milano, Italy). Air was transferred through plastic pipes connected to air-stones in each tank. The air-conditioning system and two heating elements were used to maintain the water temperature at 28°C, regarded as optimal temperature for African catfish species (Britz & Hecht, 1987), through the workings of a thermostat (Danfoss EKC 102). Recirculating water was continuously supplied to the tanks at a similar and constant flow rate (mean flow rate of 0.27 ± 0.05 standard deviation (SD) L/s) and filtered using a static mechanical filter, followed by a trickling biofilter. Filters were cleaned and a third of the water in the system replaced every two weeks.

The experimental design consisted of four dietary treatments (one control and three test diets) containing the same basal diet. Each test treatment was supplemented with a specific inclusion level of liquid xylanase. The treatment without xylanase addition (0 PPM) served as the control group. The experimental layout is provided in Table 6 below. All dietary treatments were replicated six times and thus six tanks were randomly allocated to each of the treatments. The African catfish was chosen as the preferred species due to their being a hardy, robust, omnivorous and easy to handle species with a large economic importance as a candidate species for the aquaculture sector in South Africa. Each tank received six randomly allocated, mixed sex, African catfish with initial mean weight and length ± SD of 513.0 ± 94.2 g and 419.2 ± 22.6 mm, respectively. All fish were sourced from registered hatcheries and acclimatized to the RAS system for two weeks prior to trial commencement. During these two weeks, all fish received the same control feed. During the trial, all fish were fed twice per day, at 09:00 and 16:00, and, depending on water visibility, at *ad-libitum* feeding levels. During periods of low water visibility fish were fed at a rate of 2% body mass per day, that was determined every two weeks. This was based on an initial 2% specific growth rate (SGR) at trial commencement.

Table 6 Treatment design and xylanase inclusion levels

Treatments	**Inclusion level (PPM)**
Xylanase 0 (Control)	0
Xylanase 100	100
Xylanase 150	150
Xylanase 200	200

3.2.2. Water quality parameters

Water quality parameters included water temperature, dissolved oxygen (DO), pH, total ammonia-nitrogen (nitrogen from NH_4^+/NH_3 mg/L), nitrite (NO_2^- mg/L) and nitrate (NO_3^- mg/L). Water temperature, DO and pH levels were measured daily, whereas total ammonia-nitrogen, nitrite and nitrate were measured twice a week. Water temperature and DO were measured using a YSI ProODO, optical dissolved oxygen and temperature instrument, while pH was measured using a Hanna instruments pH 211 microprocessor meter. Total ammonia-nitrogen, nitrite and nitrate were measured using a Hach DR/850 colourimeter in conjunction with Hach permachem reagents. Mean water temperature, DO and pH level ± SD was 27.7 ± 1.6°C, 6.02 ± 1.23 mg/L and 7.45 ± 0.43 respectively, which is within the acceptable range for catfish culture (Tucker, 1985; Viveen, 1985; Hoffman *et al*., 1991). Mean ammonia, nitrite and nitrate level ± SD was 6.11 ± 4.49 mg/L, 1.88 ± 1.52 mg/L and 2.49 ± 1.81 mg/L, respectively. To contain ammonia and nitrite levels at a low concentration, a third of the water in the system

was exchanged every two weeks. Concurrent with the water exchange, filters were also cleaned every two weeks. During extreme spikes in ammonia and nitrite levels, a natural adsorbent silicate, zeolite, was used to absorb ammonia during high concentrations. Zeolite was administered in the water sump where the water of all treatments was collected. This was to ensure that all treatments received the same amounts of zeolite-treated water, so as to prevent any potential external effect of zeolite on any one of the treatments.

3.2.3. Feed ingredient and diet preparation

The basal diet was formulated by Nutritionhub (Pty) LTD, South Africa, using the Winfeed 2.8 (Winfeed Ltd, Cambridge, UK) formulation software, and was designed to best elucidate the efficacy of endo-(1,4)-β-xylanase by formulating a below-optimum digestible energy content diet based on literature information (Table 3, 4 and 5). The diet formulation contains a large portion of plant-based ingredients, which contains high amounts of soluble and insoluble NSP (Bach Knudsen, 2001). Maize, wheat and soybean meal, three of the major plant ingredients in the present study, contains between 0.1 - 0 .9, 2.4 - 2.5 and 2.7 - 6.3 g/kg dry matter soluble NSP, respectively, and between 6.6 – 8.0, 7.4 – 9.0 and 9.2 – 16.5% dry matter insoluble NSP, respectively (Henry, 1985; Choct, 1997; Bach Knudsen, 2001). Apart from lowering the apparent digestible energy in the diets, these NSP-containing ingredients also serve as the substrate necessary for xylanase hydrolysis. The specific xylanase strain used in the current study was chosen as a strain with a more inclined affinity concerning the insoluble fractions of NSP, because of the large content of insoluble NSP in soybean meal especially. The endo-(1,4)-β-xylanase blend (Nutrase Xyla) was supplied by Nutrex Belgium.

Furthermore, the basal diet was formulated according to the nutritional requirements of African catfish (Table 3 and 4). Conforming to the aim of this research study, the basal diet was formulated without any FM or FO ingredients. Alternative plant protein ingredients were chosen to replace FM and FO in diets of African catfish. Fishmeal, normally being the major constituent of the protein fraction of a fish feed, was completely replaced by soybean oilcake, maize, wheat bran, alfalfa meal, blood meal and poultry by-product meal. Methionine and lysine were added to supplement the lacking essential amino acids in the diet. The high starch content of the diet replaced the use of oil as the major pellet binding material. No oil (neither of plant or animal origin) was used as a strategic reduction in the digestible energy content of the feed to avoid possible ‘masking’ of the beneficial effects of xylanase. The use of matrix values or restricted feeding regimes have been suggested by many authors regarding the use of dietary enzymes in animal feeds (Ogunkoya *et al*., 2006; Farhangi & Carter, 2007; Cowieson & Bedford, 2009; Adeola & Cowieson, 2011). The basal diet formulation is provided in Table 7.

Table 7 Basal diet feed formulation

Ingredient	%
Soybean oilcake 46	30.16
Maize	26.00
Poultry by-product meal 65 (Nobless, TICSA)	15.04
Wheat bran	11.56
Blood meal	5.00
Lucerne meal (17% protein)	4.00
Monocalcium phosphate	2.90
Limestone	1.65
DL methionine	1.20
L-lysine HCl	1.18
VitMin Premix - Standard	0.80

Table 8 Feed proximate analysis of all treatment groups ('As is', g/100g)

Nutrient analysis	Control	X 100	X 150	X 200
Moisture	5.01	6.25	5.66	5.02
Crude protein	37.0	36.1	36.3	37.2
Crude fat	5.14	5.67	5.67	4.76
Crude fibre	3.84	3.96	3.90	3.82
Ash	11.1	10.5	10.7	11.0
NFE[a]	38.0	37.6	37.7	38.2
Gross energy (MJ/kg)[b]	17.2	17.2	17.2	17.2

[a]Nitrogen free extract was determined by difference as 100 – moisture – crude protein – crude fat – crude fibre – ash. [b]Gross energy determined by 23.4*crude protein + 39.8*crude fat + 17.2*carbohydrates

All the diets were made at Welgevallen Experimental Farm, Stellenbosch, while most of the feed ingredients were sourced from local feed ingredient suppliers. All diets were formulated to be iso-nitrogenous and iso-caloric. Due to the rough texture of the soybean oilcake and

maize meal it had to be put through a hammer mill (Drotsky S8) to achieve a more suitable particle size for pellet extrusion. Diets were prepared by weighing and adding the appropriate amounts of each ingredient and mixed in 10 kg batches in a commercial dough mixer (Macadams baking systems SM-401).

Table 9 Amino acid analysis of all treatment groups and major feed protein ingredients (% m/m dry solid)

Amino acid	Control	X 100	X 150	X 200	PBP	BM	SBM
Histidine*	0.78	0.50	1.35	2.07	0.52	0.75	0.82
Serine	1.92	1.01	2.04	2.97	1.09	1.54	1.66
Arginine*	1.74	0.88	2.04	2.85	1.04	1.52	1.57
Glycine	2.85	2.36	5.64	8.14	2.54	3.49	3.92
Aspartic acid	2.17	1.00	2.12	3.26	1.13	1.64	1.81
Glutamic acid	5.03	2.35	3.84	6.20	2.58	3.77	4.10
Threonine*	1.13	0.63	1.70	2.52	0.67	1.05	1.12
Alanine	1.20	0.94	2.71	3.94	1.02	1.51	1.62
Proline	1.56	1.05	2.04	2.88	1.16	1.59	1.79
Lysine*	1.82	1.31	2.78	4.05	1.53	2.57	2.56
Tyrosine	2.06	1.08	2.34	3.33	1.21	1.67	1.83
Methionine*	0.88	1.30	1.21	1.42	1.33	1.7	1.66
Valine*	1.01	0.61	1.57	2.22	0.69	0.99	1.09
Isoleucine*	2.23	1.08	2.62	3.80	1.18	2.04	2.31
Leucine*	0.84	0.56	1.40	1.89	0.63	0.91	0.96
Phenylalanine*	2.26	1.49	2.75	3.34	1.56	1.80	1.91
Sum of EAA	12.69	8.36	17.4	24.2	9.15	13.3	14.00

*Essential amino acid. PBP: poultry by-product meal; BM: blood meal; SBM: soybean meal

For each 10 kg of feed, three litres of water were gradually added while ingredients were being mixed until a homogenous paste was obtained. This mixture was then extruded through a custom built single-screw extruder (Reomach Engineering, South Africa) with a 5 mm diameter die. Liquid endo-(1,4)-β-xylanase was mixed with distilled water (room temperature) in the appropriate concentrations and sprayed on the pellets after extrusion pelleting. Consequently, pellets were dried for 24 hours in a convection drying oven (CFW Envirowatch 5) at 60°C. After the pellets have been sufficiently dried they were stored in airtight containers and out of direct sunlight. The proximate analysis of all the treatment groups is given in Table 8, while the amino acid analysis of the treatment groups and the major protein ingredients (soybean

oilcake, blood meal and poultry by-product meal) is given in Table 9. The discrepencies regarding the amino acid anlysis in the current trial may be attributed to the nonhomogeneous sampling and/or mixing of the feed samples prior to the amino acid analysis. This rationale is based due to the relatively similar results obtained from the protein fraction of the feed proximate analysis.

3.2.4. Experimental and sampling procedures

Fish were fed *ad-libitum* twice a day until visual observation of satiation to avoid any wastage of feed. During periods of poor water visibility fish were fed 2% of their tank average body mass per day. The average body mass of the tanks was calculated every two weeks as necessary. The amount of feed administered between every sampling date was recorded and used to calculate the various growth performance parameters. Mortalities were removed daily and recorded accordingly. Six sampling dates (including initial and terminal samplings) were spread throughout the 91-day feeding trial, each separated by approximately two weeks. All fish were fasted for a minimum of 12 hours and anaesthetised with Tricaine Methanesulfonate (MS-222, Sigma) prior to all sampling parameters to ensure ease of handling, lowered stress levels and accurate measurements being taken. During all sampling times, fish were weighed, and their length taken accordingly. Care was taken to ensure that the same person responsible for a specific sampling parameter finished all the data capture that was subjected to that specific sample being taken. The relevance of this was to minimise any variability that could have existed between different persons responsible for a specific sample being taken.

Growth performance parameters were determined at each of the six sampling dates and included the individual weight and total length (from the tip of snout to the tail end) of every fish in all the tanks. Total length was measured to the closest mm on a measuring board while the body mass was recorded to 0.01 g on a balanced electronic scale (DIGI DS-673). The total number of fishes per tank, total length, body mass and feed consumption were used to calculate the following growth parameters: survival, average daily gain (ADG), specific growth rate (SGR), weight gain, body mass growth, relative feed intake (RFI) and feed conversion ratio (FCR). These performance parameters were calculated as follows:

Equation 1: Survival (%) $= \frac{Nf}{Ni}\ x\ 100$

Where:

Nf = number of fish at day 91,

Ni = number of fish at day 1.

Equation 2: SGR (%/day) $= \frac{(Ln(Wavg_f) - Ln(Wavg_i))}{t}\ x\ 100$

Where:

$Ln(Wavg_f)$ = the natural logarithm of the final weight average,

$Ln(Wavg_i)$ = the natural logarithm of the initial weight average,

t = number of days between initial and final measurements.

Equation 3: Weight gain (%) $= \frac{(Wave_f - Wavg_i)}{Wavg_i}\ x\ 100$

Where:

$Wavg_f$ = final weight average (g),

$Wavg_i$ = initial weight average (g).

Equation 4: Body mass growth (g) $= \left(BM_f + BM_{mortalities}\right) - BM_i$

Where:

BM_f = Body mass final (g),

$BM_{mortalities}$ = Body mass of mortalities (g),

BM_i = Body mass initial (g).

Body mass growth considers the weight of mortalities and can therefore be used as an adjusted value to correct for mortalities in calculations such as feed intake and FCR.

Equation 5: RFI $= \left(\frac{Feed\ intake}{\left(\frac{\left(BM_f + BM_i\right)}{2}\right)}\right)/t * 100$

Where:

BM_f= Body mass final (g),

BM_i = Body mass initial (g),

t = number of days between initial and final measurements.

Equation 6: FCR $= \frac{Feed\ intake}{\left(\left(BM_f + BM_{mortalities}\right) - BM_i\right)}$

Where:

BM_f = Body mass final (g),

$BM_{mortalities}$ = Body mass of mortalities (g),

BM_i = Body mass initial (g).

Meat proximate analysis involved the sampling of fish fillets at the termination of the trial. One randomly selected fish per tank was euthanized by subjecting to an overdose of MS-222 followed by the severing of the spinal cord. Fish were dissected following the removal of the intestinal organs and the skin using a disinfected standard dissection kit. Fillets were cut from the spine and frozen at -20°C in plastic zip-lock bags until further analysis.

Samples for the determination of the visceral somatic index (VSI) were taken at trial termination. Two randomly selected fish per tank were euthanized by subjecting to an overdose of MS-222 followed by the severing of the spinal cord. Fish were dissected using a disinfected standard dissection kit. The intestinal organs were removed and weighed. The viscera weight was used as a ratio compared to the full body mass of the fish prior to dissection. VSI = 100 x (g wet intestinal weight/g wet body mass).

Blood serum samples for non-specific immunity assays were taken at each of the six sampling dates: Day 0, Day 18, Day 41, Day 56, Day 75 and Day 91. At the start of the trial, Day 0, blood was taken from 17 randomly selected fish through puncturing of the caudal vein with a non-heparinized needle (21 G) and a 3 mL syringe. All fish were anaesthetised with MS-222 prior to taking of blood. After sampling, fish were allowed to recover in aerated, clean water and returned to their original tanks. These samples were used to evaluate the relative health status of the fish prior to treatment. During the remaining five sampling dates fish blood was taken from two randomly selected fish per tank using non-heparinized needles (21G) and 3 mL syringes. All blood was left to clot for 24 hours at 4°C in untreated Eppendorf tubes (1.5 mL). Thereafter, blood was centrifuged using an A & E Lab mini centrifuge (MC 14) at 500 x g for 15 minutes. The supernatant was collected as the blood serum and stored at -20°C in Eppendorf tubes until further analysis.

Haematocrit values were obtained from fish blood collected at the start and termination of the trial using 21-gauge non-heparinized needles and 3 mL syringes. At trial commencement, blood from 17 randomly selected fish were taken (same blood as for non-specific immunity titers) and used to fill micro haematocrit capillary tubes to circa ¾ and were sealed at the end with Critoseal. Consecutively, the haematocrit tubes were centrifuged for 5 minutes using a micro haemocytometer and read using a standard matrix. At the termination of the trial, blood from two randomly selected fish per tank was taken and immediately centrifuged using the same equipment and procedure as mentioned above. Haematocrit values are expressed as erythrocyte volume as a proportion of total blood volume and denominated as packed cell volume (PCV).

Microbial diversity analysis involved taking empty caeca intestine (hindgut) samples at the start and at the termination of the trial. Fish were fasted for 12 hours prior to sampling and euthanized by subjecting to an overdose of MS-222 followed by the severing of the spinal cord. At the start of the trial, three randomly selected fish were dissected using a disinfected standard dissection kit. The entire gastrointestinal tract was removed and unwound on a smooth disinfected surface to prevent tissue damage and infection. One section (± 2 cm) of the distal intestine (2 - 3 cm from the anus) of each fish was removed and placed in 10 mL sterile saline solution (9 g/L NaCl) containing acid-washed glass beads (Sigma, South Africa) and stored at -20°C until further analysis. These samples were used to determine the relative

hindgut microbial diversity status of the fish prior to trial commencement. At the end of the trial, one randomly selected fish per tank was dissected and used to sample one section (± 2 cm) of the distal intestine (2 - 3 cm from the anus) for microbial diversity analysis using the same procedures as described above and subsequently stored at -20°C until further analysis.

Determination of SCFAs involved hindgut digesta samples taken at trial termination. Fish were fed the morning before sampling to ensure adequate digesta in the gastrointestinal tract of the fish. One randomly selected fish per tank was euthanized by subjecting to an overdose of MS-222 followed by the severing of the spinal cord and dissected using a disinfected standard dissection kit. The large intestine was carefully removed and unwound on a smooth and disinfected surface. Samples were obtained through gently stripping the hindgut digesta into plastic containers. Thereafter, the containers were properly sealed and kept at -20°C until further analysis.

3.2.5. Analytical procedures

The following section describes the analytical procedures and the materials used to analyse xylanase enzyme activity, feed proximate analysis, feed and major feed ingredient amino acid compositions, meat proximate analysis, non-specific immunity assays, hindgut microbial diversity analysis and hindgut SCFA analysis.

I) Enzyme activity

The enzyme activity of Nutrase Xyla was not determined in the current study. However, in a previous trial conducted by the same authors as in the current study, the enzyme activity of endo-xylanase (Nutrase Xyla) was determined by the Industrial Engineering Faculty of the University of KU Leuven. The same application method and inclusion levels were used in the previous trial as in the current trial. The results obtained indicated that the measured enzyme activity was lower than the initial inclusion levels. This, however, could be expected due to reasons including, unhomogenised spraying of liquid enzyme onto the feed pellets after extrusion-processing (post-extrusion, spray-on equipment is very expensive), inactivation of enzymes due to the drying of the pellets in a convection oven, and/or unhomogenised mixing and/or sampling of the enzyme and feed samples, respectively. Therefore, due to the similarity between the two studies, and the same enzyme being used, the results pertaining the enzymatic activity could be seen as intercorrelated between the two studies. The results regarding the enzymatic activity of xylanase are provided in Appendix B.

II) Feed proximate analysis

Feed proximate analysis followed laboratory techniques that are based on the Weende nutrient system and involves the determination of crude protein, crude fat, crude fibre, moisture and ash fractions. The nitrogen free extract (carbohydrate) fraction was obtained as

the difference of moisture + crude protein (CP) + crude lipid + crude fibre (CF) + ash subtracted from a 100. The gross energy values were obtained by multiplying CP by 23.4, crude lipid by 39.8 and the carbohydrate fraction by 17.2. Care was taken to present samples that were representative as well as homogenous. All feed samples were gound with a hammer mill (1.5 mm sieve) prior to analytical procedures and were measured in duplicate.

Moisture analysis was done according to the AOAC (934.01) International method for moisture analysis and involved drying the feed sample in an oven at 100 -105°C for 24 hours (AOAC, 2002). Thereafter, the moisture fraction was calculated as follows:

$$\%\text{Moisture} = \frac{(crucible + feed\ sample\ weight) - (moisture\ free\ sample + crucible\ weight)}{feed\ sample\ weight}$$

Ash analysis was done according to the AOAC International method for ash analysis (942.05) and involved placing the already moisture free sample in a furnace at 500°C for 6 hours (AOAC, 2002). Thereafter, the ash fraction was calculated as follows:

$$\%\text{Ash} = \frac{(ash\ weight - moisture\ free\ weight)}{feed\ sample\ weight}$$

Crude protein analysis was done according to the Dumas method using a LECO FP 528 (AOAC, 2002). The method involved the combustion of a sample at ± 900°C in the presence of oxygen, causing the release of carbon dioxide, water and nitrogen. The gases are then passed over columns that selectively absorb the carbon dioxide and water. A column containing a thermal conductivity detector at the end is then used to separate the nitrogen from any residual carbon dioxide and water whereafter the remaining nitrogen content is measured. The nitrogen concentration of the sample is then converted to crude protein content by using a conversion factor depending on the protein's origin. All feed ingredients, except soybean meal, used a nitrogen (N) conversion factor of 6.25, while soybean meal used a factor of 5.71. The crude protein fraction was calculated as follows:

%Crude protein = %nitrogen x protein conversion factor

Crude fibre analysis was determined gravimetrically according to the AOAC (962.09) International method for fibre analysis (AOAC, 2002). The analyses involved an Ankom fibre analyser and the addition of diluted sulphuric acid to remove free sugars and starch. Subsequently, sodium hydroxide was added to remove proteins and some carbohydrates. The resulting components left after acid and alkali hydrolysis are cellulose, hemicellulose (pentosans), lignin and pectic substances, in different ratios depending on the fibre source analysed. The crude fibre fraction was calculated as follows:

$$\%\text{Crude fibre} = \frac{(weight\ of\ organic\ matter - (empty\ bag\ weight\ x\ blanko))}{sample\ weight}\ x\ 100$$

Crude fat was determined by acid hydrolysis according to the AOAC (920.39) International method for fat analysis (AOAC, 2002). This involved the addition of hydrochloric acid followed by extraction of hydrolysed lipid materials with mixed ethers. After the ether evaporated, the residue was measured and expressed as %crude fat. The crude fat fraction was calculated as follows:

$$\%\text{Crude fat} = \frac{(weight\ of\ fat\ cup + fat) - (weight\ of\ fat\ cup)}{weight\ of\ sample}\ x\ 100$$

III) Amino acid analysis

The amino acid analysis was determined using the LC-MS method according to the AOAC (994.12) International method for feed amino acid analysis (AOAC, 2003). Feed samples were required to be hydrolysed prior to analysis in a LC-MS lab. The hydrolysis involved mixing the sample with phenol and hydrochloric acid in a vacuum tube. Tubes were then hydrolysed in an oven at 110°C for 24 hours. After the hydrolysis, samples were transferred to Eppendorf tubes and sent to a LC-MS lab for further analysis. All amino acid samples were measured in duplicates.

IV) Meat proximate analysis

The following techniques were used to determine the meat proximate analysis and included moisture, ash, total fat and crude protein fractions. Care was taken to present samples that were representative as well as homogenous. Therefore, all feed samples were homogenised, prior to proximate and chemical analysis, using a meat blender. All meat samples were measured in duplicate.

Moisture was determined according to the AOAC (934.01) International method for moisture analysis (AOAC, 2002). Samples were placed in an oven at 100 – 105°C for 24 hours and measured as the difference between the before and after drying weight. Moisture was calculated as follows:

$$\%\text{Moisture} = \frac{(crucible + sample\ weight) - (moisture\ free\ sample + crucible\ weight)}{sample\ weight}$$

Ash was determined according to the AOAC (942.05) International method for ash analysis by placing the moist free sample in a furnace at 500°C for 6 hours and the residue weighed accordingly (AOAC, 2002). Ash was calculated as follows:

$$\%\text{Ash} = \frac{(ash\ weight - moisture\ free\ weight)}{sample\ weight}$$

Total fat was measured by mixing 50 mL of chloroform/methanol solution (1:2) with samples using a Bamix mixer. The mixture was then filtered, and the resulting solution mixed with 0.5% sodium chloride. After chloroform/methanol evaporation the residue was weighed accurately (Lee *et al*., 1996). Total fat was calculated as follows:

$$\%Fat = \frac{(weight\ of\ fat\ cup+fat)-(weight\ of\ fat\ cup)}{weight\ of\ sample}\ x\ \frac{chloroform\ volume}{5}\ x\ 100$$

Crude protein was determined using the Dumas method involving a LECO FP 528 according to the AOAC International method for protein analysis (AOAC, 2002). Samples used were already moist free. The method involved the combustion of a sample at ± 900°C in the presence of oxygen, causing the release of carbon dioxide, water and nitrogen. The gases are then passed over special columns that absorb the carbon dioxide and water. A column containing a thermal conductivity detector at the end is then used to separate the nitrogen from any residual carbon dioxide and water whereafter the remaining nitrogen content is measured. The nitrogen concentration of the sample is then converted to crude protein content by using a conversion factor depending on the protein's origin. The N conversion factor was taken as 6.25. Crude protein was calculated as follows:

%CP = %nitrogen x protein conversion factor of 6.25

V) Non-specific immunity assays

Non-specific immunity assays involved the analysis of serum lysozyme activity, serum total protein and total immunoglobulin levels. All non-specific immunity parameters were done in duplicates. Additionally, each duplicate was measured in triplicate on the microplates.

Serum lysozyme activity was determined spectrophotometrically according to (Sankaran & Gurnani, 1972), where 50 µL blood serum was added in triplicates to a 250 µL suspension of 0.075% (w/v) *Micrococcus lysodeikticus* (Sigma, M3770) and a phosphate buffer (0.05 M at pH 6.2), in a 96-well microplate. Absorbance was read after 5 minutes and again after 20 minutes at 530 nm using a microplate reader (BioTek ELx 800™). During the interval between readings, the microplate was kept a stir using a microplate shaker. A standard solution was prepared by using standard lysozyme (Sigma, L6876) instead of the blood serum as described above. The difference in absorbance at 5 and 20 minutes of the standard solution was used to draw a standard curve. Consecutively, blood serum lysozyme activity was calculated from the standard curve.

Serum total protein was determined spectrophotometrically using the linearized Bradford assay (Zor & Selinger, 1996) where a protein standard (Lysozyme, Sigma, L6876) was used to prepare a standard curve using known concentrations. Protein standard and blood serum concentrations were prepared using 0.85% NaCl solution as a dilution agent. 50 µL of standard/serum was added, in triplicates, with 200 µL of Bradford dye reagent to a 96-well microplate. The absorbance was measured at 450 nm and 630 nm. Serum total protein concentrations were calculated from a standard curve based on the ratio of absorbances (630 nm/450 nm).

Immunoglobulin concentrations of blood serum were determined using the method of Siwicki *et al*., (1994). Blood serum and 12% (w/v) polyethylene glycol (PEG, Sigma, with 10 000 Dalton average molecular weight) were added, in triplicates, in equal weights (100 µL of each) to Eppendorf tubes and left to incubate at room temperature for 2 hours. Tubes were then centrifuged at 14 000 rpm for 5 minutes, resulting in a white pellet forming at the bottom of the tube. The PEG pellet was re-solubilised with 0.85% (w/v) NaCl, maintaining the same total volume, and was used to determine the total immunoglobulin concentration, using the linearised Bradford method as described above.

VI) Microbial diversity analysis of hindgut microbiota

For the microbial diversity analysis, DNA was extracted from fish hindgut samples using a Quick-DNA Fungal/Bacteria miniprep kit (Zymo Research, California, USA). Hindgut samples were directly put into plastic tubes containing acid-washed glass beads (Sigma, South Africa) and 10 mL sterile saline solution (9 g/L NaCl). Tubes were kept at -20°C until further analysis. The microbial diversity analysis was determined by the Department of Microbiology at Stellenbosch University, South Africa. The microbial community structure was quantified using Automated Ribosomal Intergenic Spacer Analysis (ARISA). The ARISA method is used as a community fingerprinting method and is based on polymerase chain reaction (PCR) where fluorescently labelled primers are used to amplify the 16rRNA region of the specific microbial sample. Resultingly, different fragment lengths are produced that are separated using capillary electrophoreses. A genetic analyser (ABI 3010XL) was used to obtain electropherograms containing different fragment lengths and fluorescent intensity. An internal standard (LIZ1200) was used to run the bacterial samples on ARISA. Different DNA fragment lengths were obtained from the fluorescent data using Genemapper 5 software. These different DNA fragment lengths, suggesting different species, and are also known as operational taxonomic units (OTU's). Peak heights obtained from the Genemapper 5 software are plotted against the standard curve to calculate fragment lengths. Fragments sizes needed to be between 100 and 1000 base pairs in length and above 150 fluorescent units in height to be used for analysis as OTU's. Inaccuracies of the ARISA profile was minimized using a bin size of 3 base pairs. The analysis includes the calculation of species richness and alpha and beta diversity indices that are used to compare microbial communities with one another (Brown *et al*., 2005; Slabbert *et al*., 2010).

Species richness is an indication of the relative species at a particular sampling site and was determined as the number of unique OTU's of each sample. Alpha diversity is the diversity of a specific group of organisms within a specific sampling unit and was determined, for each site, using the Shannon-Weaver (H) and the Simpson's diversity (D) index. The Shannon diversity index is commonly used to determine species diversity in a community by

measuring the degree of the entropy of a community (Slabbert, 2008). The higher the Shannon index the higher the richness of the specific species community. However, the Shannon index is not a linear measure of diversity, and can be calculated as follows (Krebs, 1989):

Equation 7: The Shannon-Weaver index

$$H = -\sum_{i=1}^{s} Pi \ln Pi$$

Where:

- Pi is the proportion of organisms found in species i,
- S is the total number of species found,
- Σ is the sum of species 1 to S,
- ln is the natural logarithm.

The Simpson's diversity index tells us about bacterial dominance in a community. The higher the Simpson index the greater the effect of dominance/degree of concentration in a community. This index gives us the value that ranges between 0 and 1, where 1 represents the greater sample diversity and 0 an absence of sample diversity. The Simson index is based on the more abundant species in a community and is therefore not dramatically influenced by the presence or absence of minor species (Slabbert, 2008), and can be calculated as follows:

Equation 8: The Simpson index

$$D = 1 - \frac{\Sigma n(n-1)}{N(N-1)}$$

Where:

- Σ is the sum of,
- n is the total number of organisms of a particular species,
- N is the total number of organisms of all species.

Beta diversity is defined as the variation of species composition among sampling units (Anderson *et al*., 2006). Beta diversity is measured as the average distance, or dissimilarity, from a specific entity to the group mean (Anderson *et al*., 2006). The Bray-Curtis measure is used to measure the dissimilarity between the sampling unit and the group centroid by focusing on community structure variation (Bray & Curtis, 1957), and can be calculated as follows:

Equation 9: The Bray-Curtis dissimilarity

$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$$

Where:

- Cij is the sum of the lesser values for the species that are present at both sites,

- Si and Sj are the total numbers of species counted at site i and j respectively.

The Bray-Curtis dissimilarity index is bound between values 0 and 1, where 0 indicates identical species similarity (same species composition) between the two sites and 1 indicates the complete lack of any similarity between the two sites. The Bray-Curtis dissimilarity index has been extensively used to determine the beta diversity in microbial studies (Danovaro *et al*., 2006; Wood *et al*., 2008b; a; Wakelin *et al*., 2010).

VII) Short-chain fatty acid analysis

Fish hindgut digesta samples were deproteinized and the sugars removed following a procedure modified from Siegfried *et al*. (1984). Prior to analysis, 1 mL of digesta samples were homogenized by adding 1 mL of de-ionized water and were then centrifuged for 10 min at 4 000 rpm at 4°C. Out of this mixture, 1.5 mL was transferred to a 2 mL Eppendorf tube. The mixture was again centrifuged at the same specifications where after 600 µL of the supernatant was used as the sample representative. These samples were then mixed with calcium hydroxide and cupric sulphate solutions to remove proteins and sugars, respectively. Thereafter, samples were mixed with sulphuric acid and diethyl ether and the supernatant collected for analysis via gas-liquid chromatography (Agilent 6890 N GC - FID). A standard curve was created using an SCFA stock standard solution to calculate the amount of SCFA in the samples. Data was analysed on a gas chromatography (GC) ChemStation. The instrument and method details of the GC procedure are provided in Appendix C.

3.2.6. Statistical analysis

The experimental design of the study represented that of a completely randomised design with xylanase treatment as the main effect. Data were compared using a one-way analysis of variance (ANOVA) and descriptive statistics. All data were analysed using Statistica version 13.3 (Tibco Software Inc.). The assumptions for normality and homogeneity were checked and tested using the Shapiro-Wilk's test and Levene's tests, respectively. At instances where the homogeneity assumption was not tenable, Welch's test was used to ensure the accuracy of the analysis. At instances were the normality assumption was not tenable, Kruskal-Wallis was used as the preferred non-parametric test and performed on the residuals of the variables. Differences were regarded as significant when $P<0.05$, and Fischer's LSD post-hoc test was used to indicate significant differences between treatment means. Pre-treatment variables were included in the non-specific immunity and microbial analysis and were subjected to a one-way ANOVA. All data are presented as means ± standard error (SE). Average daily gain (ADG) was calculated by use of a linear regression of body mass compared to the time for each treatment. The gradient of each regression was then used to represent ADG. One-way ANOVA was used to compare ADG between treatments.

3.3. Results and discussion

3.3.1. Production performance parameters

The following production performance parameters were evaluated to determine the growth performance and feed efficiency of African catfish fed diets containing four increasing levels (0, 100, 150 and 200 PPM) of xylanase and include: initial body weight average ($BWavg_i$), final body weight average ($BMavg_f$), survival, SGR, weight gain, body mass growth, ADG, RFI and FCR.

The initial and final body weight average for African catfish of all the treatments are provided in Table 10. No significant difference (P>0.05) in the initial and final body weight averages existed between any of the treatment groups. The overall (Day 0-91) period for survival, SGR, weight gain, body mass growth, ADG and RFI are provided in Table 11. Similarly, no significant difference in any of these production performance parameters existed between any of the treatment groups. The following performance parameters were calculated for each of the five sampling intervals, i.e. Day 0-18, Day 18-41, Day 41-56, Day 56-75 and Day 75-91, and includes: SGR, weight gain, body mass growth, cumulative FCR and RFI. These values are provided in Tables 12, 13, 14, 15 and 16, respectively. No significant differences were found for any of these parameters between any of the treatment groups.

Table 10 Initial and final body weight average of all treatment groups

	Control	Xylanase 100	Xylanase 150	Xylanase 200	P value
$\boldsymbol{BWave_i}$ **(g)**	562 ± 70.7	496 ± 14.0	471 ± 18.0	524 ± 19.3	0.399
$\boldsymbol{BWave_f}$ **(g)**	808 ± 52.7	821 ± 62.0	731 ± 30.0	786 ± 60.8	0.648

Data presented as mean ± SE. P>0.05 for all parameters.

Table 11 Summary of production performance parameters of all treatment groups between day 0 and day 91

	Control	Xylanase 100	Xylanase 150	Xylanase 200	P value
Survival (%)	94.4 ± 3.51	97.2 ± 2.78	86.1 ± 10.90	94.4 ± 5.56	0.844
SGR (%)	1.17 ± 0.014	1.19 ± 0.008	1.18 ± 0.005	1.17 ± 0.012	0.531
Weight gain (%)	56.3 ± 11.8	57.3 ± 4.04	55.5 ± 3.77	57.0 ± 16.0	0.585
Body mass growth $(\boldsymbol{g.g^{-1}})$	1.64 x 10^3 ± 235	1.78 x 10^3 ± 158	1.46 x 10^3 ± 68.9	1.82 x 10^3 ± 206	0.247
ADG	2.65 ± 0.58	3.59 ± 0.57	2.89 ± 0.19	2.95 ± 0.57	0.605
RFI	2.34 ± 0.14	2.31 ± 0.13	2.58 ± 0.07	2.39 ± 0.22	0.606

Data presented as mean ± SE. P>0.05 for all parameters.

Table 12 Summary of SGR of all treatment groups for all five sampling intervals

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	0.61 ± 0.16	0.60 ± 0.25	0.48 ± 0.12	0.02 ± 0.08	0.39 ± 0.10
Xylanase 100	0.80 ± 0.10	0.69 ± 0.15	0.72 ± 0.12	0.08 ± 0.05	0.66 ± 0.15
Xylanase 150	0.77 ± 0.10	0.67 ± 0.09	0.54 ± 0.05	0.11 ± 0.06	0.28 ± 0.28
Xylanase 200	0.72 ± 0.04	0.65 ± 0.16	0.48 ± 0.26	0.12 ± 0.07	0.29 ± 0.08
P value	0.636	0.984	0.597	0.675	0.279

Data presented as mean ± SE. P>0.05 for all parameters.

Table 13 Summary of weight gain of all treatment groups for all five sampling intervals

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	11.9 ± 3.15	15.8 ± 6.58	7.63 ± 1.99	0.34 ± 1.49	6.59 ± 1.79
Xylanase 100	15.6 ± 2.12	17.6 ± 3.88	11.5 ± 2.09	1.63 ± 0.88	11.2 ± 2.58
Xylanase 150	15.0 ± 2.01	16.8 ± 2.37	8.51 ± 0.74	2.18 ± 1.25	11.3 ± 4.26
Xylanase 200	13.9 ± 0.81	16.5 ± 4.31	7.84 ± 3.96	2.25 ± 1.27	4.78 ± 1.28
P value	0.658	0.994	0.601	0.682	0.196

Data presented as mean ± SE. P>0.05 for all parameters.

Table 14 Summary of body mass growth of all treatment groups for all five sampling intervals

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	359 ± 109	477 ± 211	313 ± 80.0	58.7 ± 52.5	209 ± 39.3
Xylanase 100	463 ± 64.0	617 ± 150	483 ± 112	160 ± 106	511 ± 147
Xylanase 150	433 ± 71.9	423 ± 153	314 ± 32.9	29.0 ± 46.7	405 ± 134
Xylanase 200	437 ± 28.6	535 ± 141	348 ± 155	101 ± 63.2	229 ± 58.4
P value	0.785	0.860	0.629	0.600	0.145

Data presented as mean ± SE. P>0.05 for all parameters.

Table 15 Summary of cumulative FCR of all treatment groups for all five sampling intervals

	Day 0-18	Day 0-41	Day 0-56	Day 0-75	Day 0-91
Control	1.71 ± 0.27	2.06 ± 0.27	2.67 ± 0.48	4.27 ± 0.71	5.38 ± 0.70
Xylanase 100	1.49 ± 0.15	2.14 ± 0.41	2.37 ± 0.39	3.53 ± 0.60	4.30 ± 0.54
Xylanase 150	1.76 ± 0.35	2.73 ± 0.57	3.00 ± 0.42	4.85 ± 0.79	5.56 ± 0.30
Xylanase 200	1.57 ± 0.10	2.29 ± 0.72	2.38 ± 0.31	3.68 ± 0.51	4.73 ± 0.57
P value	0.832	0.672	0.639	0.485	0.349

Data presented as mean ± SE. P>0.05 for all parameters.

Table 16 Summary of RFI of all treatment groups for all five sampling intervals

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	1.09 ± 0.09	1.55 ± 0.09	2.04 ± 0.10	2.57 ± 0.14	0.68 ± 0.05
Xylanase 100	1.12 ± 0.04	1.54 ± 0.07	1.94 ± 0.15	2.55 ± 0.17	0.66 ± 0.05
Xylanase 150	1.18 ± 0.04	1.65 ± 0.07	2.19 ± 0.11	2.71 ± 0.28	0.75 ± 0.04
Xylanase 200	1.12 ± 0.05	1.57 ± 0.06	2.03 ± 0.16	2.64 ± 0.26	0.67 ± 0.07
P value	0.731	0.704	0.622	0.956	0.612

Data presented as mean ± SE. P>0.05 for all parameters.

The reputed nutrient-enhancing effect of dietary xylanase in plant-based diets warrants more study as a functional feed additive in the aquaculture industry. Therefore, for the first time, dietary xylanase has been included as the sole enzyme in a fishmeal-free diet for *C. gariepinus*. The current trial could not demonstrate any beneficial effects on the growth performance of *C. gariepinus* as a result of xylanase supplementation. It is, however, difficult to determine the variable(s) responsible for the lack of significant growth effects in any aquaculture study involving exogenous enzyme supplementation due to the wide variability of factors that may have had an influence on the efficacy of the enzyme. Compared to the previous studies that will be discussed below, the following variables have been identified as being potentially related to the significantly similar growth effects seen in the current trial. These variables include fish species, enzyme combination, enzyme inclusion levels, feeding strategy, enzyme-substrate specificity, plant ingredients and size of the fish used, and/or a combination of one or more of these variables. Based on the relatively similar results obtained from the proximate analysis regarding the protein fraction of the treatments diets the discrepencies regarding the amino acid anlysis in the current trial may be attributed to the nonhomogeneous sampling and/or mixing of the feed samples prior to the amino acid analysis.

The fish used in the current study was significantly larger than fish used in similar exogenous enzyme studies. In the current study, the average initial weight of C*. gariepinus* was 513.00 ± 94.22 g. In one of the few other studies that involved the sole inclusion (0.1 - 0.417 g/kg) of xylanase, significant growth results were reported in juvenile (7.99 ± 0.02 g) Jian carp fed plant protein-enriched diets (Jiang *et al*., 2014). Larger fish, especially omnivorous fish such as *C. gariepinus*, may better utilise alternative plant-protein products, such as soybean meal and maize meal, due to their more developed gastrointestinal tract (GIT) and intestinal microbial community, compared to smaller, less mature fish. Furthermore, it is known that the maturation of the GIT and the intestinal microbial communities of animals improve as they grow older (Choct & Kocher, 2000). It is therefore possible that the NSPs present in the treatment diets in the current trial were highly digestible to the fish which may have masked the positive effects of xylanase on growth performance. Compared with the current study, the enzyme inclusion levels of the above-mentioned study were relatively similar. The author reported the optimal level of enzyme activity to be between 0.175 – 0.250 g/kg of dietary xylanase, which is similar to the inclusion levels used in the current trial. It is possible that the slower growth rate of the large fish in the current study may have had an effect on the efficacy of the enzyme treatments.

Leeson & Caston (1996) studied the effects of a multi-enzyme complex (Roxazyme) in diets of turkey and chicken broilers and found that the enzyme had a more significant effect on growth and carcass weight during the earlier part of the growth period. Similarly, the NSP-degrading effect of dietary enzymes had a less profound effect in older broiler chickens than on younger ones (Chesson, 2001), while the negative effects of soluble NSP are more noticeable in younger than older, more developed birds (Marquardt *et al*., 1979; Veldman & Vahl, 1994). Therefore, despite the high plant-protein component in the current diets, the combination of relatively mature fish and the inherent capability of *C. gariepinus* to utilise plant ingredients may have caused any possible action by the xylanase insufficient to cause a significant effect on growth performance. It is, therefore, possible that enhanced growth effects due to xylanase supplementation may be apparent in younger, smaller fish, although this has to be tested.

Most aquaculture studies involving xylanase, however, have incorporated it as part of a multi-enzyme complex and, therefore, it is difficult to distinguish the sole effect of xylanase in such studies. Dietary xylanase supplementation has shown to significantly improve the production performance of C*. gariepinus*, although these studies involved the addition of xylanase in combination with other exogenous enzymes (Babalola, 2006; Yildirim & Turan, 2010a). The size of the fish used in the above-mentioned studies were either of larvae size (Babalola, 2006) or had an average initial size of 46.32 ± 0.29 g (Yildirim & Turan, 2010a), while the inclusion levels used ranged from 0.1 g/kg and between 0.25 – 0.75 g/kg,

respectively. The separate inclusion of a multi-enzyme complex, aimed at hemicellulose, protease and α-galactosidase did not significantly affect the feed utilisation indices of rainbow trout, except when all of the enzymes were supplemented in combination did the protein efficiency ratio significantly improve (Farhangi & Carter, 2007). A similar effect of enzyme synergy has also been reported in diets of Japanese sea bass (Ai *et al*., 2007) and Caspian salmon (Zamini *et al*., 2014).

Similar significant effects on production performance were found in multi-enzyme complex supplemented diets of *Oreochromis spp*. (Lin *et al*., 2007; Yildirim & Turan, 2010b; Goda *et al*., 2012; Adeoye *et al*., 2016b; Hlophe-ginindza *et al*., 2016; Saputra *et al*., 2016; Maas *et al*., 2018), *C. carpio* (Vajargah *et al*., 2018), *S. canaliculatus* (Zhang *et al*., 2018), *S. salar* (Carter *et al*., 1994; Jacobsen *et al*., 2018), *L. rohita* (Ranjan *et al*., 2018), *D. sargus* (Magalhães *et al*., 2016), *L. japonicus* (Ai *et al*., 2007; Zhang *et al*., 2009), *H. huso* (Ghomi *et al*., 2012), *S. trutta caspius* (Zamini *et al*., 2014) and *P. crocea* (Zhang *et al*., 2006). All of the above-mentioned studies involved the supplementation of either multi-enzyme carbohydrase complexes or carbohydrase enzymes in combination with other enzymes, such as protease and/or phytase, which may have caused a synergistic effect between the different enzymes. The authors attributed the positive effects of growth performance to the increased activity of endogenous digestive enzymes, nutrient transport and absorptive processes, enhanced brush-border enzyme activities, and beneficial microbial communities. It was suggested that these effects may have been stimulated by the hydrolysis products formed by the multi-enzyme complexes, the improvement of the gastrointestinal tract, decreasing digesta viscosity and lowering of the intestinal pH levels.

The different plant ingredients and inclusion levels used in exogenous enzymes studies may influence the efficacy of an enzyme as it determines the enzyme-substrate relation. The enzymatic supplementation (a mixture of protease and carbohydrase) of a soybean meal diet (33% inclusion level) significantly enhanced the FCR compared to the control group. However, when a soybean meal-based diet (46% inclusion level) was supplemented with a combination of phytase and a multi-enzyme complex containing carbohydrase and protease, no significant differences in the feed utilisation were observed for Atlantic salmon (Carter, 1998). Although the basal diet of the current study contained only 30.17% soybean meal, other plant ingredients constituted a large portion of the rest of the diet, viz. maize meal (26%) and wheat bran (11.56%). Xylanases (and β-glucanase) have shown to have a more profound effect when added to diets with a high amount of soluble NSP, such as wheat, rye and barley diets, than in diets with a relatively low amount of these substances such as maize and SBM diets (Marquardt *et al*., 1994; Persia *et al*., 2002; Kocher *et al*., 2003; Willamil *et al*., 2012). Soybean meal contains lower amounts of viscous β-glucans, xylans and AX than cereal grains (Bach Knudsen, 1997) and may, therefore, have less

substrate for carbohydrase enzymes to hydrolyse. This is, however, dependent on the type of enzyme used and its inclusion level.

It has been showed that xylanases differ in their ability to degrade AX, depending on their structure and substrate affinity (Choct *et al*., 2004). Therefore, xylanases may either target the soluble or the insoluble fraction of NSP or even both, depending on its substrate specificity (Bartelt *et al*., 2002). Additionally, it has also been suggested that the digestibility status of raw materials used should be known as highly digestible plant ingredients may be more difficult to improve through the action of exogenous enzymes (Ogunkoya *et al*., 2006; Dalsgaard *et al*., 2012). Leenhouwers *et al*. (2007) found low NSP digestibility (4%) for a 400 g/kg wheat and relatively high NSP digestibility (48.4%) for 400 g/kg maize in diets for African catfish. They concluded that African catfish could better utilise the NSP fraction of maize that the NSP fraction of wheat. This supports the probability that the diets in the current feeding trial could have had a high digestibility concerning African catfish, hence not relying on the NSP-degrading mechanisms of xylanase.

The nutrient-enhancing effect of carbohydrase enzymes does not always result in improved growth performance. In diets of rainbow trout, a multi-enzyme complex significantly improved the apparent digestibility coefficient of dry matter, crude protein and gross energy, without significantly enhancing the feed utilisation of the fish (Farhangi & Carter, 2007). A similar scenario was reported by Dalsgaard *et al*. (2012) where dietary xylanase significantly improved the apparent digestibility of protein without it resulting in improved growth performance of rainbow trout. Reports pertaining the non-significant effect of exogenous enzyme addition on fish growth performance are not uncommon, even for carnivorous species which are likely to benefit most from such treatments. Similar non-significant production performance results were found in studies involving carbohydrase in diets of rainbow trout (Ogunkoya *et al*., 2006; Farhangi & Carter, 2007; Denstadli *et al*., 2011; Dalsgaard *et al*., 2012; Diler *et al*., 2012), gilt-head bream (Ayhan *et al*., 2008), silver perch (Stone *et al*., 2003), Nile tilapia (Yigit & Olmez, 2011; Mahmoud *et al*., 2014; Adeoye *et al*., 2016a), common carp (Kazerani & Shahsavani, 2011), Atlantic salmon (Carter, 1998) and mullet (Ramos *et al*., 2017).

Although it was not undertaken in the current study, future studies involving exogenous enzymes in aquaculture nutrition should aim to incorporate nutrient digestibility trials. Future studies should also aim to determine the enzyme substrate specificity in order to determine if the right enzyme strain is used in conjunction with the ingredients used in the trial. Digestibility trials, however, are often difficult to undertake manually (due to the often-unsuccessful stripping of fish for digesta samples) and future studies could well benefit from new and improved methods (such as automatic faeces collectors) for the determining of nutrient digestibility in fish feeding trials.

3.3.2. Meat proximate analysis

The meat proximate analysis, i.e. moisture, crude protein (CP), crude fat (CF) and ash, for all the treatment groups are provided in Table 17. No significant differences were found for any of the indices between any of the treatments.

Table 17 Summary of the body proximate analysis for all treatment groups ('As is' (g/100g))

	Control	Xylanase 100	Xylanase 150	Xylanase 200	P value
Moisture	74.9 ± 0.98	75.5 ± 0.91	75.0 ± 0.60	74.2 ± 1.49	0.840
CP	19.2 ± 0.42	19.0 ± 0.59	19.2 ± 0.43	19.5 ± 0.84	0.940
CF	3.00 ± 0.32	2.82 ± 0.46	2.91 ± 0.57	3.21 ± 0.51	0.945
Ash	1.18 ± 0.10	1.10 ± 0.04	1.17 ± 0.05	1.21 ± 0.17	0.996

Data presented as mean ± SE. P>0.05 for all parameters.

It is clear from Table 17 that the supplementation of dietary xylanase at inclusion levels of 100, 150 and 200 PPM did not significantly affect (positive or negative) the fillet proximate analysis of African catfish. In the current trial, fish fillet analysis was chosen rather than whole body analysis because of the consumer preference adhered to it. The lack of significant difference in body proximate analysis due to exogenous enzyme supplementation is not uncommon and have been reported in previous aquaculture studies. Similar, non-significant effects of dietary xylanase were also found on the body composition of Nile tilapia. Dietary xylanase had no significant effect on the dry matter, crude protein and lipid content of tilapia fed plant-based diets (Adeoye *et al*., 2016; Maas *et al*., 2018). Lin *et al*. (2007) found that addition of a commercial enzyme complex (protease, β-glucanase and xylanase) had no significant effect on whole body moisture, ash, protein and lipid fractions of hybrid tilapia. Similar non-significant effects of exogenous enzyme supplementation were also seen in Japanese sea bass (Zhang *et al*., 2009), large yellow croaker (Zhang *et al*., 2006) and Atlantic salmon (Jacobsen *et al*., 2018).

Differences between the analysis values found in the current trial compared to previous aquaculture-enzyme studies could be due to the following influencing factors: meat analysis (fillet vs whole body), fish species, size of fish, dietary constituents (absence of fishmeal and dietary lipid source in the current trial), and enzyme combinations and concentrations. The lipid content of the body proximate analysis in the current trial (2.36 – 3.72%) was somewhat lower than the values obtained from Yildirim & Turan (2010a), who found lipid values ranging from 2.12 – 5.72% when supplementing a multi-enzyme complex (Farmazyme®) to commercial trout diets of *C. gariepinus*. It is known that body fat percentage is closely related to the lipid content in a diet (Shearer, 1994) and, therefore, the low percentage of body fat observed in the meat proximate analysis could be attributed to the lack of the lipid content of

the diet used in the current trial compared to the 18% lipid fraction in the diet of the previous-mentioned study.

Dietary xylanase has shown to significantly affect protein efficiency ratio, net protein utilisation and, as a result, body protein content of fish. In diets of *C. gariepinus* supplemented with a multi-enzyme complex (Farmazyme®), fish that received treatments containing 0.5 and 0.75 g/kg of a multi-enzyme complex shown significantly higher body protein contents compared to a 0.25 g/kg enzyme treatment and a control group (Yildirim & Turan, 2010a). Similar results were reported on *O. aureus* where fish fed 0.5 and 0.75 g/kg of a multi-enzyme complex (Farmazyme®) had a significantly higher protein content than a 0.25 enzyme treatment and a control group (Yildirim & Turan, 2010b). The high inclusion of plant-based feedstuffs used in the current study may explain the lack of significant difference compared to the commercial, and highly digestible, diets used in the above-mentioned studies. The sole inclusion of dietary xylanase (0.25 g/kg), significantly increased the body protein content of juvenile Jian carp fed plant-based diets compared to the control group (Jiang *et al*., 2014). In the current trial, no significant differences were observed at 0.2 g/kg (highest inclusion level) and therefore, together with the larger, slower growing fish used in the current study, may have rendered the efficacy of xylanase on the meat proximate analysis of *C. gariepinus* unsuccessful. Results regarding the effect of xylanase addition on the meat proximate analysis may be more promising if smaller fish or higher inclusion levels are used, although this has to be studied.

The combination of more than one exogenous enzyme may have a synergetic effect upon the meat proximate analysis of fish. Statistically significant results were seen in *L. rohita* where the protein content of the body proximate analysis significantly increased after xylanase and phytase supplementation (Ranjan *et al*., 2018). Apart from increasing the protein content in fish, xylanase supplemented with phytase in diets of juvenile red tilapia, led to significant increases in fat, ash, phosphorous, calcium and gross energy between enzyme treatments and the control group (Wallace *et al*., 2016). Moreover, the supplementation of a carbohydrase enzyme complex (Natugrain® TS) significantly increased the protein and lipid content of white seabream fed the enzyme treatments, compared to the control (Magalhães *et al*., 2016). Significant increases in body ash and lipid content have been reported in diets of *L. rohita* fed diets supplemented with phytase and cellulase (Xavier *et al*., 2012). Furthermore, the addition of a multi-enzyme complex significantly increased the lipid deposition of mullet fed enzyme treatments compared to the control group (Ramos *et al*., 2017). Thus, the significant differences in the meat and mineral proximate analysis seen in the above-mentioned studies may have been due to a synergic effect between the enzymes used.

3.3.3. Visceral somatic index

The visceral somatic index (VSI) for all the treatment groups is provided in Table 18. No significant differences existed between any of the treatment groups.

Table 18 Summary of the visceral somatic index for all treatment groups (% of total body weight)

	Control	Xylanase 100	Xylanase 150	Xylanase 200	P value
VSI (%)	12.7 ± 1.20	12.8 ± 0.71	12.9 ± 1.03	11.5 ± 2.16	0.881

Data presented as mean ± SE. P>0.05 for all parameters.

For the first time, the effects of dietary xylanase on the VSI of African catfish has been studied. Although dietary xylanase treatment had no significant effect on the VSI in the current study, similar results have been reported on the hepatosomatic index and intestinal somatic index of Nile tilapia (Adeoye *et al*., 2016a), white seabream (Magalhães *et al*., 2016) and *L. rohita* (Ranjan *et al*., 2018) fed plant-based diets supplemented with carbohydrase enzymes. The separate and combined effect of exogenous enzymes (phytase, protease and xylanase) and probiotics (*B. subtilis, B. licheniformis* and *B. pumilus*) also yielded no significant impact on the hepatosomatic index and visceral somatic index of Nile tilapia (Adeoye *et al*., 2016b).

It is possible that the effect of dietary xylanase on the VSI of *C. gariepinus* in the current study may have been influenced by the low viscosity of the diet ingredients used, although this remains to be tested. It is known that maize or soybean meal-based diets have a relatively lower viscosity index compared to wheat or rye-based diets (Singh *et al*., 2012) and, therefore, the critical viscosity levels needed for xylanase to have a meaningful effect might have been absent. Although the viscosity of the ingredients was not determined in the current study, it is advised that future studies should report on the viscosity level of plant-based diets used.

The effect of dietary xylanase on the somatic indices in aquaculture studies are unclear. Xylanases supplementation have shown to both increase and decrease somatic indices. Carbohydrase enzymes, such as xylanase, are associated with improved nutrient utilisation in animals. This improved nutrient utilisation may be caused by an increase in the growth and development of intestinal digestive organs, such as the pancreas (Gisbert *et al*., 2004). Previous studies have shown that the supplementation of xylanase significantly increased the hepatosomatic index, hepatopancreas weight and hepatopancreas protein content of juvenile Jian carp fed plant-based diets (Jiang *et al*., 2014). Furthermore, Kumar *et al*. (2006) tested the effects of α-amylase supplemented in maize-based diets of rohu carp and found that the enzyme addition significantly increased the hepatosomatic index and liver glycogen.

Contrary to the above results, significant decreases in viscera ratio, hepatosomatic index and liver lipid were also found in hybrid tilapia fed plant-based diets supplemented with

a multi-enzyme complex (Lin *et al*., 2007). Highly viscous ingredients may cause digestive organs to enlarge due to slower digestive rates (Gao *et al*., 2008) and limit interaction between nutrients and digestive enzymes (Edwards *et al*., 1988; Ikegami *et al*., 1990). Therefore, in order to increase nutrient assimilation, more digestive secretions are produced which may cause the digestive organs to enlarge (Ikegami *et al*., 1990). Leenhouwers *et al*. (2006) investigated the effects of high NSP-containing guar gum-based feedstuffs on African catfish. They reported that the NSP-containing feedstuffs stimulated the weights of digestive organs, such as the stomach and intestines. Through improving nutrient digestion and alleviating high viscous solutions, xylanase may be able to induce a decrease in digestive organs such as the GIT and pancreas (Esteve-Garcia *et al*., 1997; Wu *et al*., 2004; Kies *et al*., 2005; Gao *et al*., 2008), reducing the energy needed for maintenance and homeostasis. However, in the current study, no such an effect was noted in the VSI in xylanase treatments of African catfish. The effect of exogenous enzyme supplementation on the VSI may also be attributed to different diets compositions, substrate-enzyme interaction, enzymes and the species involved.

3.3.4. Haematocrit and non-specific immunity analysis

Blood titers for determining the non-specific immunity include serum lysozyme activity, total serum protein and immunoglobulin levels. Serum lysozyme activity, total serum protein and immunoglobulin levels were measured at Day 0, 18, 41, 56, 75 and 91. Haematocrit was only measured at Day 0 and 91 and is expressed as packed cell volume (PCV). Sampling values for Day 0 are depicted as the pre-treatment values.

The values for haematocrit, serum lysozyme activity, total serum protein and immunoglobulin for the period between Day 0 (pre-treatment) and Day 91 (xylanase 0, 100, 150 and 200 treatments) are expressed in Table 19. Hence, these values are compared among the treatments before trial commencement and after the trial had been terminated. The rationale behind this is to see if any of the above-mentioned parameters might have been significantly influenced by any of the treatment groups over the 91-Day trial period in order to establish the impact of dietary xylanase supplementation on the innate immune response of *C. gariepinus*. Statistically significant differences in serum lysozyme activity and immunoglobulin levels did occur between Day 0 and Day 91, although no significant differences existed between the treatment groups. However, no significant differences were recorded for haematocrit or total serum protein between Day 0 and Day 91. Serum lysozyme activity, total serum protein and immunoglobulin levels sampled during the course of the trial are provided in Tables 20, 21 and 22, respectively. No significant differences existed for any of these parameters between the treatment groups.

Table 19 Summary of non-specific immunity and haematocrit values for all treatment groups between Day 0 and Day 91

	Pre-treatment	Control	Xylanase 100	Xylanase 150	Xylanase 200	P value
Haematocrit (% PCV)	36.4 ± 2.07	35.2 ± 2.48	33.8 ± 1.99	30.4 ± 1.89	34.4 ± 0.27	0.361
Lysozyme (µl/ml)	1.59 ± 0.19^{b}	5.70 ± 0.45^{a}	5.01 ± 0.28^{a}	5.72 ± 0.58^{a}	4.75 ± 0.36^{a}	0.000
Total protein (mg/ml)	15.1 ± 1.12	11.5 ± 0.78	13.3 ± 1.33	12.6 ± 0.88	12.5 ± 0.65	0.300
Immunoglobulin (mg/ml)	3.40 ± 0.28^{b}	6.17 ± 1.29^{a}	6.29 ± 0.79^{a}	7.20 ± 1.19^{a}	7.48 ± 0.50^{a}	0.000

Data presented as mean ± SE. P>0.05 for haematocrit and total protein. Statistically significant differences in serum lysozyme and immunoglobulin levels between treatments (P<0.05). Different superscripts (e.g. $^{a, b}$) in the same row indicate significant different means (P<0.05).

Table 20 Summary of serum lysozyme activity of all treatment groups for all five sampling dates

	Day 18	Day 41	Day 56	Day 75	Day 91
Control	2.31 ± 0.77	5.59 ± 0.36	5.70 ± 0.58	3.40 ± 0.92	5.70 ± 0.45
Xylanase 100	1.50 ± 0.43	5.01 ± 0.18	5.20 ± 0.45	3.34 ± 0.87	5.01 ± 0.28
Xylanase 150	2.05 ± 0.54	5.11 ± 0.38	4.86 ± 0.21	3.88 ± 0.77	5.72 ± 0.58
Xylanase 200	1.75 ± 0.30	5.29 ± 0.35	6.07 ± 0.78	3.63 ± 0.74	4.75 ± 0.36
P value	0.735	0.621	0.384	0.883	0.518

Data presented as mean ± SE. P>0.05 for all parameters.

Table 21 Summary of total serum protein of all treatment groups for all five sampling dates

	Day 18	Day 41	Day 56	Day 75	Day 91
Control	15.0 ± 1.40	10.9 ± 1.83	14.4 ± 0.71	16.6 ± 0.23	11.5 ± 0.78
Xylanase 100	16.3 ± 1.38	9.16 ± 1.86	14.2 ± 0.46	15.4 ± 0.76	13.3 ± 1.33
Xylanase 150	15.2 ± 1.68	8.73 ± 1.15	13.1 ± 1.32	15.7 ± 0.52	12.6 ± 0.88
Xylanase 200	15.5 ± 1.56	10.4 ± 1.32	13.2 ± 1.11	14.6 ± 0.94	12.5 ± 0.65
P value	0.881	0.744	0.757	0.153	0.595

Data presented as mean ± SE. P>0.05 for all parameters.

Table 22 Summary of immunoglobulin of all treatment groups for all five sampling times

	Day 18	Day 41	Day 56	Day 75	Day 91
Control	2.75 ± 0.25	3.83 ± 0.60	3.39 ± 0.58	2.14 ± 0.20	6.17 ± 1.29
Xylanase 100	3.36 ± 0.49	3.69 ± 0.82	3.85 ± 0.49	2.89 ± 0.28	6.29 ± 0.79
Xylanase 150	3.56 ± 0.46	4.30 ± 1.18	3.57 ± 0.39	2.22 ± 0.35	7.20 ± 1.19
Xylanase 200	4.35 ± 0.75	3.77 ± 0.51	3.04 ± 0.33	2.10 ± 0.43	7.48 ± 0.50
P value	0.221	0.970	0.651	0.295	0.733

Data presented as mean ± SE. P>0.05 for all parameters.

To the author's knowledge, this is the first study investigating the sole effect of xylanase on the non-specific immunity of African catfish. The innate-immunity of fish species is regarded as the first line of defence and therefore considered to be the more dependent immune response (Saurabh & Sahoo, 2008). The non-specific immunity response includes properties such as serum lysozyme activity, immunoglobulin and total protein. Blood is a good indicator of the health status, physiological responses to stress and the wellbeing of fish due to it being a pathophysiological reflector of the whole body (Goda *et al*., 2012; Adeoye *et al*., 2016a). Serum lysozyme activity and immunoglobulin levels significantly increased between Day 0 and Day 91 for all treatments, although no significant differences existed between the dietary treatments. It is, therefore, possible that the increase in these parameters might be due to the increase in body weight and/or maturity of the fish over the 91-Day period. However, the increase in immunoglobulin levels at Day 18 and Day 91, although not significant, seems to have a direct relationship towards the increasing xylanase levels (Table 22).

It is clear from Table 20 that the significant increase in serum lysozyme activity occurred between Day 18 and Day 41, compared to Day 0. It could be possible that the increase in serum lysozyme activity in all of the treatments during this period could have been caused by the feeding of a fishmeal-free and high plant ingredient-containing diet seeing that serum lysozyme activity is an effective way of measuring various stresses that may influence the animal, such as water quality, handling, sickness and nutritional stressors (Magnadotir, 2006; Kiron, 2012). The immunoglobulin levels of all the treatments, on the other hand, significantly increased between Day 75 and Day 91, compared to Day 0 (Table 22). Immunoglobulin acts as antibodies in the innate immune system and has shown to facilitate the fast and broad defence mechanism of the non-specific immune response in fish (Vilain *et al*., 1984; Gonzalez *et al*., 1988, 1989). Thus, it is clear that more antibodies were activated to facilitate the innate immune system during the end period of the trial.

The spike in the immunoglobulin levels of all treatments between Day 75 - 91 could be attributed to the continual deterioration of the water quality parameters, especially the ammonia and nitrite levels. Although African catfish are known to tolerate relatively high levels of ammonia (Ip *et al*., 2004), it has been advised that, for optimal African catfish production, the water ammonia and nitrite concentration should not exceed levels of 0.34 mg/L (Schram *et al*., 2010) and 0.6 mg/L (Roques *et al*., 2015), respectively. From section 3.3.2. it is stated that the water ammonia and nitrite levels amounted to an average of 6.11 ± 4.49 mg/L and 1.88 ± 1.52 mg/L, respectively, both concentrations well in excess of the prescribed maximum levels described above. The water nitrate levels of the current trial (average of 2.49 ± 1.81 mg/L), were, however, below the advised concentrations of 140 mg/L for the optimal production of African catfish (Schram *et al*., 2014). The water ammonia and nitrite levels experienced an exponential increase towards the end of the trial, possibly due to the large biomass of the system during this period which resulted in the increased accumulation of bio-waste.

Non-digestible oligosaccharides produced by enzymatic (xylanase) hydrolysis of NSP are known to stimulate the immune system in multiple ways (Kiron, 2012). The primary area of affection after NDO supplementation, and which may be the direct link between functional feed additives and the immune system, is the gut-associated lymphoid tissue (GALT). The intestinal microbiota and their metabolites, such as SCFAs, also play a crucial role in the immune system and may, through interacting with the GALT (Watzl *et al*., 2005), enhance non-specific immune responses (Bailey *et al*., 1991). Therefore, most of the effects of prebiotics on immunity parameters are indirect and can be attributed to the benevolent change in the intestinal microbiota which may directly enhance the immune response (Hoseinifar *et al*., 2015). However, since, in the current trial, there was no significant difference between the xylanase treatments and the control group regarding the innate immunity parameters tested, it remains unclear if the hydrolysis action of xylanase had produced enough NDOs to have any effect on the GALT or the intestinal microbiota in order to significantly enhance the non-specific immunity of African catfish.

Previous studies involving exogenous enzyme application in aquaculture diets and their effects on the innate immunity of fish is scarce while the limited amount of studies available yield variable results. In a study on Nile tilapia fed plant-based diets supplemented with a carbohydrase mixture (xylanase, β-glucanase and cellulase) administered at 300 mg/kg, the level of circulatory erythrocyte was higher in fish fed the carbohydrase treatment compared to the control diet, although the haematocrit and serum lysozyme activity were unaffected by the dietary enzyme treatments (Adeoye *et al*., 2016a). However, it has been reported that an increase in red blood cells may enhance the immune response of fish (Jiang *et al*., 2007). Similar results were seen when the separate inclusion of exogenous enzymes

(phytase, protease and xylanase) and the combination of enzymes and probiotics (*Bacillus subtilis, Bacillus licheniformis* and *Bacillus pumilus*) were added to diets of Nile tilapia. However, a significant increase in the serum lysozyme activity was seen with the sole inclusion of the probiotic treatment compared to the control group (Adeoye *et al*., 2016b). Zamini *et al*. (2014) found no significant difference in haematocrit values between exogenous carbohydrase enzyme treatments (Natuzyme® and Hemicell®) and a control treatment in diets of Caspian salmon. However, both enzyme treatments did stimulate the number of white blood cells and were therefore suggested to be able to enhance the innate immunity of Caspian salmon. The authors stipulated that multi-enzyme complexes containing enzymes such as β-mannans and xylanase are potent stimulators of the innate immune system, that may positively affect the numbers of macrophages, monocytes and cytokine production. The difference in the results obtained between these studies and the current study may be attributed to the combination of exogenous enzymes and probiotics used, the dietary constituents and the species involved.

3.3.5. Hindgut microbial diversity analysis

The alpha diversity is comprised out of the following indices: Shannon diversity, Simpson diversity and the species richness (based on the number of OTU's). The Shannon diversity, Simpson's diversity and species richness indices were compared between Day 0 (pre-treatment) and Day 91 (xylanase 0, 100, 150 and 200 treatments) and are provided in Table 23. The pre-treatment sampling was done at trial commencement (Day 0), while the sampling for the xylanase treatment groups was taken at trial termination (Day 91). This was to determine if any xylanase treatment may have had any significant effect on the alpha diversity parameters over the 91 Day period. Significant effects were found for the Shannon diversity and the Simpson diversity, however, no significanct differences existed for the species richness between Day 0 and Day 91. For the Shannon diversity, the pre-treatment group was significantly different from the control and the xylanase 150 treatment group, while significant difference also existed between the control and the xylanase 200 treatment group. Significant differences also existed for the Simpsons diversity index where the pre-treatment group had a significantly lower Simpson diversity score compared to the control and the xylanase 150 treatment groups.

Table 23 Summary of alpha diversity indices for all treatment groups between Day 0 and Day 91

	Pre-treatment	Control	Xylanase 100	Xylanase 150	Xylanase 200	P value
Shannon diversity	1.63 ± 0.27^{c}	2.78 ± 0.21^{a}	2.23 ± 0.21^{abc}	2.54 ± 0.65^{ab}	2.15 ± 0.13^{bc}	0.041
Simpson diversity	0.73 ± 0.06^{b}	0.90 ± 0.02^{a}	0.82 ± 0.04^{ab}	0.87 ± 0.03^{a}	0.81 ± 0.04^{ab}	0.050
Species richness	12.3 ± 3.93	28.5 ± 5.30	20.5 ± 3.85	23.5 ± 4.48	16.7 ± 2.11	0.134

Data presented as mean ± SE. P>0.05 for species richness. Statstically significant differences for Shannon diversity index and borderline significant differences for Simpson's diversity index. Different superscripts (e.g. $^{a, b}$) in the same row indicate significant different means (P<0.05).

The Beta diversity was calculated according to the Bray-Curtis dissimilarity index. The Bray-Curtis dissimilarity index for the xylanase trial is displayed in Figure 5 and is depicted as a cluster dendrogram. This index explains the difference in species similarity between treatment groups and is bounded between values 0 and 1, where 0 states that the relevant sites (treatments) have the same species composition and 1 state that the relevant sites do not have any species in common. Once again, the pre-treatment (Day 0) is compared to the xylanase treatments (Day 91), each denominated by the prefix of PT or V, respectively. This is to determine the effect of the treatment groups on the microbial species diversity over the 91-Day period. Figure 6 contains the meta-data additionally to the Bray-Curtis dissimilarity index. The dissimilarity (distance) between the treatment groups are, therefore, based on the Bray-Curtis dissimilarity index score. Figures 5 and 6 illustrate the significant difference in the species diversity between the pre-treatment (measured at Day 0) and the rest of the treatments (measured at Day 91). The permanova of the Bray-Curtis dissimilarity index for all the treatment groups between Day 0 and Day 91 is provided in Table 24. The permanova indicates that a significant difference exists in the beta diversity between the pre-treatment (Day 0) and the xylanase treatments (Day 91), although no significant differences existed between any of the xylanase and control treatments.

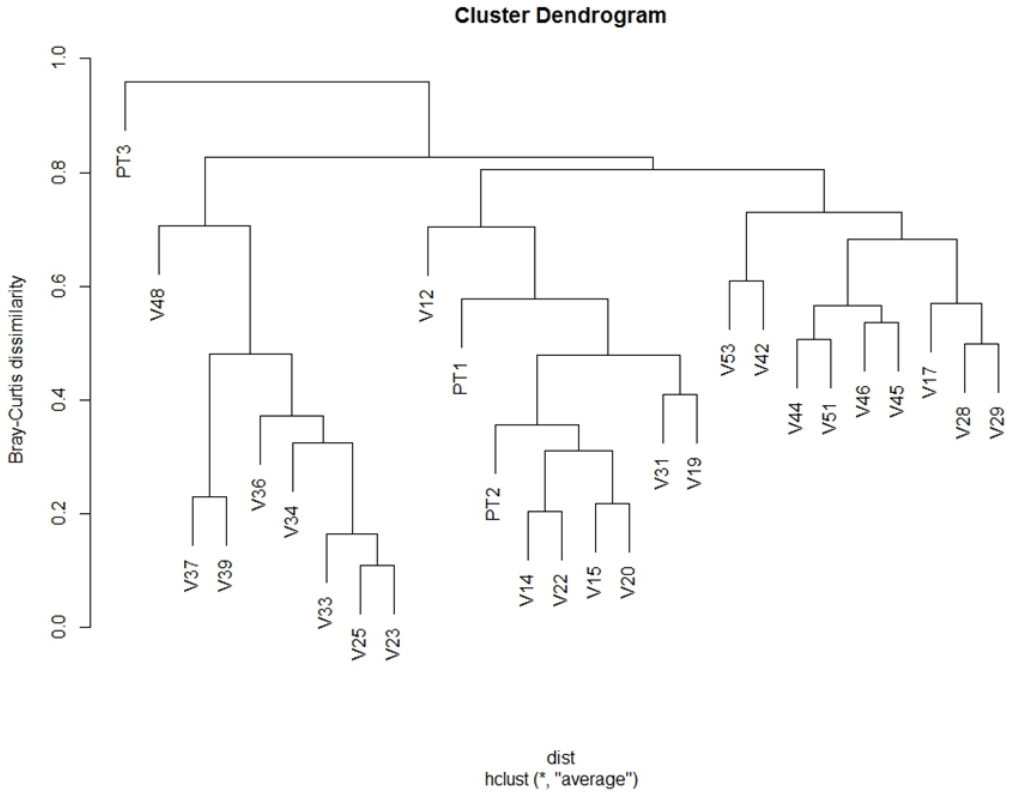

Figure 5 Cluster dendrogram of the Bray-Curtis dissimilarity between pre-treatment (PT) and dietary treatment groups (V)

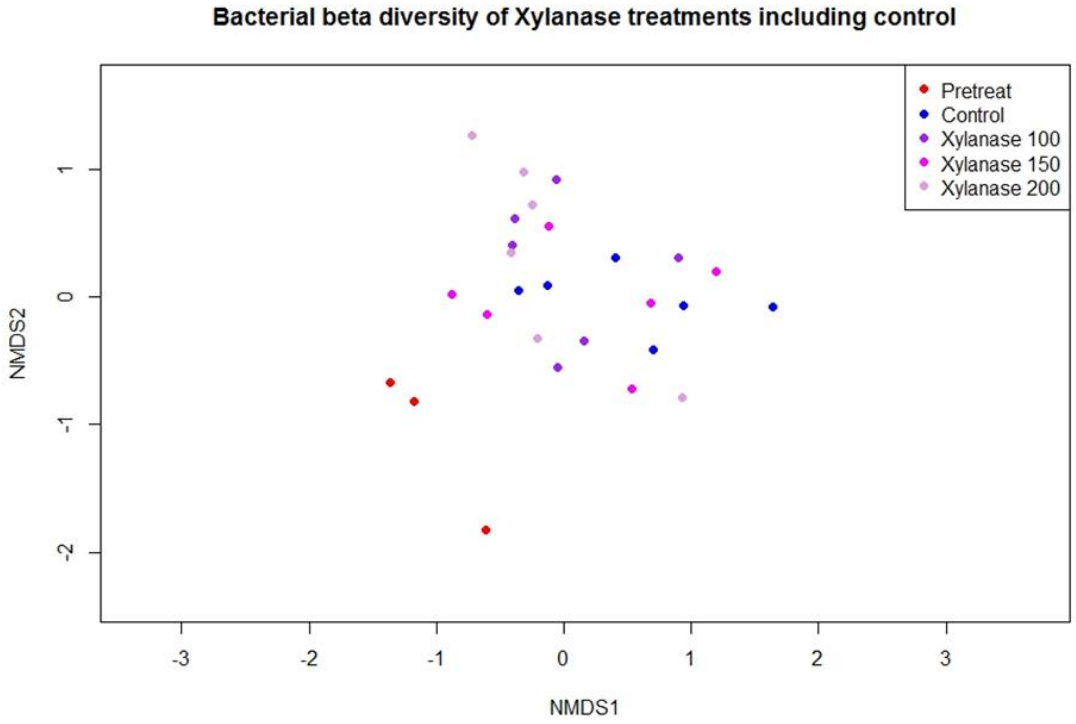

Figure 6 Bacterial beta diversity between pre-treatment (Day 0) and dietary treatment groups (Day 91)

Table 24 Permanova of Bray-Curtis dissimilarity index between pre-treatment (Day 0) and dietary treatment groups (Day 91)

	Degrees of freedom	Sum of Squares	F	Pr(>F)
Site	4	1.84	1.69	0.026
Residual	22	5.98	NA	NA

Statistically significant difference (P<0.05) in beta diversity index between Day 0 and Day 91.

There is clear evidence that the xylanase treatments affected the intestinal microbial biodiversity in the distal intestine of African catfish. Statistically significant differences were found for the alpha diversity indices which include the Shannon diversity index (P=0.041) and the Simpson diversity index (P=0.050). The Shannon diversity index showed significant differences between dietary treatments (Table 23). The Shannon diversity index is commonly used to determine species diversity in a community by measuring the degree of entropy of a community (Slabbert, 2008). Therefore, the higher the Shannon index the higher the richness of the specific species community. A significant difference in the Shannon index occurred between the control group and the xylanase 200 treatment (Table 23). Although there is no linear trend between species diversity and xylanase inclusion level, it is evident that the xylanase 200 treatment had a significantly lower species diversity compared to the control group. This suggests strong evidence that the xylanase 200 treatment resulted in a selective increase for a particular group of intestinal microbes while decreasing the presence of other, non-selective microbiota. The control group and the xylanase 150 treatment had a significantly higher Shannon diversity than the pre-treatment group.

Additionally, the control and xylanase 150 treatments had significantly higher Simpson diversity scores compared to the pre-treatment group (Table 23). The Simpson's diversity index tells us about bacterial dominance in a community and the higher the index value the greater the effect of dominance/degree of concentration in a community (Slabbert, 2008). This index gives us the value that ranges between 0 and 1, where 1 represents the greater sample diversity and 0 an absence of sample diversity. The result from the Simpson diversity index conforms with the result from the Shannon diversity index in that the control and xylanase 150 treatments had a significantly higher intestinal microbial diversity compared to the pre-treatment group.

To the author's knowledge, this is the first study investigating the effect of dietary xylanase on the hindgut microbial diversity of African catfish. Nevertheless, the microbial modulating effect of xylanase, seen in the current trial, corresponds with findings from previous aquaculture studies involving different species and exogenous enzymes. The microbial modulating effect of xylanase has only once been previously studied on juvenile Jian carp. Dietary xylanase significantly decreased the amounts of *E. coli* and *Aeromonas* (which are

regarded as potential fish pathogens, Ringø *et al*., 2010; Song *et al*., 2014; Torrecillas *et al*., 2014) in diets fed to juvenile Jian carp, while a significant increase was observed for *Lactobacillus* (generally assumed to be a beneficial intestinal organism, Burr *et al*., 2005; Song *et al*., 2014) up to a certain level of xylanase inclusion. The authors suggested that xylanase may affect the intestinal microbiota through the hydrolysis of arabinoxylans and the subsequent formation of degradation products (Jiang *et al*., 2014). It is known that these hydrolysis products, such as xylooligosaccharides and arabinoxylan-oligosaccharides, may act as fermentation substrates for health-benefitting bacteria such as lactobacilli and bifidobacteria (Van Laere *et al*., 2000; Garde *et al*., 2002).

Similar effects were seen in a study on Nile tilapia fed plant-based diets supplemented with a carbohydrase mixture (xylanase, β-glucanase and cellulase) supplemented at (300 mg/kg) where, through the means of permanova analysis, the intestinal bacterial community profile of the fish fed the carbohydrase treatment was significantly different from the bacterial community of the control group. However, treatments had no significant effect on the microbial diversity parameters. The authors suggested that the specific enzyme and diet composition used in the study may have caused the modulating effect upon the intestinal microbiota (Adeoye *et al*., 2016a). The addition of exogenous cellulase had a significant effect on the species density of the intestinal microbial community in grass carp fed duckweed and wheat flour diets compared to a control group. Cluster analysis showed that different bacterial species were found in the control group compared to the cellulase treatments, showing little similarity between the two groups. The authors suggested that the stimulatory effect of exogenous cellulase on endogenous digestive enzymes led to an increase in substrate specificity for certain microbial species (Zhou *et al*., 2013). However, contrasting results, that showed no microbial modulating effect, have also been reported regarding the use of exogenous enzyme application in aquaculture studies (Adeoye *et al*., 2016b).

The findings from the above-mentioned studies, as well as the results from the current study, indicate that exogenous enzymes, and in particular xylanase, have a modulating effect upon the intestinal microbial community of fish. This may be due to the NDO formation during enzymatic hydrolysis and the effect of these NDOs to stimulate the growth of specific carbohydrate fermenting microbes while inhibiting the growth of other potentially pathogenic bacteria in the GIT of fish. The intestinal microbiota plays a direct role in the immunity of fish and a beneficial gut community may improve FCR, growth rate, confer protection against pathogens and produce organic acids, such as SCFAs, antibiotics and lysozyme which may help to improve physiological and immunological responses in fish (Nayak, 2010). It is clear from the above-mentioned studies, that the intestinal bacterial community profile and the intestinal bacterial diversity parameters are not always intercorrelated. Thus, in order to have a more complete understanding of the effect of exogenous enzymes on the intestinal

microbiota, future studies should aim to evaluate both of these indices as opposed to only one.

Additionally, there is a clear shift in microbial diversity for all treatments between the beginning and towards the end of the trial. This is to be expected as, at the start of the trial, all treatments were shifted to a new, FM-free and high plant-ingredient diet, which is likely to impact on the intestinal microbiome. Previous studies have reported that diet may have an impact on the intestinal microbiota of fish (Ringø & Olsen, 1994; Yang *et al*., 2007; Cerezuela *et al*., 2012). The beta diversity index was determined using the Bray-Curtis dissimilarity index which measures the dissimilarity between the sampling unit and the group centroid by focusing on community structure variation (Bray & Curtis, 1957). The Bray-Curtis dissimilarity index is bound between values 0 and 1, where 0 indicates identical species similarity (same species composition) between the two sites and 1 indicates the complete lack of any similarity between the two sites (Figures 5 and 6). The permanova (Table 24) of the Bray-Curtis dissimilarity index, describing the beta diversity, also indicated that there is a significant difference in species diversity between the pre-treatment groups, measured at Day 0, and the rest of the dietary treatments, measured at Day 91. The significant difference found in the alpha and beta diversity indices shows that a definite increase in the microbial species diversity occurred between Day 0 and Day 91 (Table 23 and 24).

3.3.6. Short-chain fatty acid analysis

The analysis of SCFAs include acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid. The resulting SCFA concentrations for all the treatment groups are provided in Table 25. No statistically significant differences were found between any of the treatment groups.

Table 25 Summary of SCFA analysis for all the treatment groups (mM)

	Control	Xylanase 100	Xylanase 150	Xylanase 200	P value
Acetic acid	33.8 ± 3.07	29.5 ± 2.73	22.4 ± 4.78	30.6 ± 4.49	0.237
Propionic acid	4.04 ± 1.50	3.40 ± 1.23	1.09 ± 0.47	1.37 ± 0.45	0.138
IsoButyric acid	0.32 ± 0.14	0.13 ± 0.05	0.08 ± 0.03	0.12 ± 0.04	0.169
Butyric acid	7.06 ± 1.65	6.09 ± 1.44	3.36 ± 1.11	4.48 ± 1.53	0.313
IsoValeric acid	0.28 ± 0.12	0.09 ± 0.03	0.09 ± 0.03	0.12 ± 0.03	0.184
Valeric acid	0.03 ± 0.01	0.02 ± 0.01	0.01 ± 00	0.01 ± 00	0.117
Total SCFA	45.51	39.2	27.04	36.65	

Data presented as mean ± SE. P>0.05 for all parameters.

It is clear from Table 25 that dietary xylanase treatment had no significant effect on SCFA production. Short-chain fatty acid production is often used as a measurement of the response to an exogenous enzyme's supplementation in terrestrial animals (Bedford & Cowieson, 2012). However, to the author's knowledge, this is the only study that focused on the potential of any exogenous enzyme to alter SCFA production in any fish species. The total SCFA concentrations obtained in the current study were similar to other non-enzyme supplemented aquaculture studies involving African catfish. Leenhouwers *et al*. (2007) fed African catfish diets containing cereal grains (maize, wheat, barley and rye) with different viscosities and reported a total SCFA concentration of 25 - 34 mmol/L, whereas the total SCFA concentration of the present study was between 27.04 – 45.51 mmol/L (Table 25). Acetic acid had the highest concentration in all treatment groups followed by butyric acid, propionic acid, isobutyric acid, isovaleric acid and valeric acid, in a descending order. Acetic acid normally constitutes the majority (60%) of SCFAs produced (Clements & Choat, 1995; Kihara & Sakatag, 1997; Choct *et al*., 1999; Amirkolaie *et al*., 2006; Cloetens, 2009) followed by propionate and butyrate at 20% each (Wong *et al*., 2006), which is in accordance with the values obtained in the current trial. The low levels of butyrate and propionate corresponded with concentrations found in tilapia (Titus & Ahearn, 1988; Amirkolaie *et al*., 2006), but much lower than marine herbivorous species (Clements & Choat, 1995).

It is known that exogenous carbohydrase enzyme may alter the intestinal microbial community of fish by hydrolysing NSP into NDOs, which serves as a fermentable substrate to the carbohydrate fermenting bacteria (Bedford & Cowieson, 2012). The proliferation of beneficial, carbohydrate fermenting bacteria, such as lactic acid bacteria, increases the amounts of SCFAs being produced (Gibson, 1998; Manning & Gibson, 2004). Therefore, SCFAs may serve as indicators of the intestinal microbial community (Nisbet *et al*., 1996; Nisbet, 2002). However, although xylanase treatment significantly altered the intestinal microbial diversity, the effect of xylanase treatment on the SCFA production remains unclear. The absence of any significant effect of xylanase on the SCFA profile of African catfish may be attributed to the dietary constituents and their inclusion levels, the specific enzyme and fish species used. Due to these variables, discrepancies in literature exists.

The results from the current trial are mostly in contrast to other terrestrial studies that proved that dietary carbohydrase significantly increased SCFA concentrations. In broiler chickens, the addition of an enzyme (β-glycanase) in diets, containing a high amount of NSP, caused a decrease in the intestinal digesta viscosity as well as in SCFA production in the ileum of the broilers. The fermentation was rather shifted to the caeca of the chickens where SCFA production increased (Choct *et al*., 1996). In low-metabolizable energy wheat-based diets of broiler chickens, dietary xylanase addition significantly decreased the ileum fermentation rate while significantly increasing the rate of fermentation in the caeca of the

chickens fed the enzyme-treated diets (Choct *et al*., 1999). Similarly, dietary xylanase significantly increased caeca SCFA concentrations of broiler fed maize and wheat-based diets (Masey-O'Neill *et al*. 2014a). Contradictory results have also been reported where dietary xylanase supplementation in maize-soybean-based diets of broiler chickens, significantly decreased caeca SCFA production (Singh *et al*., 2012).

3.4. Conclusion

The relatively good growth performance parameters achieved in this study supports the feasibility of rearing *C. gariepinus* on diets completely devoid of fishmeal and fish oil despite the lack of significant growth improvements regarding dietary xylanase supplementation. The statistically similar results found for the growth and feed utilisation parameters may be attributed to the large size of the fish at trial commencement. The slower growth rate of the fish may have had a masking effect on xylanase supplementation. Dietary xylanase did, however, significantly decrease the hindgut microbial diversity based on an inclusion level dependency. Statistically significant differences regarding the Shannon diversity index were seen between the control and xylanase 200 treatments, whereas the latter had a less diverse microbial community than the former. Furthermore, regarding the same index, the control and xylanase 150 treatment groups also had a significantly higher score compared to the pre-treatment group. The xylanase 200 and xylanase 100 treatment groups had a statistically similar Simpson diversity score, whereas the control and the xylanase 150 treatment groups had a significantly higher Simpson diversity score compared to the pre-treatment group. Therefore, this study provides strong evidence that dietary xylanase treatment results in the selective benefit for a particular group of intestinal bacteria while inhibiting the growth of a greater number of other bacteria. Additionally, there is a clear shift in the microbial beta diversity for all treatments between the beginning toward the end of the trial. Similarly, significant increases in serum lysozyme activity and total immunoglobulin levels existed between the start and end of the trial, although no significant differences were reported between dietary treatments. The significant increase in the serum lysozyme levels of all the treatments occurred between Day 18 and Day 41. This is to be expected as, at the start of the trial, all treatments were shifted to a new, fishmeal-free and highly plant-based diet which is likely to have had an impact on the GIT microbiome. However, the significant increase in the total immunoglobulin levels occurred between Day 75 and Day 91. This increase in the immunoglobulin levels of all the treatments may be attributed to the worsening water quality during the end of the trial.

The results of this study merit further investigation regarding the microbial modulating effect of xylanase in plant-based diets of aquaculture species. Moreover, information regarding the link between innate immunity and the intestinal microbial community is

warranted. Therefore, in order to better understand the link between host health and its resident microbiota, it is recommended that future studies should consider using gnotobiotic environments during exogenous enzyme feeding trials. The use of smaller, faster-growing fish is also advised in order to potentially optimise the effect of dietary xylanase on African catfish growth performance and health status.

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Chapter 4

The effect of arabinoxylan-oligosaccharides on the production performance, non-specific immunity, hindgut microbial diversity and hindgut short-chain fatty acid production of African catfish, *Clarias gariepinus*, fed fishmeal-free diets

Abstract

Arabinoxylan-oligosaccharides (AXOS) are non-digestible oligosaccharides (NDOs) that have prebiotic properties and modulates the intestinal microbiota through acting as a fermentable substrate to specific, beneficial bacteria. In addition to indirectly enhancing the innate immunity by influencing the microbial species community and their fermentation products, AXOS may directly interact with the gut-associated lymphoid tissue (GALT), thereby stimulating the innate immune response. Brewer's spent grains, which are usually regarded as a waste product, were thermochemically processed by means of steam explosion and thereby used to produce AXOS-containing compounds. However, the high temperatures involved in the steam explosion processing of brewer's spent grains may also result in semi-antinutritional factors being produced. This study aimed to test whether AXOS, produced through the steam explosion of brewer's spent grains, can be used as a functional feed additive in aquaculture diets and if any positive or at worst a non-negative response could be achieved. A 91-day feeding trial was conducted to investigate the effects of an AXOS-containing component on the production performance parameters (growth performance and feed efficiency), fillet proximate analysis, visceral somatic index, innate immunity, hindgut microbial diversity and the production of SCFAs in the hindgut of African catfish fed highly plant-based diets. The trial consisted of four dietary treatments, each containing the same basal diet. The treatments included one control group and three test diets whereas each test diet was supplemented with an increasing level (0.3, 0.6 and 1.2%) of an AXOS-containing compound. All treatments were replicated six times and received six randomly placed, mixed sexed African catfish, with an initial average body mass of 547.3 ± 93.1 g (mean ± SD), at the start of the trial. Results from the trial indicated that the supplementation of an AXOS-containing compound had no significant effect (positive or negative) on the growth performance and feed efficiency of African catfish. Similar results were seen regarding the fillet proximate analysis, the visceral somatic index and the hindgut SCFA production. Statistically significant differences existed for the Simpson's microbial diversity index and serum lysozyme activity between the start and end of the trial. All dietary treatments had statistically higher Simpson's diversity scores and serum lysozyme activities at Day 91 compared to the initial samples taken at the start of the

trial. However, no significant differences regarding the Simpson's diversity index and serum lysozyme activity existed between the dietary treatments. Additionally, a numerically indirect relationship existed between the microbial species richness index and AXOS inclusion levels. Statistically significant differences existed for the immunoglobulin levels taken at Day 18 (P=0.013) and Day 75 (P=0.038) between dietary treatments. The AXOS 0.6 treatment differed significantly from the control and AXOS 0.3 treatments, while the AXOS 1.2 treatments had significantly higher immunoglobulin levels compared to the AXOS 0.3 treatment on Day 18. At Day 75, the AXOS 1.2 treatment had significantly higher (P=0.004) immunoglobulin levels than all the other treatments. From the results, it can be gathered that AXOS had no negative impact on the production performance and fillet proximate analysis of African catfish which showed that the thermochemical processing of brewer's spent grains can produce AXOS-containing compounds without the presence of semi-antinutritional factors such as polyphenolic furfural-like compounds. Furthermore, the supplementation of an AXOS-containing compound significantly improved the immunoglobulin levels of African catfish based on a dose-dependent manner.

4.1. Introduction

The use of antibiotics in fish feeds have become controversial due to the development of antibiotic-resistant bacteria, the elimination of intestinal microbial communities and possible antibiotic residues in the aquatic environment (Ringø *et al*., 2010). Prebiotics are seen as viable alternatives to enhance the immunity and growth of aquatic animals without the negative effects of antibiotics (Ringø *et al*., 2014). Prebiotics are feed ingredients that are indigestible to monogastric animals but provide beneficial effects to the host by being selectively fermented by favourable bacteria in the gastrointestinal tract (Gibson & Roberfroid, 1995; Roberfroid, 2007). Intestinal microbiota greatly influences the health (Gomez & Balcazar, 2008) and plays a crucial role in the nutritional efficiency of aquatic animals (Nayak, 2010a). Recently, NDOs have emerged as a new class of fermentable prebiotics that have shown to possess physiological and physiochemical properties (Cummings & Macfarlane, 1991; Manning & Gibson, 2004).

Non-digestible oligosaccharides are low molecular weight carbohydrates that are classified between simple sugars and complex polysaccharides. Dietary NDOs creates a shift in the intestinal bacterial community and involves the stimulation of beneficial, carbohydrate fermenting bacteria, such as lactobacilli and bifidobacteria, while simultaneously inhibiting the growth of potential pathogens and harmful, proteolytic fermenting bacteria (Macfarlane *et al*., 2006; Sang *et al*., 2011). Accompanying the increase of carbohydrate fermenters in the intestine is the higher production of SCFAs in the gastrointestinal tract (GIT) which acts as an additional energy source for the host and colonocytes alike (Rombeau & Kripke, 1990; Hamer

et al., 2008). Moreover, SCFAs may improve the absorptive qualities of the intestinal mucosa and, through lowering the intestinal pH levels, may increase mineral solubility and inhibit the growth of pathogenic bacteria (Topping & Clifton, 2001; Van Loo, 2004; Kolida & Gibson, 2011), which ultimately benefits the health of the host (Tremaroli & Backhed, 2012).

Arabinoxylan-oligosaccharides are the hydrolysis products of arabinoxylans (AX), the latter being one of the major non-starch polysaccharides (NSP) in cereal grains (Swennen *et al*., 2006; Grootaert *et al*., 2007). Arabinoxylan-oligosaccharides have recently been described as potential immunosaccharides that possess microbial- and immunomodulatory properties (Schley & Field, 2002; Swennen *et al*., 2006; Grootaert *et al*., 2007; Broekaert *et al*., 2011). Immunosaccharides may stimulate innate immunity in more than one way (Watzl *et al*., 2005). Fermentable oligosaccharides may directly activate the innate immunity of fish through the gut-associated lymphoid tissue (GALT) by activating carbohydrate receptors on leucocytes (Watzl *et al*., 2005; Song *et al*., 2014). Additionally, fermentable prebiotics may indirectly activate the innate immune response through stimulating the growth of beneficial, carbohydrate fermenting microbiota and their subsequent metabolites in the intestines of fish (Kocher, 2004; Roberfroid, 2005) resulting in improved disease resistance, immune response, growth performance and feed conversion efficiency (Nayak, 2010b; Ringø *et al*., 2014).

Dietary AXOS have been shown to stimulate the innate immune response, SCFA production and to modulate the intestinal microbial community of Siberian sturgeon (Delaedt *et al*., 2008; Rurangwa *et al*., 2008; Geraylou *et al*., 2012, 2013a; b). However, literature regarding the application of prebiotics in diets of catfish species is severely lacking. Despite the paucity of AXOS studies, the only other prebiotic substance incorporated into diets of catfish species is mannanoligosaccharides (MOS) (Welker *et al*., 2007; Peterson *et al*., 2010; Hernández *et al*., 2012). Although no significant improvements were seen regarding growth performance, immune response or feed conversion rate, mannanoligosaccharides (MOS) significantly enhanced the survival of channel catfish against the bacterium *Edwardsiella ictaluri* (Peterson *et al*., 2010).

The aim of the current trial was to determine the effects of AXOS at i) different dietary inclusion levels on the ii) production performance, iii) non-specific immunity, iv) hindgut microbial diversity and v) hindgut SCFA production of African catfish fed fishmeal-free diets.

4.2. Materials and methods

This section includes the experimental design, production of the dietary treatments, equipment used, experimental procedures followed, and the analytical methods used to determine the various parameters studied on African catfish. Parameters tested on African catfish include production performance parameters (growth and feed utilisation), fillet proximate analysis, visceral somatic index, non-specific immunity assays, haematocrit values, hindgut microbial

diversity status and hindgut SCFA production. All dietary treatments were subjected to a feed proximate analysis (Weende nutrient system) where moisture, ash, crude protein, crude fibre and crude fat values were obtained. African catfish fillet samples were subjected to a meat proximate analysis where moisture, ash, total fat and crude protein values were obtained. All diets and major feed ingredients were also subjected to an amino acid analysis. The proximate composition of feed and fish fillet samples were analysed according to the methods described by AOAC (2002).

4.2.1. Experimental design, animals and system

This research study applied and obtained ethical clearance from the Stellenbosch University Research Ethics Committee: Animal Care and Use (Protocol # 2820 ACU-2018-2820, attached in Appendix A) before the commencement of this trial and, therefore, all experimental procedures have been approved by the above-mentioned committee.

A 91-day feeding trial was designed to evaluate the effects of an AXOS-containing compound on the production performance, non-specific immunity, hindgut SCFA production and hindgut microbial species diversity of African catfish. The trial commenced on the 23rd of November 2017 and carried through until the 22nd of February 2018. This experiment was conducted in an indoor, heated, recirculating aquaculture system (RAS) located at Welgevallen Experimental Farm, Stellenbosch University. The system consisted out of 88 x 75-litre plastic tanks. Each tank received continuous aeration supply through a 1.1 kW air blower (Model SCL V4, Incorezzo, Milano, Italy). Ambient and water temperature were regulated through the workings of a thermostat (Danfoss EKC 102) in conjunction with two heating elements, an extraction fan and an air-conditioning system. Water temperature was maintained at 28°C; regarded as optimal water temperature suited for African catfish culture (Britz & Hecht, 1987). Recirculating water was continuously supplied to the tanks at a similar and constant flow rate (mean flow rate of 0.26 ± 0.07 (SD) L/s) and filtered using a static mechanical filter, followed by a trickling biofilter. Filters were cleaned and a third of the water in the system replaced every two weeks.

The experimental design consisted out of four dietary treatments containing the same basal diet. All treatments were supplemented with an increasing level of AXOS (i.e. 0, 0.3, 0.6 and 1.2%). Table 26 depicts the experimental design where AXOS 0% serves as the control treatment. All treatment groups were replicated six times. The African catfish was chosen as the preferred species due to their being a hardy, robust, omnivorous and easy to handle species with a large economic importance as a candidate species for the aquaculture sector in South Africa. Each tank received six randomly selected African catfish. A total of 144 mixed sexed African catfish were used with an initial mean body mass and length (± SD) of 547.3 ± 93.1 g and 426.9 ± 22.1 mm, respectively. All fish were sourced from registered hatcheries

and acclimatized to the RAS system for two weeks prior to trial commencement. During these two weeks, all fish received the same control feed. Fish were fed twice per day (09:00 and 16:00) at ad-libitum feeding levels depending on the water visibility. During periods of poor water visibility, fish were fed at a rate of 2% body mass per day which was determined every two weeks. This feeding rate was initially based on the 2% SGR of the fish at the time of trial commencement.

Table 26 Treatment design and AXOS inclusion levels

AXOS treatments	Inclusion levels (%)
Control	0.00
AXOS 0.3	0.30
AXOS 0.6	0.60
AXOS 1.2	1.2

4.2.2. Water quality parameters

Water quality parameters included water temperature, dissolved oxygen (DO), pH, total ammonia-nitrogen (nitrogen from NH_4^+/NH_3 mg/L), nitrite (NO_2^- mg/L) and nitrate (NO_3^- mg/L). Water temperature, DO and pH levels were measured daily, whereas total ammonia-nitrogen, nitrite and nitrate were measured twice a week. Water temperature and DO were measured using a YSI ProODO optical dissolved oxygen and temperature instrument, while pH was measured using a Hanna instruments pH 211 microprocessor meter. Total ammonia-nitrogen, nitrite and nitrate levels were measured using a Hach DR/850 colourimeter in conjunction with Hach permachem reagents. Mean water temperature, DO and pH level ± SD, was 27.7 ± 1.6°C, 6.02 ± 1.23 mg/L and 7.45 ± 0.43 respectively, which is within the acceptable range for catfish culture (Tucker, 1985; Viveen, 1985; Hoffman *et al*., 1991). Mean ammonia, nitrite and nitrate level ± SD, was 6.11 ± 4.49 mg/L, 1.88 ± 1.52 mg/L and 2.49 ± 1.81 mg/L, respectively. To help contain ammonia and nitrite levels at a low concentration, a third of the water in the system was exchanged every two weeks. Concurrent to the water exchange, filters were also cleaned every two weeks. During extreme spikes in ammonia and nitrite levels, a natural adsorbent silicate, zeolite, was used to absorb ammonia during high concentration fluctuations.

4.2.3. Feed ingredient and diet preparation

The basal diet was formulated by Nutritionhub (Pty) LTD, South Africa, using the Winfeed 2.8 (Winfeed Ltd, Cambridge, UK) formulation software, according to the nutrient requirements of African catfish (Table 3 and 4). The basal diet was designed to best elucidate the efficacy of AXOS by formulating a below-optimum digestible energy content diet based on information of previous studies (Table 5). The relevance of the complete substitution of fishmeal fits in with

the sustainable approach of today's aquafeeds but is also used to best elucidate the microbial- and immunomodulatory effect of AXOS in a somewhat nutrient challenging diet. Accordingly, the beneficial effects of AXOS should be most noticed against the backdrop of a low nutrient available and high ANF-containing diet. Even though African catfish is an omnivorous fish species known for its ability to utilise plant ingredients quite efficiently (Clay, 1981), high amounts of NSP may still have a negative or stunted effect on their health and growth performance.

Table 27 Feed formulation of basal diet

Ingredient	**%**
Soybean oilcake 46	30.16
Maize	26.00
Poultry by-product meal 65 (Nobless, TICSA)	15.04
Wheat bran	11.56
Blood meal	5.00
Lucerne meal (17% protein)	4.00
Monocalcium phosphate	2.90
Limestone	1.65
DL methionine	1.20
L-lysine HCl	1.18
VitMin Premix - Standard	0.80

Table 28 Feed proximate analysis of AXOS treatments ('As is' (g/100g))

Nutrient analysis	**AXOS 0 (Control)**	**AXOS 0.3**	**AXOS 0.6**	**AXOS 1.2**
Moisture	5.01	5.75	5.9	6.03
Crude protein	37.0	37.0	37.3	37.3
Crude fat	5.14	5.06	5.26	4.99
Crude fibre	3.84	3.89	4.07	3.78
Ash	11.1	10.6	9.84	9.57
NFE[a]	38.0	37.6	37.6	38.4
Gross energy (MJ/kg)[b]	17.2	17.2	17.3	17.3

[a]Nitrogen free extract was determined by difference as 100 – moisture – crude protein – crude fat – crude fibre – ash. [b]Gross energy determined by 23.4*crude protein + 39.8*crude fat + 17.2*carbohydrates

Fishmeal, normally being the major constituent of the protein fraction of a fish feed, was completely replaced by soybean oilcake, maize, wheat bran, alfalfa meal, blood meal and poultry by-product meal. Methionine and lysine were added to supplement the lacking essential amino acids in the diet. No oil (neither plant or animal origin) was used as a strategic reduction in digestible energy content of the feed with the aim of elucidating the positive effects AXOS on the health and growth parameters tested. The high starch content of the diet replaced the use of oil as the major pellet binding material. The basal diet information is given in Table 27. All treatment diets were subjected to a feed proximate analysis and amino acid analysis. The feed proximate analysis of all treatment groups is given in Table 28, while the amino acid analysis of the treatment groups and the major protein ingredients (soybean oilcake, blood meal and poultry by-product meal) is given in Table 29.

Table 29 Amino acid analysis of all treatment groups and major feed protein ingredients (% m/m dry solid)

	Control	A 0.3	A 0.6	A 1.2	PBP	BM	SBM
Histidine*	0.78	0.56	0.48	0.56	0.52	0.75	0.82
Serine	1.92	1.09	0.98	1.16	1.09	1.54	1.66
Arginine*	1.74	1.05	0.94	1.11	1.04	1.52	1.57
Glycine	2.85	2.48	2.35	2.68	2.54	3.49	3.92
Aspartic acid	2.17	1.14	1.09	1.25	1.13	1.64	1.81
Glutamic acid	5.03	2.66	2.45	2.73	2.58	3.77	4.10
Threonine*	1.13	0.67	0.70	0.68	0.67	1.05	1.12
Alanine	1.20	0.98	0.95	1.12	1.02	1.51	1.62
Proline	1.56	1.12	1.06	1.22	1.16	1.59	1.79
Lysine*	1.82	1.56	1.44	1.80	1.53	2.57	2.56
Tyrosine	2.06	1.14	1.08	1.28	1.21	1.67	1.83
Methionine*	0.88	1.36	1.25	1.48	1.33	1.70	1.66
Valine*	1.01	0.66	0.63	0.72	0.69	0.99	1.09
Isoleucine*	2.23	1.16	1.21	1.44	1.18	2.04	2.31
Leucine*	0.84	0.6	0.57	0.68	0.63	0.91	0.96
Phenylalanine*	2.26	1.52	1.41	1.57	1.56	1.80	1.91
Sum of EAA	12.69	9.14	8.63	10.00	9.15	13.3	14.00

*Essential amino acid. PBP: poultry by-product meal; BM: blood meal; SBM: soybean meal

The discrepencies regarding the amino acid anlysis in the current trial may be attributed to the nonhomogeneous sampling and/or mixing of the feed samples prior to the amino acid analysis. This rationale is based due to the relatively similar results obtained from

the protein fraction of the feed proximate analysis. All treatment diets were made at Welgevallen Experimental Farm, Stellenbosch, while most of the feed ingredients were sourced from local animal feed ingredient suppliers. Diets were formulated to be iso-nitrogenous and iso-caloric. The soybean oilcake, maize and maize-AXOS ingredients had to be put through a hammer mill (Drotsky S8) to achieve a more suitable particle size for extrusion. Diets were prepared by weighing and adding the appropriate amounts of each ingredient and mixed in 10 kg batches in a commercial dough mixer (Macadams baking systems SM-401). For each 10kg feed, three litres of water were gradually added while ingredients were being mixed until a homogenous paste was obtained. This paste was then extruded through a custom built single-screw extruder (Reomach Engineering, South Africa) with a 5 mm diameter die. The resulting pellets were then dried for 24 hours in a convection drying oven (CFW Envirowatch 5) at 60°C. After the pellets have been sufficiently dried they were stored in airtight containers and away from direct sunlight.

Liquid AXOS was produced through the thermochemical treatment (steam explosion) of a waste product (brewer's spent grains) by the Department of Process Engineering at Stellenbosch University (Álvarez et al., 2019). Brewer's spent grains were obtained as a waste product from a local beer brewery. The treatment of brewer's spent grains through steam explosion may have resulted in different nutritional fractions being processed due to the high temperatures involved. It is possible that semi-antinutritional factors, such as polyphenolic furfural compounds, may have been produced alongside positive compounds, viz. AXOS. It is, however, unclear if any antinutritional compounds were formed during the steam explosion process and, therefore, the product was evaluated based upon the response, positive or at worst a non-negative response, achieved. The positive oligosaccharide fractions and their amounts produced are given in a chromatogram attached as Appendix D.

To ensure ease of handling and to maintain the iso-nitrogenous and isocaloric status of the treatments, liquid AXOS were mixed with commercial maize meal to form an AXOS-maize blend that was used to substitute the maize fractions in the AXOS treatments. This AXOS-maize ingredient was obtained by soaking the maize with AXOS at the appropriate concentrations. This mixture was then dried in a convection drying oven (CFW Envirowatch 5) at 60°C for 24 hours. The dried ingredient was then milled using a hammer mill (Drotsky S8) to obtain a fine powder suitable for extrusion pelleting. The AXOS-maize ingredient replaced the maize fraction in the AXOS diets at the relevant inclusion levels to ensure a quantifiable measure of application of AXOS in the diets.

4.2.4. Experimental and sampling procedures

Fish were fed *ad-libitum* twice a day until visual observation of satiation to avoid uneaten feed being fed. During periods of poor water visibility fish were fed 2% of their tank average body

mass per day. The average body mass of the tanks was calculated every two weeks as necessary. The amount of feed used between every sampling period was calculated for each tank and used to calculate the performance parameters. Mortalities were removed daily and recorded accordingly. The experimental trial contained six sampling dates, including initial and terminal samplings, each occurring approximately every two weeks. All fish were fasted for a minimum of 12 hours prior to all sampling procedures to ensure accurate measurements as well as minimising stress during handling. Fish were anaesthetised with Tricaine Methanesulfonate (MS-222, Sigma) to ensure ease of handling and lowered stress levels before any sampling measurements were taken. The weighed and length of all fish were taken during all sample times. Care was taken to ensure that the same person responsible for a sampling parameter finished all the data capture subjected to that specific sample being taken. This minimized any variability that could have existed between different persons responsible for a specific sample being taken.

Growth performance parameters were determined at every one of the six sampling dates and included the individual weight and total length (from the tip of snout to the tail end) of all fish. Total length was measured to the closest mm on a measuring board while the body mass was recorded to 0.01 g on a balanced electronic scale (DIGI DS-673). Total number of fish per tank, total length, body mass and feed consumption were used to calculate the following growth parameters: survival, average daily gain (ADG), specific growth rate (SGR), weight gain, body mass growth, relative feed intake (RFI) and feed conversion ratio (FCR). These performance parameters were calculated as follows:

Equation 1: Survival (%) $= \frac{Nf}{Ni}\ x\ 100$

Where:

Nf = number of fish at day 91,

Ni = number of fish at day 1.

Equation 2: SGR (%/day) $= \frac{(Ln(Wavg_f) - Ln(Wavg_i))}{t}\ x\ 100$

Where:

$Ln(Wavg_f)$ = the natural logarithm of the final weight average,

$Ln(Wavg_i)$ = the natural logarithm of the initial weight average,

t = number of days between initial and final measurements.

Equation 3: Weight gain (%) $= \frac{(Wavg_f - Wavg_i)}{Wavg_i}\ x\ 100$

Where:

$Wavg_f$ = final weight average (g),

$Wavg_i$ = initial weight average (g).

Equation 4: Body mass growth (g) $= \left(BM_f + BM_{mortalities}\right) - BM_i$

Where:

BM_f = Body mass final (g),

$BM_{mortalities}$ = Body mass of mortalities (g),

BM_i = Body mass initial (g).

Body mass growth considers the weight of mortalities and can therefore be used as an adjusted value to correct for mortalities in calculations such as feed intake and FCR.

Equation 5: RFI $= \left(\frac{feed\ intake}{\left(\frac{\left(BM_f + BM_i\right)}{2}\right)}\right)/t * 100$

Where:

BM_f = Body mass final (g),

BM_i = Body mass initial (g),

t = number of days between initial and final measurements.

Equation 6: FCR $= \frac{Feed\ intake}{\left(\left(BM_f + BM_{mortalities}\right) - BM_i\right)}$

Where:

BM_f = Body mass final (g),

$BM_{mortalities}$ = Body mass of mortalities (g),

BM_i = Body mass initial (g).

The meat proximate analysis involved the sampling of fish fillets at the end of the trial. One randomly selected fish per tank was euthanized by subjecting to an overdose of MS-222 followed by the severing of the spinal cord. Fish were dissected, and the intestinal organs and the skin removed using a disinfected standard dissection kit. Fillets were cut from the spine and frozen at -20°C in plastic zip-lock bags until further analysis.

Samples for the determination of the visceral somatic index (VSI) were taken at trial termination. Two randomly selected fish per tank were euthanized by subjecting to an overdose of MS-222 followed by the severing of the spinal cord. Fish were dissected using a disinfected standard dissection kit. The intestinal organs were removed and weighed. The viscera weight was used as a ratio compared to the full body mass of the fish prior to dissection. VSI = 100 x (g wet intestinal weight/g wet body mass).

Blood samples for determining of non-specific immunity parameters were taken at each one of the six sampling dates: Day 0, Day 18, Day 41, Day 56, Day 75 and Day 91. At the start of the trial, Day 0, blood was taken from 17 randomly selected fish through caudal

vein puncture with a non-heparinized needle (21G) and a 3 mL syringe. All fish were anaesthetised with MS-222 prior to taking of blood. After sampling, fish were allowed to recover in aerated, clean water and returned to their original tanks. During the remaining five sampling dates blood was taken from two randomly selected fish per tank using non-heparinized needles (21G) and 3 mL syringes. All blood was left to clot for 24 hours at 4°C in untreated Eppendorf tubes (1.5 mL). Thereafter, blood was centrifuged using an A & E Lab mini centrifuge (MC 14) at 500 x g for 15 minutes. The supernatant was collected as the blood serum and stored at -20°C in Eppendorf tubes until further analysis.

Haematocrit values were obtained from fish blood collected at the start and termination of the trial using 21-gauge non-heparinized needles and 3 mL syringes. Micro haematocrit capillary tubes were filled to ¾ and sealed at the end with Critoseal. Blood from 17 randomly selected fish was taken (same fish as for innate immunity) and immediately centrifuged for 5 minutes using a micro haemocytometer. Haematocrit values were read and determined using a standard matrix. At the termination of the trial, blood from two randomly selected fish per tank was taken and immediately centrifuged using the same equipment and procedure as mentioned above. Haematocrit values are expressed as packed cell volume (PCV).

The microbial diversity analysis involved taking empty fish hindgut samples at Day 0 and Day 91 of the trial. Fish were fasted for 12 hours prior to sampling and euthanized by subjecting to an overdose of MS-222 followed by the severing of the spinal cord. At the start of the trial, three randomly selected fish were dissected using a disinfected standard dissection kit. The entire gastrointestinal tract was removed and unwound on a smooth disinfected surface to prevent tissue damage and infection. One section (± 2 cm) of the distal intestine (2-3 cm from the anus) was removed and placed in 10 mL sterile saline solution (9 g/L NaCl) containing acid-washed glass beads (Sigma, South Africa) and stored at -20°C until further analysis. At the end of the trial, one randomly selected fish per tank was dissected and used to collect one section (± 2 cm) of the distal intestine (2-3 cm from the anus) for microbial diversity analysis using the same procedures as described above and stored at -20°C until further analysis.

The SCFA analysis samples were taken from hindgut digesta at Day 91 of the trial. Fish were fed the morning before sampling to ensure adequate digesta in the hindgut. One randomly selected fish per tank was euthanized by subjecting to an overdose of MS-222 followed by the severing of the spinal cord. The fish was dissected using a disinfected standard dissection kit. The large intestine was carefully removed and unwound on a smooth and disinfected surface. Samples were obtained through gently stripping the hindgut digesta into plastic containers. Thereafter, the containers were properly sealed and kept at -20°C until further analysis.

4.2.5. Analytical procedures

The following section describes the analytical procedures and the materials used to analyse feed proximate analysis, feed and major feed ingredient amino acid compositions, meat proximate analysis, non-specific immunity assays, hindgut microbial diversity analysis and hindgut SCFA analysis.

I) Feed proximate analysis

Feed proximate analysis followed laboratory techniques that are based on the Weende nutrient system and involves the determination of crude protein, crude fat, crude fibre, moisture and ash fractions. The carbohydrate fraction was obtained as the difference of moisture + crude protein (CP) + crude lipid + crude fibre (CF) + ash subtracted from a 100. The gross energy values were obtained by multiplying CP by 23.4, crude lipid by 39.8 and the carbohydrate fraction by 17.2. Care was taken to present samples that were representative as well as homogenous. All feed samples were ground with a hammer mill (1.5 mm sieve) prior to analytical procedures and were measured in duplicate.

Moisture analysis was done according to the AOAC (934.01) International method for moisture analysis and involved drying the feed sample in an oven at 100 -105°C for 24 hours (AOAC, 2002). Thereafter, the moisture fraction was calculated as follows:

$$\%\text{Moisture} = \frac{(crucible + feed\ sample\ weight) - (moisture\ free\ sample + crucible\ weight)}{feed\ sample\ weight}$$

Ash analysis was done according to the AOAC International method for ash analysis (942.05) and involved placing the already moisture free sample in a furnace at 500°C for 6 hours (AOAC, 2002). Thereafter, the ash fraction was calculated as follows:

$$\%\text{Ash} = \frac{(ash\ weight - moisture\ free\ weight)}{feed\ sample\ weight}$$

Crude protein analysis was done according to the Dumas method using a LECO FP 528 (AOAC, 2002). The method involved the combustion of a sample at ± 900°C in the presence of oxygen, causing the release of carbon dioxide, water and nitrogen. The gases are then passed over columns that selectively absorb the carbon dioxide and water. A column containing a thermal conductivity detector at the end is then used to separate the nitrogen from any residual carbon dioxide and water whereafter the remaining nitrogen content is measured. The nitrogen concentration of the sample is then converted to crude protein content by using a conversion factor depending on the protein's origin. All feed ingredients, except soybean meal, used a nitrogen (N) conversion factor of 6.25, while soybean meal used a factor of 5.71. The crude protein fraction was calculated as follows:

%Crude protein = %nitrogen x protein conversion factor

Crude fibre analysis was determined gravimetrically according to the AOAC (962.09) International method for fibre analysis (AOAC, 2002). The analyses involved an Ankom fibre analyser and the addition of diluted sulphuric acid to remove free sugars and starch. Subsequently, sodium hydroxide was added to remove proteins and some carbohydrates. The resulting components left after acid and alkali hydrolysis are cellulose, hemicellulose (pentosans), lignin and pectic substances, in different ratios depending on the fibre source analysed. The crude fibre fraction was calculated as follows:

$$\%\text{Crude fibre} = \frac{(weight\ of\ organic\ matter - (bag\ weight\ x\ blanko))}{sample\ weight}\ x\ 100$$

Crude fat was determined by acid hydrolysis according to the AOAC (920.39) International method for fat analysis (AOAC, 2002). This involved the addition of hydrochloric acid followed by extraction of hydrolysed lipid materials with mixed ethers. After the ether evaporated, the residue was measured and expressed as %crude fat. The crude fat fraction was calculated as follows:

$$\%\text{Crude fat} = \frac{(weight\ of\ fat\ cup + fat) - (weight\ of\ fat\ cup)}{weight\ of\ sample}\ x\ 100$$

II) Amino acid analysis

The amino acid analysis was determined using the LC-MS method according to the AOAC (994.12) International method for feed AA analysis (AOAC, 2003). Feed samples were required to be hydrolysed prior to analysis in LC-MS lab. The hydrolysis involved mixing the sample with phenol and hydrochloric acid in a vacuum tube. Tubes were then hydrolysed in an oven at 110°C for 24 hours. After the hydrolysis, samples were transferred to Eppendorf tubes and sent to LC-MS lab for further analysis. All amino acid samples were measured in duplicates.

III) Meat proximate analysis

The following techniques were used to determine the meat proximate analysis and included moisture, ash, total fat and crude protein fractions. Care was taken to present samples that were representative as well as homogenous. Therefore, all feed samples were homogenised, prior to proximate and chemical analysis, using a meat blender. All meat samples were measured in duplicate.

Moisture was determined according to the AOAC (934.01) International method for moisture analysis (AOAC, 2002). Samples were placed in an oven at 100 – 105°C for 24 hours and measured as the difference between the before and after drying weight. Moisture was calculated as follows:

$$\%\text{Moisture} = \frac{(crucible + sample\ weight) - (moisture\ free\ sample + crucible\ weight)}{ample\ weight}$$

Ash was determined according to the AOAC (942.05) International method for ash analysis by placing the moist free sample in a furnace at 500°C for 6 hours and the residue weighed accordingly (AOAC, 2002). Ash was calculated as follows:

$$\%\text{Ash} = \frac{(ash\ weight - moisture\ free\ weight)}{meat\ sample\ weight}$$

Total fat was measured by mixing 50 mL of chloroform/methanol solution (1:2) with samples using a Bamix mixer. The mixture was then filtered, and the resulting solution mixed with 0.5% sodium chloride. After chloroform/methanol evaporation the residue was weighed accurately (Lee *et al*., 1996). Total fat was calculated as follows:

$$\%\text{Fat} = \frac{(weight\ of\ fat\ cup + fat) - (weight\ of\ fat\ cup)}{weight\ of\ sample}\ x\ \frac{chloroform\ volume}{5}\ x\ 100$$

Crude protein was determined using the Dumas method involving a LECO FP 528 according to the AOAC International method for protein analysis (AOAC, 2002). Samples used were already moist free. The method involved the combustion of a sample at ± 900°C in the presence of oxygen, causing the release of carbon dioxide, water and nitrogen. The gases are then passed over special columns that absorb the carbon dioxide and water. A column containing a thermal conductivity detector at the end is then used to separate the nitrogen from any residual carbon dioxide and water whereafter the remaining nitrogen content is measured. The nitrogen concentration of the sample is then converted to crude protein content by using a conversion factor depending on the protein's origin. The N conversion factor was taken as 6.25. Crude protein was calculated as follows:

%Crude protein = %nitrogen x protein conversion factor of 6.25

IV) Non-specific immunity assays

Non-specific immunity assays involved the analysis of serum lysozyme activity, serum total protein and total immunoglobulin. All non-specific immunity parameters were done in duplicates. Additionally, each duplicate was measured in triplicate on the microplates.

Serum lysozyme activity was determined spectrophotometrically according to (Sankaran & Gurnani, 1972), where 50 µL blood serum was added in triplicates to a 250 µL suspension of 0.075% (w/v) *Micrococcus lysodeikticus* (Sigma, M3770) and a phosphate buffer (0.05 M at pH 6.2), in a 96-well microplate. Absorbance was read after 5 minutes and again after 20 minutes at 530 nm using a microplate reader (BioTek ELx 800™). During the interval between readings, the microplate was kept a stir using a microplate shaker. A standard solution was prepared by using standard lysozyme (Sigma, L6876) instead of the blood serum as described above. The difference in absorbance at 5 and 20 minutes of the standard solution was used to draw a standard curve. Consecutively, blood serum lysozyme concentration was calculated from the standard curve.

Serum total protein was determined spectrophotometrically using the linearized Bradford assay (Zor & Selinger, 1996) where a protein standard (Lysozyme, Sigma, L6876) was used to prepare a standard curve using known concentrations. Protein standard and blood serum concentrations were prepared using 0.85% NaCl solution as a dilution agent. 50 µL of standard/serum was added, in triplicates, with 200 µL of Bradford dye reagent to a 96-well microplate. The absorbance was measured at 450 nm and 630 nm. Serum total protein concentrations were calculated from a standard curve based on the ratio of absorbances (630 nm/450 nm).

Immunoglobulin concentrations of blood serum were determined using the method of Siwicki *et al*., (1994). Blood serum and 12% (w/v) polyethylene glycol (PEG, Sigma, with 10 000 Dalton average molecular weight) were added, in triplicates, in equal weights (100 µL of each) to Eppendorf tubes and left to incubate at room temperature for 2 hours. Tubes were then centrifuged at 14 000 rpm for 5 minutes, resulting in a white pellet forming at the bottom of the tube. The PEG pellet was re-solubilised with 0.85% (w/v) NaCl, maintaining the same total volume, and was used to determine the total immunoglobulin concentration, using the linearised Bradford method as described above.

V) Hindgut microbial diversity analysis

For the microbial diversity analysis, DNA was extracted from fish hindgut samples using a Quick-DNA Fungal/Bacteria miniprep kit (Zymo Research, California, USA). Hindgut samples were directly put into plastic tubes containing acid-washed glass beads (Sigma, South Africa) and 10 mL sterile saline solution (9 g/L NaCl). Tubes were kept at -20°C until further analysis. The microbial diversity analysis was determined by the Department of Microbiology at Stellenbosch University, South Africa. The microbial community structure was quantified using Automated Ribosomal Intergenic Spacer Analysis (ARISA). The ARISA method is used as a community fingerprinting method and is based on polymerase chain reaction (PCR) where fluorescently labelled primers are used to amplify the 16rRNA region of the specific microbial sample. Resultingly, different fragment lengths are produced that are separated using capillary electrophoreses.

A genetic analyser (ABI 3010XL) was used to obtain electropherograms containing different fragment lengths and fluorescent intensity. An internal standard (LIZ1200) was used to run the bacterial samples on ARISA. Different DNA fragment lengths were obtained from the fluorescent data using Genemapper 5 software. These different DNA fragment lengths, suggesting different species, and are also known as operational taxonomic units (OTU's). Peak heights obtained from the Genemapper 5 software are plotted against the standard curve to calculate fragment lengths. Fragments sizes needed to be between 100 and 1000 base pairs in length and above 150 fluorescent units in height to be used for analysis as OTU's.

Inaccuracies of the ARISA profile was minimized using a bin size of 3 base pairs. The analysis includes the calculation of species richness and alpha and beta diversity indices that are used to compare microbial communities with one another (Brown *et al*., 2005; Slabbert *et al*., 2010).

Species richness is an indication of the relative species at a particular sampling site and was determined as the number of unique OTU's of each sample. Alpha diversity is the diversity of a specific group of organisms within a specific sampling unit and was determined, for each site, using the Shannon-Weaver (H) and the Simpson's diversity (D) index. The Shannon diversity index is commonly used to determine species diversity in a community by measuring the degree of entropy of a community (Slabbert, 2008). The higher the Shannon index the higher the richness of the specific species community. However, the Shannon index is not a linear measure of diversity, and can be calculated as follows (Krebs, 1989):

Equation 7: The Shannon-Weaver index

$$H = -\sum_{i=1}^{S} Pi \ln Pi$$

Where:

- Pi is the proportion of organisms found in species i,
- S is the total number of species found,
- Σ is the sum of species 1 to S,
- ln is the natural logarithm.

The Simpson's diversity index tells us about bacterial dominance in a community. The higher the Simpson index the greater the effect of dominance/degree of concentration in a community. This index gives us the value that ranges between 0 and 1, where 1 represents the greater sample diversity and 0 an absence of sample diversity. The Simson index is based on the more abundant species in a community and is therefore not dramatically influenced by the presence or absence of minor species (Slabbert, 2008), and can be calculated as follows:

Equation 8: The Simpson index

$$D = 1 - \frac{\Sigma n(n-1)}{N(N-1)}$$

Where:

- Σ is the sum of,
- n is the total number of organisms of a particular species,
- N is the total number of organisms of all species.

Beta diversity is defined as the variation of species composition among sampling units (Anderson *et al*., 2006). Beta diversity is measured as the average distance, or dissimilarity, from a specific entity to the group mean (Anderson *et al*., 2006). The Bray-Curtis measure is used to measure the dissimilarity between the sampling unit and the group centroid by

focusing on community structure variation (Bray & Curtis, 1957), and can be calculated as follows:

Equation 9: The Bray-Curtis dissimilarity

$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$$

Where:

- Cij is the sum of the lesser values for the species that are present at both sites,
- Si and Sj are the total numbers of species counted at site i and j respectively.

The Bray-Curtis dissimilarity index is bound between values 0 and 1, where 0 indicates identical species similarity (same species composition) between the two sites and 1 indicates the complete lack of any similarity between the two sites. The Bray-Curtis dissimilarity index has been extensively used to determine the beta diversity in microbial studies (Danovaro *et al*., 2006; Wood *et al*., 2008b; a; Wakelin *et al*., 2010).

VI) Short-chain fatty acid analysis

Fish hindgut digesta samples were deproteinized and the sugars removed following a procedure modified from Siegfried *et al*. (1984). Prior to analysis, 1 mL of digesta samples were homogenized by adding 1 mL of de-ionized water and were then centrifuged for 10 min at 4 000 rpm at 4°C. Out of this mixture, 1.5 mL was transferred to a 2 mL Eppendorf tube. The mixture was again centrifuged at the same specifications where after 600 µL of the supernatant was used as the sample representative. These samples were then mixed with calcium hydroxide and cupric sulphate solutions to remove proteins and sugars, respectively. Thereafter, samples were mixed with sulphuric acid and diethyl ether and the supernatant collected for analysis via gas-liquid chromatography (Agilent 6890 N GC - FID). A standard curve was created using a SCFA stock standard solution to calculate the amount of SCFAs in the samples. Data was analysed on gas chromatography (GC) ChemStation. The instrument and method details of the GC procedure are provided in Appendix C.

4.2.6. Statistical analysis

The experimental design of the study represented that of a completely randomised design with treatment as the main effect. The data of the study were compared using a one-way analysis of variance (ANOVA) and descriptive statistics. All data were analysed using Statistica version 13.3 (Tibco Software Inc.). The assumptions for normality and homogeneity were checked and tested using the Shapiro-Wilk's test and Levene's tests, respectively. At instances where the homogeneity assumption was not tenable, Welch's test was used to ensure the accuracy of the analysis. At instances were the normality assumption was not tenable, Kruskal-Wallis

was used as the preferred non-parametric test and performed on the residuals of the variables. Differences were regarded as significant when P<0.05, and Fischer's LSD post-hoc test was used to indicate significant differences between treatment means. Pre-treatment variables were included in the non-specific immunity and microbial analysis and were subjected to a one-way ANOVA. All data are presented as means ± standard error (SE). Average daily gain (ADG) was calculated by use of a linear regression of body mass compared to the time for each treatment. The gradient of each regression was then used to represent ADG. One-way ANOVA was used to compare ADG between treatments.

4.3. Results and discussion

4.3.1. Production performance parameters

The following production performance parameters were evaluated to determine the growth of African catfish fed diets containing different AXOS inclusion levels and include: $BWavg_i$, $BWavg_f$, survival, SGR, weight gain, body mass growth, ADG, RFI and cumulative FCR.

The mean initial and final body weights of all fish used in all the treatment groups are provided in Table 30. No significant differences were found between initial or final mean body weight for any of the treatments. Survival, SGR, weight gain, body mass growth, ADG and RFI for the overall trial period of 91 days are provided in Table 31. Similarly, no significant differences existed between any of these parameters between any of the treatment groups. SGR, weight gain, body mass growth, RFI and cumulative FCR for all five sampling periods are presented in Tables 32, 33, 34, 35 and 36, respectively. No statistically significant differences in any of these parameters were found.

Table 30 Initial and final body weight average of all treatment groups

	Control	AXOS 0.3	AXOS 0.6	AXOS 1.2	P value
$\boldsymbol{BWave_i}$ **(g)**	562 ± 70.7	560 ± 16.2	500 ± 24.7	568 ± 12.9	0.582
$\boldsymbol{BWave_f}$ **(g)**	808 ± 52.7	811 ± 70.0	830 ± 54.1	809 ± 37.7	0.990

Data presented as mean ± SE. P>0.05 for all parameters.

Table 31 Summary of production performance parameters of all treatment groups between Day 0 and Day 91

	Control	AXOS 0.3	AXOS 0.6	AXOS 1.2	P value
Survival (%)	94.4 ± 3.51	88.9 ± 8.24	72.2 ± 15.3	80.6 ± 13.2	0.748
SGR (%)	1.17 ± 0.014	1.16 ± 0.013	1.19 ± 0.007	1.16 ± 0.01	0.284
Weight gain (%)	56.3 ± 11.8	58.5 ± 8.54	66.1 ± 6.55	49.2 ± 4.93	0.306
Body mass growth ($g.g^{-1}$)	1.64 x 10^3 ± 235	1.61 x 10^3 ± 302	1.74 x 10^3 ± 179	1.46 x 10^3 ± 184	0.858
ADG	2.65 ± 0.58	2.89 ± 0.66	3.71 ± 0.45	2.70 ± 0.49	0.508
RFI	2.34 ± 0.14	2.42 ± 0.27	2.49 ± 0.14	2.39 ± 0.13	0.941

Data presented as mean ± SE. P>0.05 for all parameters.

Table 32 Summary of SGR for all treatment groups during the five sampling periods

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	0.61 ± 0.16	0.60 ± 0.25	0.48 ± 0.12	0.02 ± 0.08	0.39 ± 0.10
AXOS 0.3	0.69 ± 0.08	0.71 ± 0.07	0.33 ± 0.16	0.14 ± 0.05	0.17 ± 0.15
AXOS 0.6	0.72 ± 0.07	0.86 ± 0.13	0.59 ± 0.13	0.16 ± 0.10	0.36 ± 0.13
AXOS 1.2	0.57 ± 0.08	0.68 ± 0.09	0.51 ± 0.19	0.21 ± 0.08	0.34 ± 0.26
P value	0.730	0.679	0.677	0.403	0.777

Data presented as mean ± SE. P>0.05 for all parameters.

Table 33 Summary of weight gain for all treatment groups during the five sampling periods

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	11.9 ± 3.15	15.8 ± 6.58	7.63 ± 1.99	0.34 ± 1.49	6.59 ± 1.79
AXOS 0.3	13.3 ± 1.54	17.8 ± 1.82	5.28 ± 2.48	2.68 ± 1.03	5.04 ± 1.29
AXOS 0.6	13.8 ± 1.47	22.3 ± 3.49	9.28 ± 2.17	3.12 ± 2.06	6.07 ± 2.31
AXOS 1.2	10.9 ± 1.53	17.1 ± 2.35	3.40 ± 5.25	4.11 ± 1.61	8.79 ± 4.48
P value	0.737	0.678	0.603	0.409	0.814

Data presented as mean ± SE. P>0.05 for all parameters.

Table 34 Summary of body mass growth for all treatment groups during the five sampling periods

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	359 ± 109	477 ± 211	313 ± 80.0	58.7 ± 52.5	127 ± 87.9
AXOS 0.3	442 ± 44.0	685 ± 75.9	216 ± 140	120 ± 55.5	233 ± 60.4
AXOS 0.6	416 ± 49.5	557 ± 127	369 ± 105	26.3 ± 60.8	170 ± 60.2
AXOS 1.2	406 ± 33.8	637 ± 103	156 ± 241	158 ± 74.8	168 ± 64.8
P value	0.845	0.751	0.766	0.448	0.827

Data presented as mean ± SE. P>0.05 for all parameters.

Table 35 Summary of RFI for all treatment groups during the five sampling periods

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	1.09 ± 0.09	1.55 ± 0.09	2.04 ± 0.10	2.57 ± 0.14	0.68 ± 0.05
AXOS 0.3	1.05 ± 0.03	1.54 ± 0.06	1.99 ± 0.12	2.58 ± 0.28	0.71 ± 0.10
AXOS 0.6	1.16 ± 0.05	1.62 ± 0.08	2.20 ± 0.18	2.62 ± 0.36	0.65 ± 0.06
AXOS 1.2	1.16 ± 0.05	1.46 ± 0.07	2.04 ± 0.13	2.55 ± 0.14	0.71 ± 0.05
P value	0.593	0.543	0.721	0.998	0.880

Data presented as mean ± SE. P>0.05 for all parameters.

Table 36 Summary of cumulative FCR for all treatment groups during the five sampling periods

	Day 0-18	Day 18-41	Day 41-56	Day 56-75	Day 75-91
Control	1.71 ± 0.27	2.06 ± 0.27	2.67 ± 0.48	4.27 ± 0.71	5.38 ± 0.70
AXOS 0.3	1.61 ± 0.17	1.96 ± 0.17	2.91 ± 0.45	4.35 ± 0.76	6.07 ± 1.11
AXOS 0.6	1.75 ± 0.27	2.24 ± 0.28	2.86 ± 0.55	4.52 ± 0.99	4.49 ± 0.59
AXOS 1.2	1.72 ± 0.12	2.03 ± 0.22	3.81 ± 1.22	4.20 ± 0.51	5.45 ± 0.46
P value	0.970	0.849	0.728	0.992	0.584

Data presented as mean ± SE. P>0.05 for all parameters.

The current trial indicated that the AXOS ingredient derived from brewer's spent grains through thermochemical processing showed no impact on the production performance of the fish. Reports where AXOS addition exerted no beneficial effect on growth performance in aquaculture studies are not uncommon. However, it is possible that the lack of growth effects may be related to differences in dietary ingredients, the initial size of the fish and possible differences in the characteristics and inclusion levels of the AXOS compound used. It is known that older animals with a more developed gut microbial community are able to utilise NSPs in feedstuffs more efficiently than younger animals with a less developed bacterial community (Choct & Kocher, 2000; Refstie *et al.*, 2006). It is, therefore, possible that the NSPs present in the treatment diets were highly digestible to the fish which may have masked the positive effects of AXOS on growth performance. Although the fish used in the current trial were significantly larger (547.28 ± 93.09 g) than fish used in previous prebiotic studies on African catfish (ca. 20 - 50 g), the results of the current trial are in accordance with most other studies that tested the effects of AXOS supplementation on the growth of Siberian sturgeon and African catfish species (Rurangwa *et al*., 2008; Geraylou *et al*., 2012, 2013a).

It is known that slow growth rate accompanies larger size fish and, therefore, the slow growth and maturity of the fish used in the current study may have influenced the effect of AXOS on growth performance. Based on the relatively similar results obtained from the proximate analysis regarding the protein fraction of the treatments diets the discrepencies

regarding the amino acid anlysis in the current trial may be attributed to the nonhomogeneous sampling and/or mixing of the feed samples prior to the amino acid analysis.

Rurangwa *et al*. (2008) studied the effects of AXOS supplementation (10 and 20 g/kg) in diets of African catfish (± 20 g) and Siberian sturgeon (± 20 g) without any significant improvement in growth performance. Furthermore, juvenile Siberian sturgeon (25.9 ± 0.9 g) fed commercial diets supplemented with 2% AXOS-32-0.30 showed improved but not significant weight gain and FCR (Geraylou *et al*., 2012, 2013a). Similar non-significant differences in growth performance were reported for other catfish species fed diets supplemented with 2 g/kg MOS (Welker *et al*., 2007; Peterson *et al*., 2010; Hernández *et al*., 2012).

In contrast to the above-mentioned studies, the cumulative effect of AXOS and *Lactococcus lactis spp. lactis* ST G45 proved to significantly enhance the growth performance of juvenile Siberian sturgeon (Geraylou *et al*., 2013b). However, in the same study, the sole inclusion of AXOS in sturgeon diets failed to significantly improve the growth performance of the fish. The stimulated growth of *L. lactis*, in the above-mentioned study, showed that AXOS can be utilised as an effective substrate for certain beneficial bacteria. The enhanced growth performance of fish fed symbiotic treatments may be related to an improved immune response, proliferation of the probiotic and increased digestibility of the prebiotic (Rodriguez-Estrada *et al*., 2009; Ai *et al*., 2011; Mehrabi *et al*., 2012), relative to the separate administration of the prebiotic or probiotic.

The supplementation of dietary AXOS in aquaculture studies seems to have a more prominent effect on the innate immunity, hindgut fermentation products and intestinal microbial community than on the growth parameters of the fish species studied. Despite the high plant-protein component in the current diets, the combination of relatively mature fish and the inherent capability of *C. gariepinus* to utilise plant ingredients may have caused any possible action by the AXOS compound insufficient to cause a significant effect on growth performance. It is, therefore, possible that enhanced growth effects due to AXOS supplementation may be apparent in younger, smaller fish, although this has to be tested. However, this trial showed, according to the statistically similar growth performance between the AXOS and control treatments, that AXOS can be produced through the steam explosion method from a waste product, viz. brewer's spent grains, without any negative, antinutritional compounds present that might compromise the growth performance of African catfish. Additionally, these results also indicate relatively good growth and feed utilisation performance of African catfish fed highly plant-based diets which were completely absent of fishmeal and fish oil.

4.3.2. Meat proximate analysis

The meat proximate analysis, i.e. moisture, crude protein, crude fat and ash, for all the treatment groups are provided in Table 37. No significant differences were found between any of the treatment groups.

Table 37 Summary of the fillet proximate analysis for all treatment groups ('As is' (g/100g))

	Control	AXOS 0.3	AXOS 0.6	AXOS 1.2	P value
Moisture	74.9 ± 0.98	74.7 ± 0.97	74.4 ± 0.82	75.4 ± 0.63	0.860
Crude protein	19.2 ± 0.42	19.0 ± 0.51	19.5 ± 0.62	19.2 ± 0.62	0.936
Crude fat	3.00 ± 0.32	3.49 ± 0.69	3.41 ± 0.44	3.12 ± 0.21	0.864
Ash	1.18 ± 0.10	1.12 ± 0.04	1.09 ± 0.05	1.18 ± 0.09	0.953

Data presented as mean ± SE. P>0.05 for all parameters.

Dietary AXOS inclusion levels had no significant effect on the meat proximate analysis of African catfish. The meat proximate analysis was analysed in order to determine whether the steam explosion processing method of brewer's spent grains may have had any effect (positive or negative) on the fillet quality of African catfish. It is possible that the high temperatures accompanied by the thermochemical method may produce negative, antinutritional compounds together with AXOS which may have a negative effect on the meat quality of fish. From Table 37 it is evident that the supplementation of AXOS-containing compounds did not have any negative effect on the fillet proximate analysis of African catfish.

4.3.3. Visceral somatic index

The VSI values for all the dietary treatments are provided in Table 38. No significant differences were found between the treatment groups. Dietary AXOS treatments did not have any significant effect (positive or negative) on the VSI of African catfish. These results indicate that no negative substances were produced during the thermochemical extraction of AXOS from brewer's spent grains.

Table 38 Visceral somatic index values for all treatment groups (% of total body weight)

	Control	AXOS 0.3	AXOS 0.6	AXOS 1.2	P value
VSI (%)	12.7 ± 1.20	9.65 ± 0.23	12.0 ± 1.16	11.5 ± 0.74	0.148

Data presented as mean ± SE. P>0.05 for all parameters.

4.3.4. Haematocrit and non-specific immunity analysis

Blood titers for determining the non-specific immunity include serum lysozyme activity, total protein and immunoglobulin levels. Serum lysozyme activity, total protein and immunoglobulin were measured at Day 0, 18, 41, 56, 75 and 91 whereas haematocrit was only measured at Day 0 and 91. Sampling values for Day 0 are depicted as the pre-treatment values. The values

for haematocrit, serum lysozyme activity, total protein and immunoglobulin for the period between Day 0 (pre-treatment) and Day 91 (AXOS 0, 0.3, 0.6 and 1.2 treatments) are expressed in Table 39. Innate immunity assays taken prior to the start of the trial are being compared to the values taken at trial termination. The rationale behind this is to see if any of the above-mentioned assays did improve for any of the treatment groups over the 91-Day trial period. Significant differences in serum lysozyme activity did occur between Day 0 and Day 91 where all treatments including the control had significantly higher serum lysozyme activity levels at the trial conclusion than at the start. However, serum lysozyme activity levels did not differ among dietary treatments. A similar trend was observed between the immunoglobulin levels although AXOS 0.6 and the pre-treatment were not significantly different. All treatments except AXOS 0.6 had significantly higher immunoglobulin levels at the trial conclusion than at the start. Additionally, AXOS 1.2 had significantly higher immunoglobulin levels compared to the rest of the AXOS treatments and the control group. No significant differences were found between the haematocrit or total protein levels between Day 0–91.

Serum lysozyme activity, total protein and immunoglobulin levels of all treatment groups sampled at the five sampling dates of the trial are provided in Tables 40, 41 and 42, respectively. No significant differences were found for serum lysozyme activity and total protein between any of the treatment groups. However, there were significant differences for immunoglobulin levels at Day 18 and Day 75. At Day 18, AXOS 0.6 was significantly different from the control and AXOS 0.3, while AXOS 1.2 was significantly different from AXOS 0.3. At Day 75, AXOS 1.2 was significantly different from the rest of the treatment groups.

Table 39 Summary of non-specific immunity and haematocrit values for all treatment groups between Day 0 and Day 91

	Pre-treatment	Control	AXOS 0.3	AXOS 0.6	AXOS 1.2	P value
Haematocrit (%)	36.4 ± 2.07	35.2 ± 2.48	38.0 ± 1.65	31.9 ± 2.13	30.8 ± 2.62	0.295
Lysozyme (µl/ml)	1.59 ± 0.19^{b}	5.70 ± 0.45^{a}	5.51 ± 0.62^{a}	6.07 ± 0.88^{a}	5.17 ± 0.47^{a}	0.000
Total protein (mg/ml)	15.1 ± 1.12	11.5 ± 0.78	14.2 ± 1.03	11.4 ± 1.22	11.6 ± 1.34	0.168
Immunoglobulin (mg/ml)	3.40 ± 0.28^{c}	6.17 ± 1.29^{b}	5.81 ± 0.80^{b}	5.08 ± 0.79^{bc}	8.69 ± 1.07^{a}	0.004

Data presented as mean ± SE. P>0.05 for haematocrit and total protein. Statistically significant differences in serum lysozyme and immunoglobulin levels existed between treatments (P<0.05). Different superscripts (e.g. $^{a, b}$) in the same row indicate significant different means (P<0.05).

Table 40 Summary of serum lysozyme activity of all treatment groups for the five sampling dates

	Day 18	Day 41	Day 56	Day 75	Day 91
Control	2.31 ± 0.77	5.59 ± 0.36	5.70 ± 0.58	3.40 ± 0.92	5.70 ± 0.45
AXOS 0.3	1.45 ± 0.45	4.98 ± 0.13	6.03 ± 0.96	3.12 ± 1.17	5.51 ± 0.62
AXOS 0.6	1.45 ± 0.76	5.14 ± 0.39	5.18 ± 0.52	3.43 ± 0.93	6.07 ± 0.88
AXOS 1.2	0.47 ± 0.18	5.04 ± 0.41	5.20 ± 0.30	3.83 ± 0.75	5.17 ± 0.47
P value	0.220	0.221	0.796	0.995	0.774

Data presented as mean ± SE. P>0.05 for all parameters.

Table 41 Summary of total serum protein of all treatment groups for the five sampling dates

	Day 18	Day 41	Day 56	Day 75	Day 91
Control	15.0 ± 1.40	10.9 ± 1.83	14.4 ± 0.71	16.6 ± 0.23	11.5 ± 0.78
AXOS 0.3	14.5 ± 1.79	10.5 ± 2.35	13.0 ± 0.84	15.0 ± 0.84	14.2 ± 1.03
AXOS 0.6	14.6 ± 1.49	9.78 ± 1.84	12.0 ± 1.17	15.0 ± 0.72	11.4 ± 1.22
AXOS 1.2	14.4 ± 1.58	11.3 ± 1.82	13.7 ± 0.88	14.7 ± 0.89	11.6 ± 1.34
P value	0.994	0.955	0.325	0.082	0.266

Data presented as mean ± SE. P>0.05 for all parameters.

Table 42 Summary of immunoglobulin of all treatment groups for the five sampling dates

	Day 18	Day 41	Day 56	Day 75	Day 91
Control	2.75 ± 0.25^{bc}	3.83 ± 0.60	3.39 ± 0.58	2.14 ± 0.20^{b}	6.17 ± 1.29
AXOS 0.3	2.34 ± 0.40^{c}	3.50 ± 0.81	3.92 ± 0.58	1.97 ± 0.18^{b}	5.81 ± 0.80
AXOS 0.6	4.39 ± 0.47^{a}	5.24 ± 0.83	2.82 ± 0.08	2.14 ± 0.33^{b}	5.08 ± 0.79
AXOS 1.2	3.66 ± 0.54^{ab}	3.97 ± 0.84	3.29 ± 0.41	3.37 ± 0.56^{a}	8.69 ± 1.07
P value	0.013	0.389	0.264	0.038	0.098

Data presented as mean ± SE. P>0.05 for all sampling dates except for Day 18 and Day 75. Statistically significant Immunoglobulin levels existed between treatments at Day 18 and Day 75 (P<0.05). Different superscripts (e.g. $^{a, b}$) in the same row indicate significant different means (P<0.05).

A paucity of information exists regarding the effects of AX and their hydrolysis products on fish immunity (Li *et al*., 2008). Therefore, the current study aimed to elucidate the effects of AXOS supplemented at different dietary inclusion levels on the innate immune response of African catfish fed plant-based diets. For the first time, dietary AXOS supplementation has shown to significantly increase the non-specific immune response of African catfish fed plant-based diets. The immunoglobulin levels differed significantly between treatments at Day 18 and Day 75 (Table 42). At Day 18, AXOS 0.6 had a significantly higher immunoglobulin level than the control and AXOS 0.3 treatments. Furthermore, the AXOS 1.2 treatment had a significantly higher immunoglobulin level than the AXOS 0.3 treatment. At Day 75, the AXOS

1.2 treatment had a significantly higher immunoglobulin level than the rest of the treatments. Thus, it is clear that dietary AXOS supplemented at 0.6% and 1.2% significantly enhanced the immunoglobulin concentration of African catfish fed plant-based diets.

The results of the current study are in accordance with previous studies where dietary AXOS supplementation proved to significantly enhance the innate immunity of juvenile Siberian sturgeon. Geraylou *et al*. (2012) reported that AXOS-32-0.30 significantly enhanced the alternative haemolytic complement activity, total serum peroxidase activity and the phagocytic activity, although no significant effects were seen regarding the serum lysozyme activity. In another study, the synergistic effect of AXOS and *L. lactis spp. lactis* ST G45 proved to significantly enhance the cellular and humoral immunity of juvenile Siberian sturgeon, although the total immunoglobulin levels were unaffected (Geraylou *et al*., 2013b).

The improvement of the innate immunity through the supplementation of prebiotics can be attributed to the stimulation of beneficial intestinal microbiota (Sang *et al*., 2011; Zhang *et al*., 2011). These commensal microbiota plays a direct role in the modulation of the immunity and may improve the immune response through enhancing feed conversion efficiency, protection against potential pathogens, the competitive exclusion for adhesion sites, production of fermentation metabolites, as well as modulate gut physiology and morphology (Nayak, 2010b). The production of SCFAs limits the proliferation of pathogenic bacteria and their harmful metabolites through lowering the intestinal pH levels and, therefore, also increases the solubility of minerals (Gibson, 2004; Cloetens, 2009). These organic acids may also act as an additional source of energy to the colonocytes and the host alike (Rombeau & Kripke, 1990; Hamer *et al*., 2008). It is also known that prebiotics may directly stimulate the innate immunity through the GALT and interaction with pattern recognition receptors and microbe-associated molecular patterns (Watzl *et al*., 2005; Song *et al*., 2014). Contradictory to the current study, no significant difference in immune response parameters were found when channel catfish were fed diets supplemented with MOS at 2% dietary inclusion levels (Welker *et al*., 2007). The discrepancies may be attributed to the different fish species, diet constituents, as well as the prebiotic substances used.

Furthermore, there exists a clear significant increase in the serum lysozyme activity between the start and end of the trial. From Table 40 it is clear that this increase occurred between Days 18 – 41, compared to Day 0. The significant increase in the serum lysozyme activity may have been caused by the fishmeal-free and highly plant-based diet fed to the fish at the start of the trial. Serum lysozyme activity is an effective way of measuring various stresses that may influence the animal, such as water quality, handling, sickness and nutritional stressors (Magnadotir, 2006; Kiron, 2012), and might, therefore, have increased due to the low digestibility and/or presence of antinutritional factors present in the diet. Therefore, it is possible that the absence of fishmeal and the high inclusion of plant-based

ingredients may have influenced the immunostimulatory effects of AXOS. In order to better understand the immunostimulatory activity of AXOS, it is necessary to investigate the effect of AXOS on the hindgut SCFA production and the hindgut microbial diversity.

4.3.5. Hindgut microbial diversity analysis

The alpha diversity is comprised out of the following indices: Shannon diversity, Simpson diversity and the species richness (based on the number of OTU's). The Shannon diversity, Simpson diversity and species richness indices were compared between Day 0 (pre-treatment) and Day 91 (AXOS 0, 0.3, 0.6 and 1.2 treatments) and are provided in Table 43. The pre-treatment sampling groups were taken at trial commencement (Day 0), while all the dietary treatments were taken at trial termination (Day 91). This was to determine if any treatment groups had any effect on the microbial diversity for the duration of the feeding trial. A statistically significant difference (P=0.038) was found for the Simpson diversity index between Day 0 and Day 91. No significant differences were found for the Shannon diversity and species richness, however, a numerically inverse relationship between species richness and the levels of AXOS inclusion can be seen which might indicate the modulating effect of dietary AXOS on the hindgut microbiota of African catfish.

Table 43 Summary of alpha diversity indices for all treatment groups between Day 0 and Day 91

	Pre-treatment	Control	AXOS 0.3	AXOS 0.6	AXOS 1.2	P value
Shannon diversity	1.63 ± 0.27	2.78 ± 0.21	2.56 ± 0.31	2.60 ± 0.26	2.52 ± 0.16	0.112
Simpson diversity	0.73 ± 0.06^{b}	0.90 ± 0.02^{a}	0.86 ± 0.05^{a}	0.88 ± 0.03^{a}	0.88 ± 0.02^{a}	0.038
Species richness	12.3 ± 3.93	28.5 ± 5.30	25.7 ± 4.64	25.0 ± 5.11	22.7 ± 4.00	0.386

Data presented as mean ± SE. P>0.05 for Shannon diversity and Species richness indices. Statistically significant differences existed for the Simpson's diversity index. Different superscripts (e.g. $^{a, b}$) in the same row indicate significant different means (P<0.05).

The Beta diversity was calculated according to the Bray-Curtis dissimilarity index. The Bray-Curtis dissimilarity index is displayed in the form of a cluster dendrogram in Figure 7. This index explains the difference in species similarity between treatment groups and is bounded between values 0 and 1, where 0 states that the relevant sites (treatments) have the same species composition and 1 state that the relevant sites do not have any species in common. The sampling for the pre-treatment took place at Day 0, while the sampling for all AXOS and control treatments took place at Day 91. This is to elucidate the potential effect of AXOS treatment on the microbial species diversity for the 91-Day duration of the feeding trial. Pre-treatment samples are displayed with the prefix 'PT' while the AXOS and control treatments are indicated with the prefix 'V', each followed by a number. Figure 8 contains the meta-data

accompanied by the dissimilarity (distance) between the treatment groups which are based on the Bray-Curtis dissimilarity index. The permanova of the Bray-Curtis dissimilarity index for all treatment groups between Day 0 and Day 91 is provided in Table 44. The permanova indicated that no significant difference existed in the beta diversity between Day 0 and Day 91.

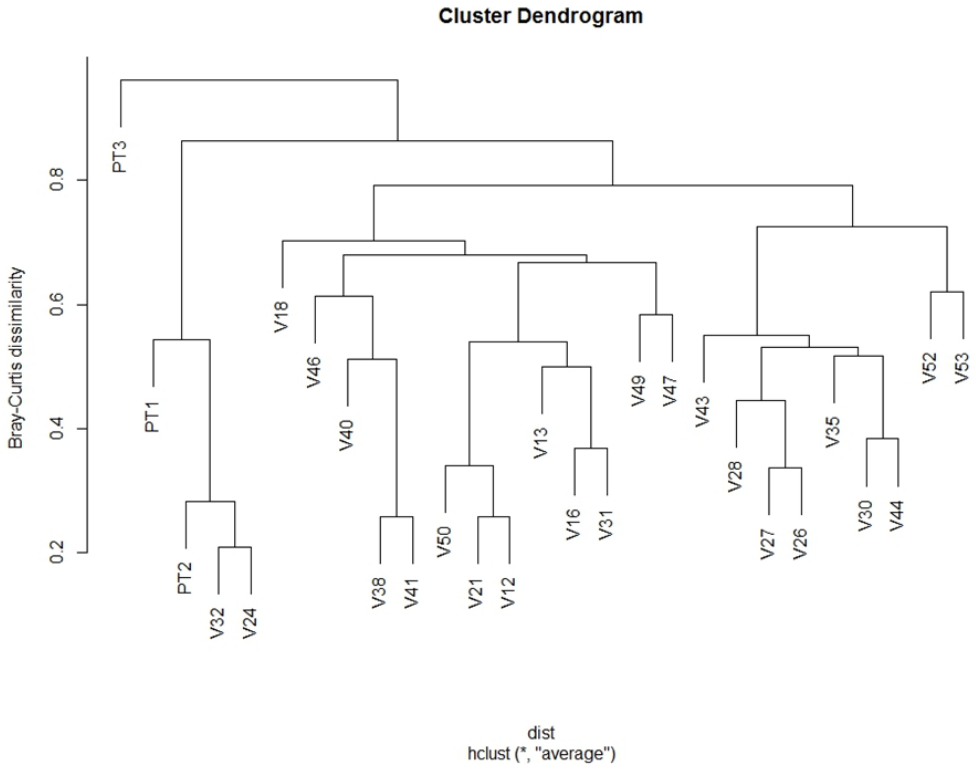

Figure 7 Cluster dendrogram of the Bray-Curtis dissimilarity of the pre-treatment (PT) and dietary treatment groups (V)

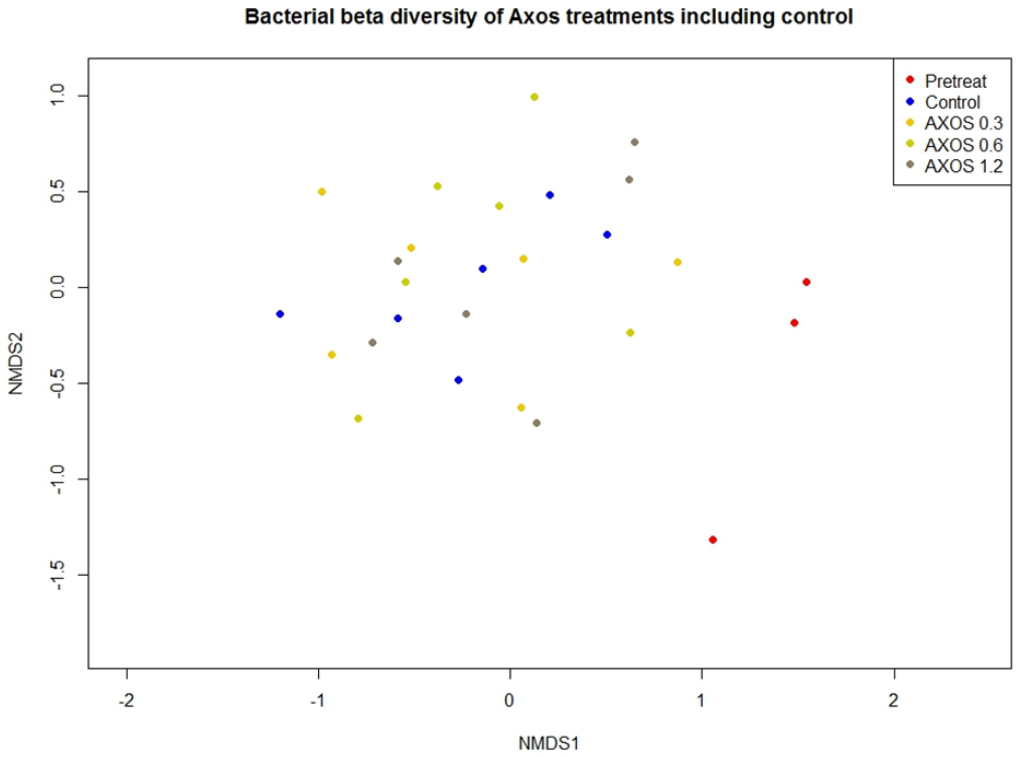

Figure 8 Bacterial beta diversity between the pre-treatment (Day 0) and dietary treatment groups (Day 91)

Table 44 Permanova of Bray-Curtis dissimilarity index between pre-treatment (Day 0) and AXOS treatment groups (Day 91)

	Degrees of freedom	Sum of Squares	F	Pr(>F)
Site	4	1.55	1.35	0.124
Residual	22	6.33	NA	NA

No significant difference existed in the microbial beta diversity between Day 0 and Day 91 (P>0.05).

To the author's knowledge, this is the first study that tested the effect of dietary AXOS on the hindgut microbial diversity of African catfish. Statistically significant differences existed for the Simpson diversity index of all treatments between the start and end of the trial, although no significant differences existed between any of the dietary treatments. From Table 43 it is clear that a shift in microbial diversity occurred for all treatments between the beginning towards the end of the trial. The AXOS and control treatments had a significantly higher Simpson diversity score, measured at Day 91, compared to the pre-treatment group, measured at Day 0. This is to be expected as, at the start of the trial, all treatments were shifted to a new, FM-free and high plant-ingredient diet, which is likely to impact on the intestinal microbiome. Previous studies have reported that diet may have an impact on the intestinal microbiota of fish (Ringø & Olsen, 1994; Yang *et al*., 2007; Cerezuela *et al*., 2012). The Simpson's diversity index tells us about bacterial dominance in a community and the

higher the index value the greater the effect of dominance (degree of concentration) in a community (Slabbert, 2008). This index gives us the value that ranges between 0 and 1, where 1 represents the greater sample diversity and 0 the absence of sample diversity. Therefore, the index shows that an increase in the microbial species diversity occurred for all treatments during the course of the trial. Additionally, a numerically indirect relationship existed between the species richness index and the control and AXOS treatments. The species richness index decreased numerically based on an AXOS inclusion level dependency (Table 43).

Past literature has shown that dietary AXOS was able to significantly modulate the intestinal microbiota of Siberian sturgeon and African catfish. Geraylou *et al*. (2012) reported that dietary AXOS stimulated the growth of certain lactic acid bacteria and several species of the *Clostridium* genus in the hindgut of Siberian sturgeon while suppressing the growth of *Aeromonas sp*., *C. freundii* and *E. coli*. The differences in the results between the current and above-mentioned study may be attributed to the different species and potentially different AXOS compounds used as well as the maturity of the animals. In another study, the combination of AXOS and *L. lactis spp. lactis* ST G45 significantly reduced the intestinal microbiota diversity of Siberian sturgeon in terms of the specific species richness and Shannon diversity index compared to the control group (Geraylou *et al*., 2013b). In the same study, the combination of AXOS and *B. circulans* ST M53 also significantly decreased the bacterial species richness. The combined use of AXOS and probiotics may have caused the significant decrease in microbial species diversity, compared to the current trial. It is known that *L. lactis* has antimicrobial properties (Balcázar *et al*., 2008) and thus may have influenced the microbial species diversity of Siberian sturgeon in combination with dietary AXOS.

A study involving Siberian sturgeon and African catfish fed dietary AXOS, oligofructose, xylose and fructose revealed that hindgut microbiota from Siberian sturgeon had a higher fermentation capacity that those of African catfish. Conversely, this might explain the difference in the effect of AXOS on the bacterial community in the current study compared to previous studies that mostly involved Siberian surgeon. However, in the same study, AXOS and its monomer xylose still had an impact on the hindgut bacteria of African catfish, although to a lesser extent than what was reported in Siberian sturgeon (Geraylou *et al*., 2014). Other variables that may also have had an effect on the intestinal microbial community include fish age, nutritional diet, environment and the complexity of the GIT of fish (Cahill, 1990; Ringø *et al*., 1995, 2006; Al-Harbi & Uddin, 2004).

Contradicting the above-mentioned studies, Delaedt *et al*. (2008) reported that dietary AXOS supplementation modulated the intestinal microbiota of Siberian sturgeon, however, AXOS treatments had a more diverse bacterial community than the control group. The lack of significant difference regarding AXOS supplementation in aquaculture studies is however not uncommon. Dietary AXOS-32-0.30 fed at 2 and 4% inclusion levels did not significantly alter

the hindgut bacterial species diversity of Siberian sturgeon. However, in the same study, AXOS-3-0.25 fed at 2% inclusion level, significantly increased the hindgut bacterial diversity (bacterial species richness and Shannon diversity index) of Siberian sturgeon. The author reported that AXOS had a significant effect on the abundance and clustering of intestinal microbiota by stimulating relative abundances of beneficial bacteria, such as *Clostridium* and LAB. Thus, the community composition of the intestinal microbiota was significantly altered by AXOS supplementation without significantly influencing the microbial diversity parameters.

It is therefore possible that dietary AXOS may have a positive effect upon the microbial community by stimulating specific beneficial bacteria without significantly affecting the intestinal microbial diversity (Geraylou *et al*., 2013a). Conversely, dietary AXOS has shown to affect the fermentation activity in the stimulator of the human intestinal microbial ecosystem without significantly affecting the intestinal microbial community (Sanchez *et al*., 2009). It is thus possible that dietary AXOS inclusion may have, in the current trial, altered the specific species composition of the hindgut microbial community without significantly affecting the species diversity parameters. Therefore, it is recommended that future studies should incorporate methods such as pyrosequencing and redundancy analysis of the operational taxonomic units in order to identify the specific bacterial species present in the gut in addition to studying the bacterial diversity parameters. Furthermore, it is known that the average degree of polymerisation (avDP) and substitution (avDS) greatly influences the growth of intestinal microflora as well as the fermentability of AXOS (Grootaert *et al*., 2007; Van Craeyveld *et al*., 2008; Cloetens *et al*., 2010). Although the avDP and avDS were not determined in the current trial, future studies may benefit from focusing on the structure-function relationship of dietary AXOS.

4.3.6. Short-chain fatty analysis

The analysis of the hindgut SCFAs included acetic, propionic, isobutyric, butyric, isovaleric and valeric acids. The SCFA concentrations of all the treatment groups are provided in Table 45. No significant differences were found between any of the treatment groups.

Table 45 Summary of SCFA analysis for all the treatment groups (mM)

	AXOS 0 (C)	AXOS 0.3	AXOS 0.6	AXOS 1.2	P value
Acetic acid	33.8 ± 3.07	31.7 ± 1.76	34.0 ± 1.86	26.8 ± 1.14	0.084
Propionic acid	4.04 ± 1.50	2.19 ± 0.63	3.01 ± 1.33	1.17 ± 0.32	0.198
Iso-Butyric acid	0.32 ± 0.14	0.12 ± 0.03	0.28 ± 0.14	0.09 ± 0.03	0.233
Butyric acid	7.06 ± 1.65	5.18 ± 1.62	6.32 ± 1.27	2.93 ± 1.03	0.146
Iso-Valeric acid	0.28 ± 0.12	0.11 ± 0.03	0.25 ± 0.11	0.09 ± 0.03	0.178
Valeric acid	0.03 ± 0.01	0.01 ± 0.00	0.03 ± 0.02	0.01 ± 0.00	0.649

Data presented as mean ± SE. P>0.05 for all parameters.

Dietary AXOS had no statistically significant effect on the hindgut SCFA production of *C. gariepinus* fed highly plant-based diets. The effects of prebiotic supplementation are not always evident in the pattern and concentration of SCFA production in the gastrointestinal tract (Cummings *et al.*, 2001; Flickinger *et al.*, 2003). However, other aquaculture studies have reported significant effects on the SCFA production caused by AXOS supplementation. The discrepancy between the current and other studies may be related to species-specific differences, diet constituents, fish size and different preparations of AXOS used. Geraylou *et al*. (2013a) reported that dietary AXOS supplementation significantly increased the relative abundance of SCFA-producing bacteria and, therefore, observed significant increases in the production of acetate, butyrate and total SCFA production in the hindgut of Siberian sturgeon compared to the control treatment. Similarly, in another study, dietary AXOS significantly increased the production of acetate, butyrate and total SCFA production in the hindgut of Siberian sturgeon compared to the control group (Geraylou *et al*., 2012).

The differences between the current study and the above-mentioned studies may be attributed to the species-specific bacteria between the two different species. Geraylou *et al*. (2014) revealed, through in vitro fermentation of AXOS by hindgut bacteria from Siberian sturgeon and African catfish, that clear substrate preferences exist between hindgut bacteria from these two fish species. They also reported that the differences in SCFA production may be related to the differences in the intestinal microbial composition between these two species. However, Rurangwa *et al*. (2008) reported that dietary AXOS significantly enhanced the production of acetate, propionate and total SCFAs in the hindgut of Siberian sturgeon and African catfish. The discrepancies may, therefore, be attributed to differences in fish size, diet composition and AXOS preparations. It is known that the microbial community of fish may be influenced by diet composition, environmental factors, fish age and species (Cahill, 1990; Ringø *et al*., 1995, 2006; Al-Harbi & Uddin, 2004). The size of the fish used in the current trial

(547.28 ± 93.09 g) was significantly larger than the fish used in the above-mentioned trials, ca. 30 g (Geraylou *et al*., 2012, 2013a). Older fish have more mature gut microbial communities than smaller, younger fish. Furthermore, it has been reported that structural differences in dietary substrates affected the fermentability of non-digestible oligosaccharides in common carp fed various structurally different nutritional compounds (Kihara & Sakata, 2002). Conversely, it is also known that the structure of an AXOS preparation affects its fermentability (Pollet et al., 2012). It is, therefore, possible that the AXOS preparation in the current study may have been structurally different from the AXOS preparations used in the above-mentioned studies.

Studies have reported that acetic acid, propionic acid and butyric acid are the main fermentation products of dietary AXOS (Kabel *et al*., 2002; Hughes *et al*., 2007; Geraylou *et al*., 2012), which are corresponding to the results of the current trial. The dominant production of acetate in the current study is in accordance with other studies involving the fermentation of carbohydrates by hindgut microbiota in fish (Bergman, 1990; Clements *et al*., 1994; Kihara & Sakatag, 1997; Geraylou *et al*., 2012, 2013a). Moreover, the SCFAs produced are indicative of the resident microbiota in the gastrointestinal tract (Nisbet *et al.*, 1996; Nisbet, 2002). The high production of acetate may be explained by the large numbers of acetate producing bacteria such as *Clostridium spp*. and LAB (Fuller & Peridigón, 2003) in the hindgut of *C. gariepinus*. In an in vitro study involving the fermentability of AXOS, Rurangwa *et al*. (2009) reported that LAB and *Bacillus spp*. mainly produced acetic acid as the fermentation product. Furthermore, it has been reported that the cell wall components of LAB may have immunostimulatory properties (Perdigón *et al*., 2001; Bricknell & Dalmo, 2005) while it is also known that SCFAs may have immunomodulatory properties as has been proven in a number of studies (Pratt *et al*., 1996; Meijer *et al*., 2010). Resultingly, the combined effect of a favourable gut microbiota and the relative abundance of SCFAs may explain the significant increase in the immunoglobulin levels witnessed.

4.4. Conclusion

This study showed that AXOS-containing compounds can be thermochemically produced from brewer's spent grains without the presence of semi-antinutritional compounds that may negatively affect the growth performance, feed efficiency and fillet composition of African catfish. Additionally, this trial showed that AXOS, processed from a waste product, can be used as a prebiotic and an innate immune response stimulant in fishmeal-free diets of *C. gariepinus*.

The statistically similar results found for the growth and feed utilisation parameters may be attributed to the large size of the fish at trial commencement. As a result, the slower growth rate of the fish may have had a masking effect on AXOS supplementation. A significant

increase in the Simpson's diversity index for all treatments between the start and termination of the trial may be attributed to the growth and maturity of the fish as well as the acclimatisation to a highly plant-based diet throughout the course of the trial. In addition to the increase of bacterial diversity throughout the trial, a numerically indirect relationship existed between the species richness index and AXOS inclusion level. It is thus possible that significant differences in the microbial diversity between dietary treatments may be apparent in smaller, less matured fish, although this has to be tested. Similarly, a significant increase in the serum lysozyme activity for all treatments existed between the start and end of the trial. This is to be expected as, at the start of the trial, all treatments were shifted to a new, fishmeal-free and highly plant-based, diet, which is likely to have had an impact on the innate immunity. Statistically significant differences between dietary treatments existed for the immunoglobulin levels at Day 18 ($P=0.013$) and Day 75 ($P=0.038$). At Day 18, the AXOS 0.6 treatment group had a significantly higher immunoglobulin level compared to the control and AXOS 0.3 treatments, while the AXOS 1.2 treatment had a significantly higher immunoglobulin level compared to the AXOS 0.3 treatment. At Day 75, the AXOS 1.2 treatment had a significantly higher immunoglobulin level compared to all the other treatments. For the overall trial period, the AXOS 1.2 treatment had a significantly higher ($P=0.004$) immunoglobulin level compared to the rest of the treatments.

This study showed that AXOS-containing compounds may improve the innate immunity of African catfish, although it is unclear if the improved immunoglobulin levels were caused by the change in the intestinal microbiota or through the direct immunostimulatory properties of AXOS. A possibility might have been that the decrease in microbial diversity could have meant a decrease in pathogenic bacteria and a subsequent increase in a specific group of beneficial bacteria that may have contributed to the significantly increased immunoglobulin levels witnessed. It is also possible that the microbial modulatory activities of AXOS may have been influenced by the communal water source that could have acted as a bacterial vector between treatments.

However, the results obtained in this study merit further investigation. It is recommended that future studies should aim to use smaller, faster growing and less microbially mature fish in order to emphasise the effect of dietary AXOS on the growth performance and health status of African catfish. Moreover, to better understand the link between host health and its resident microbiota, it is recommended that future studies should consider using gnotobiotic environments during feeding trials as this will provide for a more complete understanding of the specific microbial species involved in the microbial shift caused by prebiotic fermentation in the hindgut. Although it was not assessed in the current study, it is advised that future studies should focus more on the structure-function relationship of AXOS which greatly influences its rate of fermentability. Furthermore, it is advised that similar studies should aim

not to have a communal water source and to individually separate the water source of each dietary treatment as to ensure the integrity of the inherent microbiota of each treatment.

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Chapter 5

General conclusion and recommendation

This study showed, for the first time, the microbial modulating effect of dietary xylanase and the immunostimulatory effect of dietary AXOS on *C. gariepinus* fed highly plant-based diets. In trial one, dietary xylanase significantly decreased the Shannon diversity index of the xylanase 200 treatment compared to the control. Moreover, the control and the xylanase 150 treatments had a significantly higher Simpson's diversity index compared to the pre-treatment group (sample taken at Day 0). These results are indicative that dietary xylanase caused a significant decrease in the hindgut microbial species diversity of African catfish based on a dose-dependent manner. The significant increase in the serum lysozyme activity and immunoglobulin levels between the start and end of the trial may be attributed to the high plant-containing diets and poor water quality, respectively, given that no significant differences existed between dietary treatments. This study showed that AXOS, or similar non-digestible oligosaccharides, released by the enzymatic hydrolysis of xylanase in the GIT were produced in quantities sufficient enough to have a meaningful effect on the hindgut microbial diversity. Arabinoxylan-oligosaccharides have shown to stimulate the growth of health-benefitting intestinal bacteria while inhibiting the growth of potentially pathogenic microbiota. Therefore, the decrease in the intestinal microbial diversity, shown in the current study, may entail the increase and decrease of beneficial and potentially pathogenic intestinal bacteria, respectively, although the specific strains of bacteria need to be tested.

The increased growth and nutrient utilisation generally seen after xylanase supplementation is most likely caused by the separate or combined improvement of nutrient digestion caused by changes in the intestinal physiology, morphology and microbial community. Therefore, further investigations may benefit from studying the changes in intestinal physiology and morphology in addition to the change in the intestinal microbiota. Due to a large amount of equivocal results, more information is needed on the enzyme/substrate interaction of xylanases in aquaculture diets containing different plant ingredients. Different xylanase types which have different affinity towards NSP fractions exist and therefore care should be taken when deciding on a specific type of xylanase. Xylanases with an affinity towards the soluble as well as the insoluble NSP fractions may prove to be more beneficial as opposed to the sole inclusion of either one type.

In trial two, the supplementation of an AXOS-containing compound significantly increased the immunoglobulin levels of the AXOS 0.6 treatment compared to the control and AXOS 0.3 treatments as well as the AXOS 1.2 treatment compared to the AXOS 0.3 group at Day 18. At Day 75, the AXOS 1.2 treatment had a significantly higher immunoglobulin level

compared to all the other dietary treatments. All the dietary treatments had a significantly higher hindgut microbial diversity (Simpson's diversity index) and serum lysozyme activity at the end of the trial compared to the pre-treatment. This could be attributed to the feeding of a highly plant-based diet as no significant differences existed between the dietary treatments themselves. However, a numerically indirect relationship existed between the species richness index and the AXOS inclusion levels. Although not significant, this result conforms to the microbial modulating result of the xylanase trial and shows that dietary AXOS had a decreasing effect, although not significant, on the hindgut microbial diversity. This study demonstrated that AXOS-containing compounds possessing immune stimulating properties could be produced from a waste product. The significant increase in the immunoglobulin levels and the non-negative response gathered from the production performance and fillet composition, indicated that AXOS-containing compounds could be thermochemically produced from brewer's spent grains without the presence of semi-antinutritional compounds. Depending on the processing methods and source of origin, AXOS can have several different structural complexities and variabilities and therefore it would be beneficial to consider the structure-function relationship of AXOS, such as the average degree of polymerisation and substitution, rather than considering it as a generalised AXOS structure.

The lack of significant growth and feed efficiency performance in both trials could be related to the relatively large size and maturity of the fish at the start of the investigation. Larger fish have a slower growth rate and a more mature intestinal microbial community which may mask the positive effects of xylanase and/or AXOS supplementation in highly plant-based diets. A more mature intestinal microbial community may assist the host in nutrient digestion and therefore lead to a more efficient utilisation of NSPs. To better understand how microbiota benefits the nutritional and health aspects of the host, molecular techniques for the characterisation of microorganisms and how they react to prebiotics and interact with each other should be scrutinised. Techniques such as a high-throughput pyrosequencing approach or the use of gnotobiotic or axenic systems should allow comparison of communities at various taxonomic levels and identification of the taxa and therefore give an accurate description of the desirable microbial community.

This study showed that AXOS may be a viable alternative to xylanase and antibiotics in fish feeds. The thermolabile nature of exogenous enzymes hinders their application in aquafeeds and are, therefore, an area of concern. Dietary AXOS are more thermostable and can, therefore, be included before pelleting. The excess amount of hydrolyses products produced from xylanase, and xylanase inhibitors present in certain cereals may all be circumvented with the addition of AXOS. The principal benefit, however, may be that AXOS is effective regardless of the diet constituents. Functional feed additives, such as xylanase and AXOS, may provide benefits to feed manufacturers and aquaculture farmers such as cheaper

feed formulations, more uniformly animal production performance and less environmental impact (viz, leaching of nutrients) and holds a great deal of promise for future development of feed additives. Future investigations should aim to focus on the mechanism underlying effects of xylanase and AXOS on fish performance, immunity and microbiota and how these parameters affect each other as well as the structural integrity of xylanase and AXOS throughout the gastro-intestinal tract of various fish species as to ascertain where the area of application in the GIT is most effective.

Appendices

Appendix A. Ethical clearance approval

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Protocol Approval

Date: 27 July 2018

PI Name: Mr Stephan Gericke

Protocol #:2820

Title: POST-EXTRUSION APPLICATION OF EMULSIFIED ENDO-1,4-B-XYLANASE IN DIETS OF AFRICAN CATFISH Clarias gariepinus.

Dear Stephan Gericke ,

Your Progress Report submission , was reviewed on 16 May 2018 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report, up to a maximum of three years.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 2820 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU Secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

Page 1 of 1

Appendix B. Evaluation of Nutrase Xyla in post-extrusion application of African catfish diets

KU LEUVEN

KU Leuven | Technologiecampus Gent
Faculteit Industriële Ingenieurswetenschappen
Onderzoeksgroep Enzym-, Fermentatie- en Brouwerijtechnologie / EFBT

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(analyseaanvraag: 11.08.2017)

Bepaling van de enzymdosering in voeders - Lourens

1. Opstellen ijklijn met blancovoeder A-con

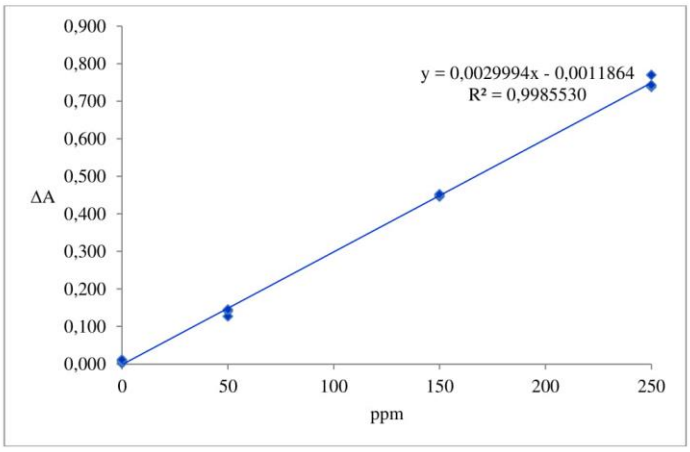
extract : 5 g voeder in 25 ml buffer (maleaatbuffer 100 mM, pH 6,0)
30 min. extraheren bij kamertemperatuur
centrifugeren
endo-xylanase activiteitsmeting : (maleaatbuffer 100 mM / pH 6,0 / 50 °C / 30 min.)

A = A-con

	ΔA
A	0,002
A + 50 ppm	0,141
A + 150 ppm	0,451
A + 250 ppm	0,770

	ΔA
A	0,012
A + 50 ppm	0,145
A + 150 ppm	0,452
A + 250 ppm	0,743

	ΔA
A	0,008
A + 50 ppm	0,127
A + 150 ppm	0,446
A + 250 ppm	0,738



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Staal	ΔA gem	ppm
L + 100 ppm NX spray / A-L100S	0.254	85
L + 200 ppm NX spray / A-L200S	0.646	216
L + 100 ppm NX emulsion / A-L100E	0.262	88
L + 200 ppm NX emulsion / A-L200E	0.567	189

2. Opstellen ijklijn met blancovoeder B-con

extract : 5 g voeder in 25 ml buffer (maleaatbuffer 100 mM, pH 6,0)
30 min. extraheren bij kamertemperatuur
centrifugeren
endo-xylanase activiteitsmeting : (maleaatbuffer 100 mM / pH 6,0 / 50 °C / 30 min.)

A = B-con

	ΔA
A	0,010
A + 50 ppm	0,148
A + 150 ppm	0,454
A + 250 ppm	0,761

	ΔA
A	0,013
A + 50 ppm	0,144
A + 150 ppm	0,435
A + 250 ppm	0,764

	ΔA
A	0,003
A + 50 ppm	0,135
A + 150 ppm	0,446
A + 250 ppm	0,765

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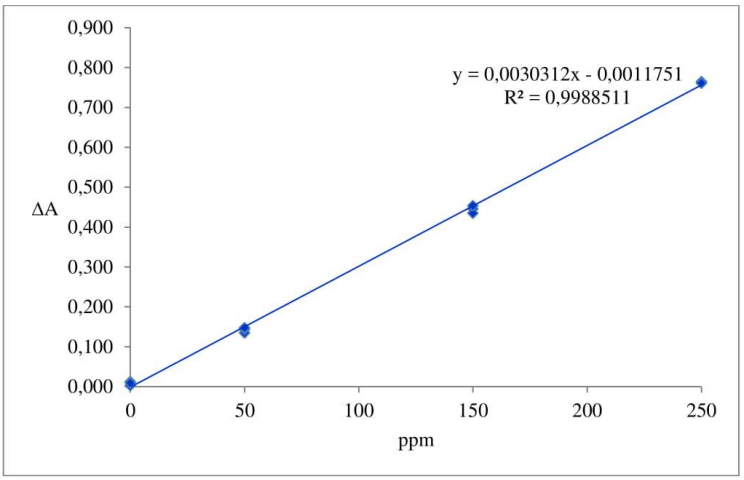

Staal	ΔA gem	ppm
L + 100 ppm NX spray / B-L100S	0.196	65
L + 200 ppm NX spray / B-L200S	0.541	179
L + 100 ppm NX emulsion / B-L100E	0.098	33
L + 200 ppm NX emulsion / B-L200E	0.231	77

3. Opstellen ijklijn met referentie L0

extract : 1.0 ml L in 4.0 ml buffer (maleaatbuffer 100 mM, pH 6,0)
centrifugeren
10x verdunnen
endo-xylanase activiteitsmeting : (maleaatbuffer 100 mM / pH 6,0 / 50 °C / 20 min.)

	ΔA
A	0,023
A + 750 ppm	0,209
A + 1500 ppm	0,414
A + 2500 ppm	0,689

A = L0

	ΔA
A	0,031
A + 750 ppm	0,215
A + 1500 ppm	0,411
A + 2500 ppm	0,675

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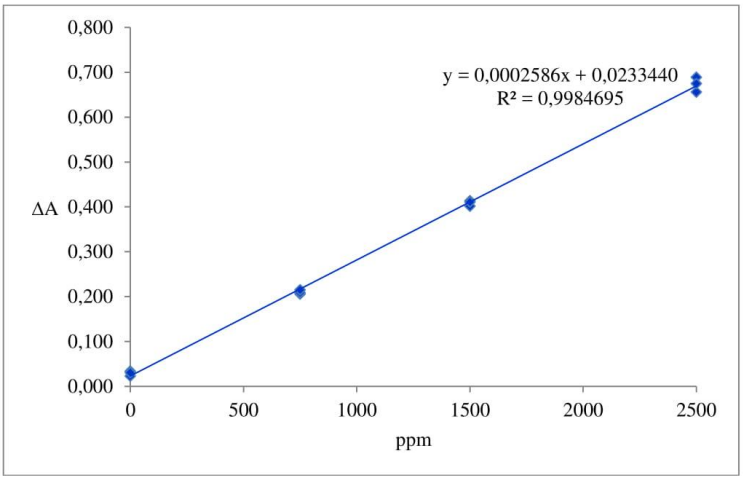
	ΔA
A	0,034
A + 750 ppm	0,206
A + 1500 ppm	0,402
A + 2500 ppm	0,656



Staal	ΔA gem	ppm
L 1000 ppm	1.419	5395
L 2000 ppm	2.150	8224

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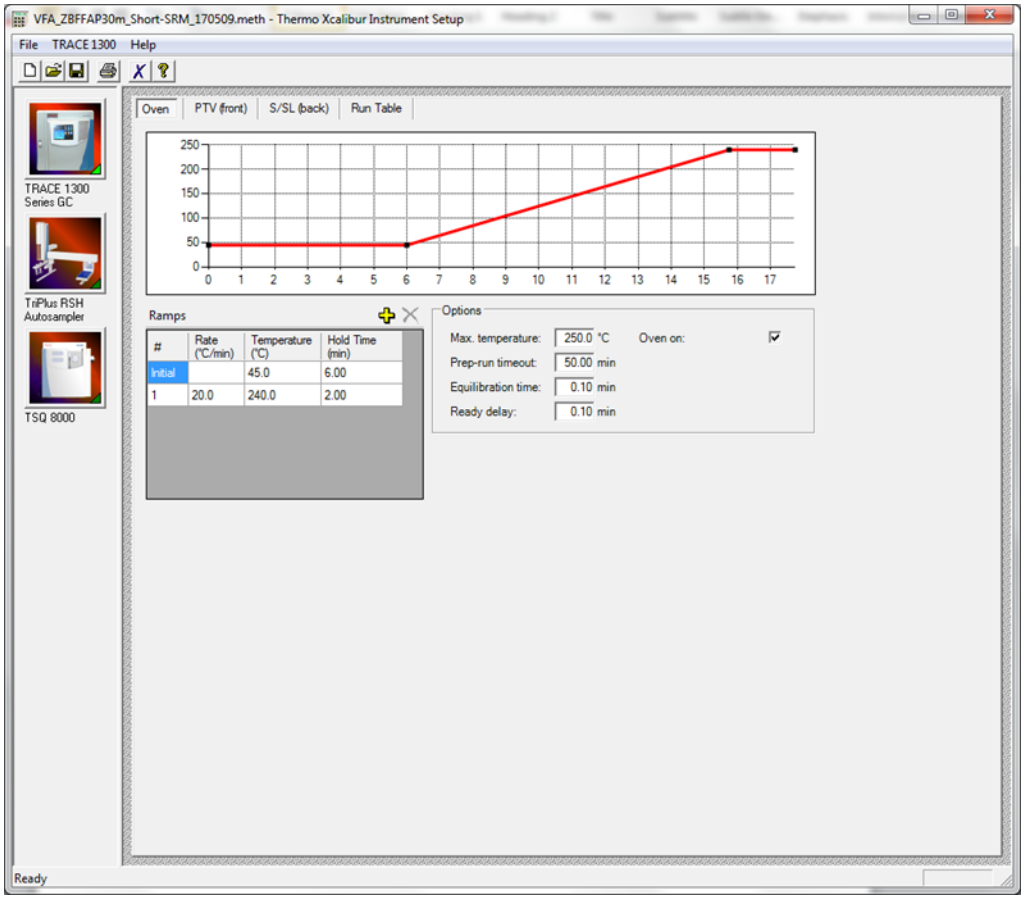
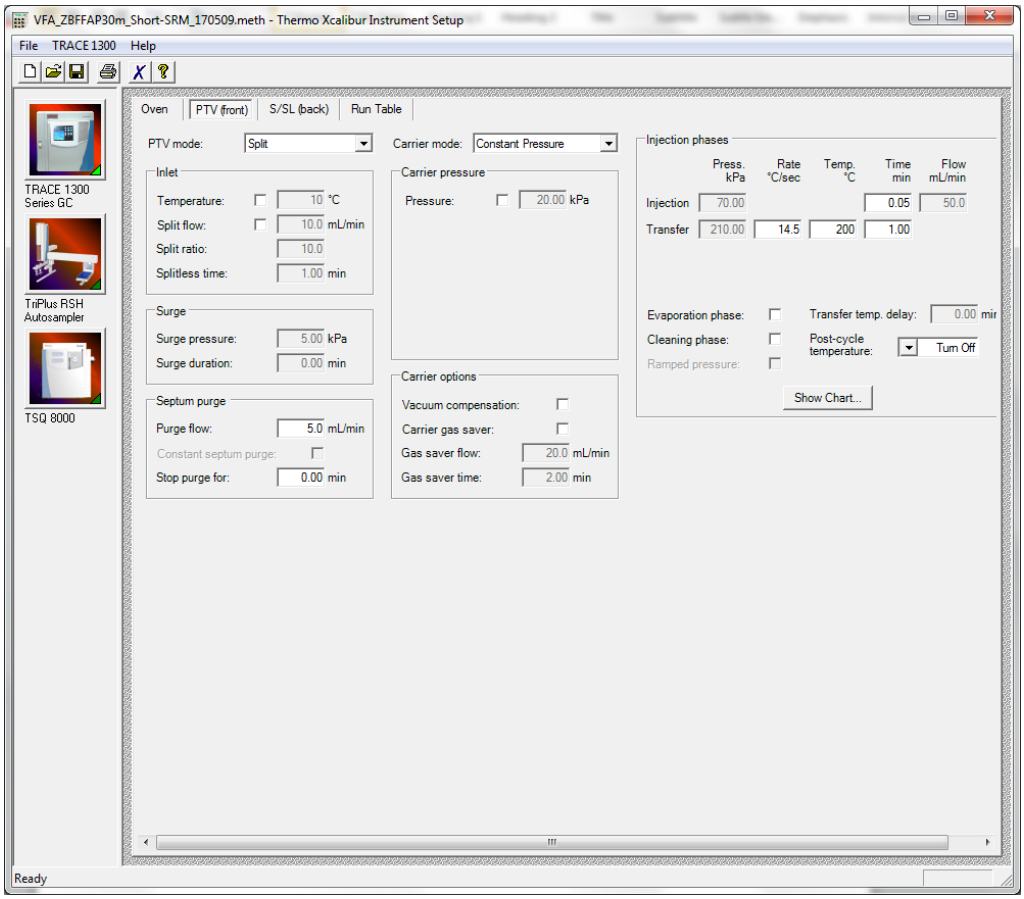
Appendix C. Short-chain fatty analysis: Instrument and method details

- Instrument: Thermo TRACE 1300 GC, fitted with Thermo TriPlus RSH Autosampler. Thermo Scientific, Made in Switzerland
- Software (for analysis and processing of data): Thermo XCalibur
- Column: Zebron ZB-FFAP, Length = 30 m x ID = 0.25 mm x Film thickness = 0.25 µm (*write as "30 m x 0.25 mm x 0.25 µm*"), Min 40˚C, max 250/260 ˚C, Part No 7HG-G009-11, Serial no 197085, Made in USA by Phenomenex
- Method: Oven
    a) Initial temp = 45˚C for 6 min
    b) Ramp 1 = 20˚C/min until temp reaches 240˚C, hold for 2 min
    c) Total Run time = ± 18 min
    d) Detection
    e) Temp = 200 ˚C
- Inlet:
    a) Inlet temp = 240˚C
    b) Mode = splitless
    c) Split flow = 30 mL/min
    d) Injection volume = 1 µL
- Thermo Xcalibur Instrument Setup:





Appendix D. Arabinoxylan-oligosaccharide chromatogram results

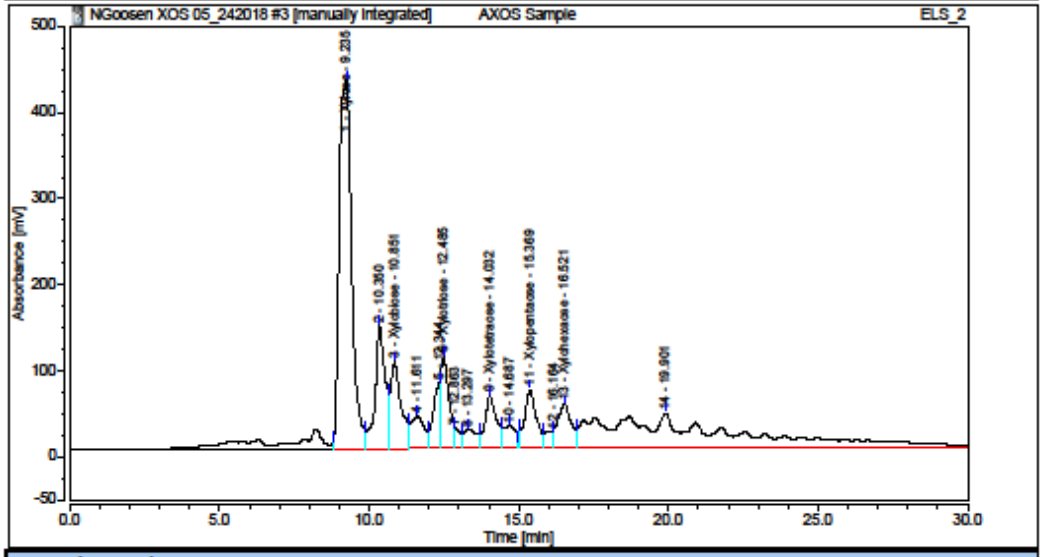
'Amount' measured in g/L

Chromatogram and Results

Injection Details

Injection Name:	**AXOS Sample**	*Run Time (min):*	**55.00**
Vial Number:	**RA1**	*Injection Volume:*	**10.00**
Injection Type:	**Unknown**	*Channel:*	**ELS_2**
Calibration Level:		*Wavelength:*	**n.a.**
Instrument Method:	**Shodex Gradient Scouting**	*Bandwidth:*	**n.a.**
Processing Method:	**FOS Sugars**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**24/May/18 17:09**	*Sample Weight:*	**1.0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount
1	Xylose	9.235	204.743	426.791	29.46	35.54	2.8475
2		10.350	55.748	142.485	8.02	11.87	n.a.
3	Xylobiose	10.851	40.538	100.501	5.83	8.37	0.1610
4		11.611	19.166	37.516	2.76	3.12	n.a.
5		12.344	16.782	76.248	2.41	6.35	n.a.
6	Xylotriose	12.485	35.586	106.298	5.12	8.85	1.0541
7		12.863	5.157	24.329	0.74	2.03	n.a.
8		13.297	11.237	21.544	1.62	1.79	n.a.
9	Xylotetraose	14.032	26.574	60.229	3.82	5.02	1.1433
10		14.687	12.475	25.789	1.79	2.15	n.a.
11	Xylopentaose	15.369	29.438	67.820	4.24	5.65	1.2410
12		16.164	6.948	20.176	1.00	1.68	n.a.
13	Xylohexaose	16.521	26.109	50.482	3.76	4.20	4.1348
14		19.901	204.560	40.635	29.43	3.38	n.a.
Total:			**695.060**	**1200.843**	**100.00**	**100.00**	**10.58**