The effect of plant-derived dietary protein sources on cultured abalone *Haliotis midae* (L. 1758) digestive physiology

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ABSTRACT

The growth and feed conversion ratio of farmed abalone *Haliotis midae* (L. 1758) fed a formulated diet varies in feeds with different protein sources. To better understand this, the effect that various combinations of dietary soybean meal (SBM), sunflower meal (SFM) and fishmeal (FM) had on the digestive efficiency of farmed *H. midae* when included in a formulated feed, were assessed. The aim of this study was to investigate the effect that plant-based proteins had on abalone digestion and gut-bacterial profile of cultured adult abalone when combined with fishmeal. This was done by analysing the chymotrypsin (protease) and cellulase (carbohydrase) digestive enzyme activities from whole-gut sections of farmed abalone that were fed one of four diets for 12 months: FM-only, SBM-only, fishmeal and soybean meal (FM-SBM), and fishmeal and sunflower meal (FM-SFM).

There were significant interactions between the diets, sampling date, and sex of the abalone, which had an influence on the cellulase activities in the digestive tracts of the animals. Those fed the SBM diet had the highest cellulase activity by the end of the sampling period (ANOVA; df = 9; F = 2.9; P = 0.005). The significant interactions between diet and sampling date influenced the chymotrypsin activities of abalone. Animals fed the FM diet had the highest activity of chymotrypsin for most of the sampling period (ANOVA; df = 9; F = 2.5; P = 0.01). Therefore, diet did have an impact on the cellulase and chymotrypsin activities of abalone fed either combination diets (FM-SBM; FM-SFM) or single protein diets (FM; SBM).

The presence of plant material such as cellulose fibres in the diets containing plant proteins may have influenced the cellulase activity levels of each treatment as it would have stimulated endogenous and exogenous cellulase production. However, treatments with the highest crude fibre content, SBM and FM-SFM, resulted in differing activities. The activity levels of chymotrypsin

may have been impacted by the protein type, presence of protein inhibitors and temporal changes in utilisation. Abalone fed the FM diet exhibited the highest chymotrypsin activities on sampling day 182, which was in summer. Animals fed the treatment diets containing fishmeal also developed their reproductive tissues, and may explain the low chymotrypsin activities in abalone fed SBM-only. Additionally, the presence of allergenic compounds would have influenced enzyme activity, as they would have negatively affected the nutrient utilisation and absorptive capacity. Fluctuations in water temperature may have had an effect on enzyme activity, as activities were highest during summer, when seawater temperatures are most variable. A change to lowered water temperatures are known to trigger the growth of digestive epithelial tissue in abalone to allow for increased enzyme secretions and nutrient absorption (Schaeffer *et al.* 2013).

The effect that plant protein inclusion in abalone diets had on the gut-microbiota was revealed by analysing the differences in gut-bacterial community profiles, through the use of metagenomic sequencing. The relative abundance of the phylum Verrucomicrobia, class Verrcomicrobiae and the genus *Rubritalea* were highest in the SBM diet. These bacterial groups were also influenced by the plant material from soybean meal, as hydrolysed cellulose is broken down by cellulases, and allowed for the proliferation of *Rubritalea* bacteria due to them using cellobiose as an energy source. The presence of Bacteroidetes was due to the phyla having bacterial species that can breakdown proteins and species that can break down carbohydrates. The animal protein present in the composition of the FM diet had a positive influence on proteolytic Bacteroidetes growth. The high levels of cellulose from the plant fibres in the soybean meal-only diet (SBM) promoted growth of saccharolytic Bacteroidetes. Tenericutes bacteria's prominence was due to the higher plant fibres from the soybean meal as some *Mycoplasma* are known to

degrade polysaccharides. Therefore, exposure to formulated feeds with high levels of these plant materials would allow for high proliferation.

The thesis established that plant-derived dietary proteins in formulated feeds result in an effect on digestive enzyme activity and a difference in the community structure of gut bacteria in *H. midae* abalone. The results from the current research highlight the importance of studying abalone gut physiology when investigating the impacts of formulated feed composition, and can be used as an additional factor when determining the efficacy of a dietary component. Further research on the gut-bacterial groups and different digestive enzymes of *H. midae* abalone is encouraged, in order to create a holistic view on their response to dietary plant proteins inclusion in formulated feeds, thus reducing the need for fishmeal protein.

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DECLARATION

I declare that the work of this thesis is my own as far as I am aware, and that it does not include information that has been published or written by others without their acknowledgement and reference.

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CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Farmed South African abalone, *Haliotis midae* (L. 1758), have an omnivorous feeding capacity and are fed formulated diets with relatively high protein levels (25 – 35%) to promote growth when farmed commercially (Knauer *et al.* 1996). The issue with formulated feed is the extensive use of fishmeal as the primary protein source, and aquaculture has been criticised for its use of fishmeal-based feeds as they are an expensive and overexploited resource (Gomes *et al*. 1995; Hardy 1996; Sugiura *et al*. 1999; Carter *et al*. 2000; Kissil *et al*. 2000; Goldburg *et al*. 2002; FAO Fisheries 2006; Palti *et al*. 2006; Tacon *et al*. 2008; Hardy 2010; Booman *et al.* 2018). Therefore, plant protein sources have been added to feeds to cut down on the percentage of fishmeal used (Sales *et al*. 2003).

Soybean meal is one of the preferred alternative protein sources used in formulated feeds, due to a high protein content and balanced amino acid profile (AA) (Sales and Britz 2001a; Banaszkiewicz 2011). As such, it results in improved overall abalone growth rate and environmental sustainability of the feed soy ingredient is applicable (Guzmán and Vianna 1998; Shipton and Britz 2001; Dlaza *et al.* 2008; Ayres 2013; Wu *et al.* 2019). The limiting factor for soybean meal and reason for not being included at any higher levels in formulated feed than currently used is due to the presence of anti-nutritional factors (ANFs) (Liener 1994; Francis *et al.* 2001; Krogdahl *et al.* 2003; Heikkinen *et al.* 2006; Murashita *et al.* 2015). These factors include tannins, phytates, fibres, glucosinolates, and saponins (Liener 1994; Francis *et al.* 2001; Krogdahl *et al.* 2003; Heikkinen *et al.* 2006; Murashita *et al.* 2015). Anti-nutritional factors may lead to inflammation

in the intestine and decreased growth rates in abalone due to metabolic costs associated with the regeneration of intestinal tissue when the inclusion level of soybean meal is too high (Dauncey *et al.* 1983; Holt *et al.* 1986; Yamauchi *et al.* 1996; Kemp 2005). Soy is also known to promote unwanted development of abalone gonads due to the presence of phytoestrogens, which can unpredictable spawning events and result in biomass loss (Sales and Britz 2001b; Ayres 2013; Wu 2015),

Formulated feed with slightly lower inclusion levels of soybean meal tend to promote the beneficial effects of certain ANFs such as tannins and fibres and are referred to as bioactive properties (Bolanho and Beléia 2012). These may contribute to improving digestive physiological efficiencies and metabolic regulation in abalone such as digestive enzyme production and the modulation of the composition of the gut-bacterial communities present, which can potentially result in better feed utilisation and growth rate of cultured abalone (Chalupa 1975; Brock *et al.* 1982; Gatesoupe 1999; Verschuere 2000; Macey and Coyne 2005; Bansemer *et al.* 2016). The impact of macroalgae inclusion on formulated feed on the gut-microbiota and digestive enzyme activities of *H. midae* has been investigated before (Nel 2016, Nel *et al.* 2017a; Nel *et al.* 2017b; Nel *et al.* 2018), however not that of terrestrial plant-based protein inclusion.

1.2 RESEARCH AIMS AND OBJECTIVES

The aim of this study was to assess the effect of dietary soybean meal on abalone digestive enzymes, in addition to the gut bacterial profile of the gastrointestinal tract of cultured adult abalone when combined with fishmeal as a protein source. To address these aims, the following objectives were presented: 1) to determine and quantify the digestive effect of abalone fed a soybean and fish meal combination diet as compared to that of abalone fed single protein sources

or a combination of fish meal and sunflower meal as an alternative to soybean meal, by analysing digestive enzyme levels in whole gut sections of farmed abalone (Chapter 2), 2) to characterise the effect of plant protein inclusion in abalone diets on the gut environment by analysing the changes and differences in gut-bacterial community profiles (Chapter 3).

In the literature, the term "gut" can refer to either the intestine or the whole digestive tract. In the current study, "gut" refers to the whole digestive tract which consists of the oesophagus, crop, stomach, style sac and intestines of an abalone.

1.3 LITERATURE REVIEW OF FORMULATED FEEDS AND PLANT-DERIVED DIETARY PROTEIN SOURCES AND ITS EFFECT ON THE ABALONE GUT ENVIRONMENT AND MICROBIOTA

To meet the nutritional requirements of an abalone species, the amino acid profiles of the feed need to be balanced (Guillaume 1997; Sales *et al*. 2003). Abalone require the same ten essential amino acids as other animal groups, with the specific dietary AA requirements of abalone being determined by analysis of the soft tissue AA profile (Allen and Kilgore 1975; Knauer *et al*. 1996; Britz and Hecht 1997; Sales *et al*. 2003). By ensuring that a formulated feed contains the correct AA ratio and protein inclusion level and sufficient digestible energy, somatic growth can be maximized (Shipton 1999; Green *et al.* 2011). By adjusting the experimental diets of juvenile *Haliotis tuberculata* (L. 1758) and *Haliotis discus hannai* (I. 1953), using their soft tissue AA profiles, Mai *et al.* (1995) were able to report optimum growth. However, the digestibility of individual protein sources was not determined in the previously mentioned study. Ensuring that an formulated feed contains the correct AA ratio and sufficient digestible energy levels will result in maximum somatic growth (Britz 1996; Shipton 1999).

Sales and Britz (2001b) concluded from their study that fish meal and *Spirulina* algae were the most appropriate protein sources to be included in an formulated diet, in terms of growth rate and feed conversion ratio (FCR). Additionally, there were no reductions in abalone growth rates when 30 % of the fish meal was replaced by soybean meal, sunflower meals and torula yeast (Shipton 1999; Sales and Britz 2001b). The sunflower and soybean meal were considered to have the most potential as a fish meal replacement due to their lower costs (Sharkie 1997; Shipton 1999). I find that affordability is not the only benefit of using soybean and sunflower meal, but that they are a more sustainable protein source with a consistent supply compared to alterative plant proteins. These results highlight that terrestrial plant-based proteins are viable for inclusion in formulated abalone feeds.

Abalone display size-specific differences in protein and energy requirements (Shipton 1999). When comparing two sizes of *H. midae*, 0.57 g and 23 g, Sales and Britz (2001b) noted that there were different responses in growth rates and feed conversion ratio (FCR) to the diets and protein to energy ratio requirements (PE). Both size classes displayed the lowest growth rates when fed a 10 % lipid level diet, compared to a 2 % and 6 % diet. The growth rates of both large and small size classes increased as the PE ratio increased, though the growth rates peaked at different protein levels. Larger abalone had peaked growth rates when fed a 44 % protein diet, whilst the smaller ones peaked when fed a 34 % protein diet. These results suggest that protein requirements increase as *H. midae* abalone age, which may be due to differences in physiological processes, such as gut microbiota maturation, and therefore different essential AA requirements (Britz and Hecht 1997; Shipton 1999; Sales and Britz 2001b; Sawabe *et al*. 2003, Tanaka *et al*. 2003; Sawabe 2006; Huang *et al*. 2010; Zhao *et al.* 2012; Nel *et al*. 2018). This makes sense as adult abalone are physically larger than younger abalone, and that a portion of their

nutritional demand goes to gonad development. Knowing the impact that protein type and inclusion has on not only abalone physiology, but gut morphology as well, will help with including another aspect in formulating feeds with improved digestibility, which has an impact on somatic growth.

1.3.1 SOYBEAN MEAL ANTI-NUTRITIONAL FACTORS AND ITS EFFECTS ON THE DIGESTIVE TRACT

The inclusion rate of soybean meal in fish and abalone feed is limited by the presence of several ANFs, such as tannins, phytates, fibres, glucosinolates, goitrogens, and saponins, which can lead to decreased growth, cause inflammation in intestinal tissue and limit protein and carbohydrate digestion in fish (Liener 1994; Francis *et al.* 2001; Krogdahl *et al.* 2003; Heikkinen *et al.* 2006; Murashita *et al.* 2015).

Due to the inclusion of phytase and heat-treated soybean meal at a 33.3 % level within a formulated diet, farmed *Haliotis laevigata* (D. 1808) abalone exhibited an increase in mucous cover over their intestinal epithelium compared to those in the wild (Kemp 2005). This inflammatory response was most likely due to the ANFs, specifically antigenic proteins such as lectins and protease-inhibitors (Kemp 2005). Lectins can bind to glycoprotein receptors on epithelial cells along the lining of the intestinal mucosa, which interferes with nutrient absorption and therefore inhibits growth (Liener 1994). Protease inhibitors have the anti-nutritional effect of causing pancreatic hypertrophy which, in turn, reduces growth (Liener 1994). The ANFs in solvent-extracted soybean meal have been shown to cause damage to the intestinal mucosa of Atlantic salmon (Van den Ingh *et al.* 1991; Baeverfjord and Krogdahl 1996; Uran *et al*. 2008; Schaefer *et al.* 2013). Having fed juvenile and sub-adult *H. laevigata* solvent-extracted soybean meal feed, Schaefer *et al.* (2013) did not find significant reductions in growth rates, yet thinning of the stomach epithelium and thickening of the crop was observed. The authors hypothesised that

by increasing the cell size of the crop epithelium, the abalone may have compensated for the lower surface areas present in the stomach.

These morphological responses were less when phytates were added to the feed (Kemp 2005). These responses would be lessened further if the soybean meal was heat-treated before being introduced into the feed (Holt *et al*. 1986; Kemp 2005). Tissue changes such as apical surface denudation, villi shrinkages (Dauncey *et al.* 1983; Yamauchi *et al.* 1996), and underdeveloped villus and crypt cells (Holt *et al.* 1986) impact digestive and nutrient uptake capabilities due to the changes in the intestinal surface area for absorption, digestive enzyme production, and macronutrient transport. While digestibility may not be as affected, the expense of intestinal tissue reparation will result in a reduction in available protein and energy for somatic growth (Kemp 2005). Factors like protease inhibitors from plant protein sources are heat-labile (Van der Poel 1989; Rumsey *et al*. 1993; Francis 2001; Kemp 2005). When the raw dietary proteins are heat-treated, it results in increases in metabolizable energy, feed utilisation, protein and starch digestibility and a decreased pancreas size in animals (Marquardt and Ward 1979; Conan and Carre 1989; Grant 1989; Brenes *et al*. 1993; Igbasan and Guenter 1996; Kemp 2005). This procedure, accompanied by dietary enzyme supplementation, may counteract the deleterious effects of some ANFs. Inactivating protease inhibitors, will allow digestive proteases produced by the digestive tract and gut-microbiota of abalone to continue to function at normal levels, allowing for better digestion, feed utilisation and therefore somatic growth.

1.3.2 Bioactive compounds in plant proteins

Farmed abalone has a relatively slow growth rate of roughly 2 mm a month depending on the stage of development, and it takes far longer for an individual to reach a marketable weight which can

vary between 40 and 200 g according to Rowan Yearsley from Aqunion Pty Ltd (Personal communication 2018). Therefore, a formulated diet needs to provide both suitable growth and maintain optimum health (Kemp 2005). Soybeans are rich in antioxidants and other bioactive compounds (Hayes *et al.* 1977; Bolanho and Beléia 2012) and may contribute to abalone health if provided at the appropriate dietary inclusion level. Therefore, the nutraceutical effects of soybean meal may ultimately improve the physiological status of abalone which may result in increased digestion efficiencies and growth over the long term; this is substantiated by improved growth when soybean meal is included in the abalone diet in combination with fishmeal (Riddin 2012; Ayres 2013). For example, soy promotes gonad development at certain times of the year, though not at the cost of growth rate (Riddin 2012; Ayres 2013; Wu 2015). An issue that may arise from high levels of bioactive compounds present in formulated feed is that it may cause dietary-induced dysbiosis, which is an occurrence where proliferation of unwanted opportunistic bacterial species outcompete the resident bacteria (Nel 2016). Dietary-disruption of the gut-bacterial community may arise if too many extra nutrients are available from a formulated feed (Nel 2016). High bioactive compound levels can potentially promote the proliferation of unwanted opportunistic bacterial species which would use up nutrients that would otherwise have been absorbed by the abalone and therefore impact somatic growth (Nel 2016).

1.3.3 Dietary supplementation and prebiotics in plant proteins

Prebiotics and bioactive compounds are important ingredients in animal diets and have shown promising benefits in mammal examples (O'Sullivan *et al.* 2010). These benefits may also be displayed in abalone as they can digest complex polysaccharides and produce substrates from undigested feed particles that allow for bacterial colonisation (Erasmus *et al.* 1997; Johnston *et al.* 2005; Garcia-Esquivel and Felbeck 2006; Dhanaraj *et al.* 2010; Kemp 2017).

Stimulating the growth of beneficial microflora may help abalone with more efficient fermentation and nutrient breakdown of feed and result in a positive systemic effect and improved growth (O'Sullivan *et al.* 2010; Kemp 2017). The soluble and insoluble fibres, phytates, and tannins in plant protein sources selectively stimulate the growth of gut bacteria in fish (Shipton 1999; Yi-You 2004; Banaszkiewicz 2011; Rijavec and Zupin 2011; Desai *et al.* 2012; Khan and Ghosh 2012; Askarian *et al.* 2013) and may, therefore, act as prebiotics which can promote the growth of enzyme-secreting bacteria in the gut and improve animal growth rate and making plant-based proteins viable substitutes for fishmeal in formulated feed (Gatesoupe 2008; Bagheri *et al.* 2008; Mazouk *et al.* 2008; Merrifield *et al.* 2010; Maity *et al.* 2011; Ray *et al.* 2012; Nel 2016). Prebiotics are not the only method of maintaining or modifying gut-microbiota in abalone, although supplementing formulated feeds is a viable method in doing so (Nel 2016).

Supplementing enzymes such as protease, cellulase, and b-glucanases into formulated diets may assist in abalone digestion, growth, and health. Work by Coote *et al.* (1996) involving the supplementation in diets seems to confirm this since it was discovered that phosphorous supplementation in an formulated diet resulted in a 7.9 % increase in the growth rate of *H. laevigata* abalone fed the treatment, even though much of the supplement leached into the water within twelve hours. This highlights the importance of the role of enzymes in abalone digestive processes and the prospect that certain ANFs at low dietary inclusion levels may be beneficial in that they stimulate the growth of enzyme-producing gut-bacteria, and therefore exhibits the viability of plant-based proteins in formulated feeds (Kemp 2005).

CHAPTER 2

2 THE EFFECT OF PLANT-DERIVED DIETARY PROTEINS IN FORMULATED FEED ON DIGESTIVE ENZYME ACTIVITY LEVELS IN CULTURED SOUTH AFRICAN ABALONE, *HALIOTIS MIDAE*

2.1 INTRODUCTION

Abalone are slow-growing marine gastropods that feed on various macroalgae in the wild. They have long digestive tracts, which accommodates the digestion of a fibrous and relatively nutrient-deficient diet (Nel 2016). The food abalone consume is masticated, ingested, and transported into the crop where the food particles are kept and mixed with digestive fluids, and undergo extracellular digestion (Campbell 1965; McLean 1970; Day and Cook 1995; Harris *et al.* 1998a; Nel 2016). The digested food particles are then transported to the digestive gland, stomach, caecum, and intestine (Campbell 1965). The crop is relatively acidic with a pH between five and six, making it a site for anaerobic bacterial growth and the fermentation of food particles (Nel 2016). The digestive gland of the abalone is a large organ that secretes digestive enzymes and is the production site of extracellular enzymes, nevertheless, it can also absorb food particles previously processed in the crop and caecum for metabolic transformations and intracellular digestion (Campbell 1965).

Haliotis midae, among other abalone species found along the Western Cape coast, feed predominantly on brown macroalgae (Nel 2016). These algae consist of many complex polysaccharides such as cellulose (crystalline b 1,4-D-glucans) (Haug *et al.* 1967; Lahaye and Kaeffer 1997; O'Sullivan *et al.* 2010). Abalone have digestive enzymes present to

degrade these complex polysaccharides, with cellulase (endo-b 1,4-D-glucanase) being one that degrades cellulose (Suzuki *et al.* 2003).

Most farmed abalone feed on nutrient-rich formulated feeds due to the faster growth rates that they yield (Knauer *et al*. 1996; Nel 2016). These formulated feeds tend to have high levels of protein even though abalone are traditionally herbivorous, though it is commonly known now that abalone have an omnivorous feeding capacity, effectively being able to digest and absorb formulated feeds with high levels of protein (Shipton and Britz 2001; Nel 2016). A combination of high costs and sustainability issues encouraged the industry to include various protein sources such as soybean meal and sunflower meal as alternatives to fishmeal or to reduce fishmeal proportions in an formulated feed (Shipton and Britz 2001). These formulated feeds have low levels of fibre and complex polysaccharides, and high levels of reserve carbohydrates and proteins from animal and terrestrial plant sources (Lahaye and Kaeffer 1997; Sales and Britz 2003; Nel 2016). While formulated feed differs greatly from natural diets, cultured abalone have shown to adjust well to formulated feed when introduced at an early developmental stage (Knauer *et al.* 1996).

An abalone's diet influences the activity levels of both endogenous and exogenous carbohydrases (Bennet *et al*. 1971; Knauer *et al*. 1996; Erasmus *et al*. 1997; Nel 2016). When comparing the dietary effect of red and brown macroalgae, Erasmus *et al.* (1997) discovered that alginate lyase, laminarinase, agarose and carrangeenase activity levels in the digestive gland of *H. midae* were considerably higher in abalone fed brown macroalgae. This illustrates that abalone can produce a range of carbohydrases, as the digestive glands had no bacteria present in the study. However, the bacterial carbohydrase activity throughout the rest of the digestive tract of the abalone suggests that resident bacteria help digest polysaccharides (Erasmus *et al*. 1997).

Protease enzymes play an important role in the digestive tract and are well-studied due to high protein levels present in abalone formulated feed (Picos-García *et al.* 2000; Garicia-Esquivel and Felbeck 2006). The high levels of protease activity levels recorded in the digestive glands of *Haliotis rubra* (L. 1814) and *Haliotis fulgens* (P. 1845) highlight how important the organ is as a site for protease secretion (Picos-García *et al.* 2000; Edwards and Condon 2001). Serviere-Zaragoza *et al.* (1997) discovered that alkaline protease activity levels were highest in intestinal and rectal fluid, followed by the digestive gland and crop-stomach in wild *H. fulgens*, with chymotrypsin activity levels being present only in the intestine and rectum. Conversely, chymotrypsin activity levels were prominent in both the digestive gland and digestive tract in juvenile *H. fulgens* (Picos-García *et al.* 2000).

Similar to carbohydrases, diet also influences protease activity levels (Garcia-Esquivel and Felbeck 2006). Interestingly, protease activity was higher in juvenile *Haliotis rufescens* (S. 1822) abalone fed kelp than in those fed two other high–protein level formulated feed treatments (Garcia-Esquivel and Felbeck 2006). Since formulated feed has higher protein bioavailability and digestibility than macroalgae, the need to utilize enzyme-facilitated digestion is not as high and protease activity levels may decrease (Nel *et al.* 2017a). Alternatively, the high reserves of carbohydrates and proteins can cause an increase in the number of opportunistic bacteria in abalone fed formulated feeds which could lead to competition for nutrients, and not a symbiotic relationship as seen within exogenous enzyme supplementation (Macey and Coyne 2005; Garcia-Esquivel and Felbeck 2006; Nel 2016).

As mentioned in the previous chapter, soybean meal and various other terrestrial plant-based proteins used for feed contain several anti-nutritional factors (ANFs), which may have severe effects on an animal's digestive tract by reducing the efficacy of digestion and nutrient absorption. An ANF present in soybean meal that may interfere with protease enzyme production and efficacy is protease inhibitors (Krogdahl *et al*. 1994; Olli *et al*. 1994; Anderson and Wolf 1995;

Takii *et al*. 1998; Refstie *et al*. 2006; Lazzari *et al*. 2010). The introduction of protease inhibitors via soybean meal disrupts protease enzyme activity by binding to chymotrypsin or trypsin, for example, and leaving the enzyme inactive (Silva *et al*. 1995; Shipton 1999). Protease inhibitor concentrations as low as 0.1 % in diets that have low protein levels have the ability to increase mortality rates due to malnutrition in *Teleogryllus commodus* (W. 1869) crickets (Burgess *et al.* 1994). Organisms can compensate for inhibition by increasing digestive protease production, as seen in Atlantic Salmon (Olli *et al.* 1994; Shipton 1999). The deleterious effects of protease-inhibitors within the plant meals of formulated feed still must be addressed to increase feed utilisation in animals (Shipton 1999). Fortunately, terrestrial plant protein digestibility can be improved through processing conditions, which destroy protease inhibitors and open protein structures to enzymatic reactions (Hsu *et al.* 1977; Shipton 1999). Plant meals undergo heat-treatment, a method used to inactivate protease inhibitors as they are heat-labile, as are several other ANFs (Hsu *et al.* 1977; Shipton 1999). This process was also performed on the plant protein meals used in the current study. Shipton (1999) discovered *H. midae* fed formulated feed with heat-treated soybean and sunflower meal resulted in better digestibility than those that were not heat-treated. This seems to have been evident in the sister study to the current one conducted by Wu (2020), as it was observed that the abalone fed FM-SBM, FM-SFM and SBM-only had significantly lower FCR values than those fed the FM-only treatment diet. Additionally, García-Carreño *et al.* (1997) observed similar digestibility results in shrimp fed heat-treated and un-treated plant materials. Analysing digestive enzyme extracts will help shed light on the effect these meals may have on the protease activities of abalone gastrointestinal tract.

The aim of Chapter 2 was to assess the effect of plant-derived dietary proteins on farmed abalone digestive enzyme activities in the gut. Thus, the aim was addressed by achieving the

following objective: to quantify the digestive effect of abalone fed a soybean and fish meal combination diet (FM-SBM) as compared to that of abalone fed single protein sources or a combination of fish meal (FM-only) and sunflower meal (FM-SFM) as an alternative to soybean meal (SBM-only), by analysing the cellulase (carbohydrase) and chymotrypsin (protease) digestive enzyme activities of whole-gut sections over time.

2.2 METHODS

2.2.1 EXPERIMENTAL SITE AND SYSTEM

The study was conducted at Whalerock Abalone Farm, owned by Aqunion (Pty) Ltd, in Hermanus, South Africa (34° 43' S; 19° 22 E). The study took place from July 2017 to July 2018. Seawater was supplied to the farm via a pump-a-shore system, where it was transported to a header tank equipped with a 90-micron drum filter and gravity-fed to the culture tanks and hatchery (Naylor *et al.* 2011). The 2800 litre tanks used for the study were the same as those used by the farm. They were made of plastic canvas with wooden frames and contained seven abalone baskets in a series. All tanks were supplied with aeration via two 20 mm polyvinyl chloride pipes with 1 mm holes, which ran perpendicular along the bottom of the tank. Each tank had one inflow and one standing pipe for outflow. The inflow rate was maintained between 400 and 600 mL/s with a tank water exchange rate between 1.30 and 1.94 hrs. The abalone used for the study were stocked in oyster mesh baskets, each containing seven vertical acrylonitrite butadiene styrene plastic plates and a horizontal floating top feeder plate to increase surface area and create shade for each basket. The tanks used for the study were included in the farm's tank cleaning schedule, which took place every two weeks. The baskets of each tank were moved during the clean-up process. This was done in accordance with farm practice and was done to reduce tank effects.

2.2.2 EXPERIMENTAL ABALONE AND STOCKING

Commercially reared sub-adult *H. midae* abalone from the same cohort, with wet-weights between 40-50 g, were used for this study. Before the study, the abalone were fed diatoms after their larval phase, Abfeed®- ES26 (26 % protein, 3.4 % lipid, Marifeed Pty Ltd, Hermanus, South Africa) and green *Ulva* algae during their weaning phase, and Abfeed®- S34 (34 % protein, 4.2 % lipid, Marifeed Pty Ltd, Hermanus, South Africa) during their grow-out phase. Prior to and throughout the study, the experimental abalone were subjected to the farm's standard husbandry practises. At the beginning of the study, abalone were added to baskets at a stocking density that complied with the current farm system at the time. Every four months, the stocking densities were reduced by dividing abalone from each basket at random so that consistent basket masses were achieved. This process is known as splitting.

2.2.3 FEED FORMULATION AND EXPERIMENTAL DESIGN

The experimental *H. midae* abalone were fed isonitrogenous and isoenergetic diets with various protein source combinations, including: (1) fishmeal only (FM); (2) soybean meal only (SBM); (3) fishmeal and soybean meal (FM-SBM); and (4) fishmeal and sunflower meal (FM-SFM), for 12 months. Each diet contained 30 % crude protein and 6 % lipid (Table 2.1). The type of soybean and sunflower meal used in the formulation was ± 45 % soya oilcake and 30 – 35 % sunflower oilcake. Each diet was randomly allocated to one of four replicate tanks of abalone, each containing seven baskets of abalone. Abalone were fed a known amount of feed daily between 16:00 and 17:00. The known amount of feed was determined by using the farm's feeding method, which was to satiation, and the amount of feed for each treatment was recorded daily. There were four treatments, with an allocation of four tanks per treatment and therefore a total of 16 tanks was used

in the trial. The Abfeed® leaf pellets remained stable and in shape for roughly 36 hours, after which they start to crumble over time. The leaf pellets are able to keep their stability for longer, however, when abalone start to feed on them, the shape starts to crumble.

Table 2.1: The percentage composition (%) of the dietary ingredients used in the diets with four different protein sources that included: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal only (SBM) and the parameters used to generate the dietary formulations.

	Treatment			
Ingredient	FM	FM-SBM	FM-SFM	SBM
Fishmeal	38.84	27.55	29.45	0.00
Soya meal	0.00	18.00	0.00	61.92
Sunflower meal	0.00	0.00	18.00	0.00
Starch	59.06	51.70	49.26	33.74
Lysi-PEARL™	0.93	0.83	1.46	0.56
Vitamin mixture	0.11	0.11	0.11	0.11
Vegetable oil	1.06	1.82	1.72	3.68
Total	100.00	100.00	100.00	100.00
Protein (%)	30.00	30.00	30.00	30.00
Lysine (% of protein)	6.90	6.90	6.90	6.90
Lipids (%)	6.00	6.00	6.00	6.00
Crude fibre (%)	0.00	0.37	1.34	1.38
Energy (MJ kg^{-1})	17.43	16.94	17.00	15.77

The complete ingredient composition of the treatment diets is proprietary information; therefore, the crude fibre values are calculated using the average fibre content of each protein meal given by the producers of Abfeed®, Marifeed Pty Ltd, Hermanus, South Africa.

2.2.4 Environmental variables

Seawater temperatures, pH and oxygen concentration (% saturation and mg/L) in the abalone tanks were recorded weekly at 08:00 with an OxyGuard® Handy Polaris oxygen meter and an OxyGuard® Handy pH meter (OxyGuard International A/S Birkerød, Denmark). To measure seasonal seawater temperatures, a temperature logger (HOBO UX100, Onset Computer Corporation, Bourne, Massachusetts, United States) was used in the header tank of the abalone farm every ten minutes for the study duration. Total ammonia nitrogen (TAN) concentrations were determined monthly by using the Solózano (1969) method: 50 mL of water from each tank was sampled and kept in darkness for one hour after the reagents were added. A spectrophotometer (Prim Light, Secomum, 30319, Ales, France) was used for the absorbance readings of the water samples, which were read at a wavelength of 640 nm. Total ammonia nitrogen (TAN) values were determined using linear regression standard curves from known ammonia chloride concentrations.

2.2.5 Abalone viscera sample preparation

During sampling, two males and two females of each treatment were collected at random from each of the four replicate tanks. A total of 64 abalone were collected every 90 days, eight males and eight females per treatment. Sampling started at 08:00 and the animals were gut purged 48 hours prior to sampling to ensure their digestive tracts are clear of food. Each sampling period lasted two days. These sampled abalone were used for the comparison of digestive enzymes between different diets. On the farm, the abalone were dissected aseptically after euthanasia via chilling in a blast freezer, according to standard farm practices. The viscera containing the digestive tract was removed and placed into numbered plastic zip lock bags on ice before being placed into a freezer and kept at -20 °C. In the laboratory, two dissected viscera samples belonging

to abalone of the same sex and replicate were defrosted and cut into small pieces using a sterile pair of surgical scissors while chilled. The fragments were then homogenised together with a sterilised mortar and pestle while chilled. Each homogenised sample was placed into a 50 mL centrifuge tube with 5 mL of chilled 0.1 M citric acid buffer (pH 5.2). The samples were then centrifuged for 45 min at 13000 RCF at 4 °C. Aliquots of each supernatant were added evenly into three 1.5 mL Eppendorf tubes and stored at -20 °C, and 4 °C during short-term storage between assays.

2.2.6 Enzyme assay protocols

The chemicals and reagents used for this study were produced by Sigma-Aldrich. The commercial enzymes used were Beta-(1- >3)-D-Glucanase from *Helix Pomatia* (L. 1758), Endoglucanase E1 from *Acidothermus* (M. 1986), Cellulase (E2164-100UN) and Chymotrypsin from bovine pancreas (C4129). These enzymes were used for the establishment and optimisation of the two enzyme assay protocols. With regards to cellulase, the production of reducing sugars was tested for assay conditions at room temperature for 30 min with a pH 5.2, which would replicate that of an abalone's gut (Nel 2016). Carboxymethyl cellulase (CMC) was used as substrate for the assay at 1 % (w/v) substrate, to determine carboxymethyl cellulase activity.

N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (S7388) (SAPNA) with a 0.2 % concentration was used as substrate for the chymotrypsin activity assay, with the pH and temperature being 7.5 and 25 °C for five minutes. Taking the activity levels of the two commercial enzymes at the specified assay conditions into account, the assays were tailored for the assays with abalone gut extracts.

2.2.7 ENZYME ASSAY PROTEIN CONCENTRATIONS

The Bradford method was used to determine soluble protein for each sample in triplicates (Bradford 1976). Bovine serum albumin (BSA) with a concentration range of 0.2 to 0.9 $mg.mL^{-1}$ was used to create a standard curve at a wavelength of 595 nm ($R^2 = 0.9446$ for 25 µl BSA volumes).

2.2.8 ENZYME ASSAYS

All enzyme assays were performed using endpoint colorimetric assays, for which absorbance of hydrolysis end-products were determined by a Powerwave X microplate reader spectrophotometer with KC Junior™ software (Bio Tek® Instruments, Winooski, VT 05404, United States). For each assay, the concentration of the end product was calculated from the absorbance at a specific wavelength using a standard curve equation ($y = mx+c$), with concentration (x-axis) plotted against absorbance (y-axis). The assays were performed in triplicate to produce three readings per sample, with the use of 96-well microplates. Specific enzyme activity (mg product. mg^{-1} protein) was expressed as the amount of end product produced per protein in the assay over a given time in order to avoid the assumption that there exists a linear relationship between the liberation of hydrolysis end products and time.

Proteases

Chymotrypsin assays were performed by using the method of Serviere-Zaragoza *et al.* (1997). The assays were prepared by adding 0.5 % (w/v) SAPNA substrate to a buffer with Tris-HCl buffer (pH 7.5) and 20 nM $CaCl_2$. A volume of 25 µl of each enzyme extract was added to 225 µl of buffered substrate into one of the 96 wells. The reaction was left to run for five minutes at 25 °C. The reaction was terminated afterward by adding 30 % acetic acid. The wells were read against a

water blank at 410 nm. The chymotrypsin activity levels were expressed as SAPNA units per mg (A410nm/min*1000* volume of the reaction mixture)/8800*mg protein.

Carbohydrases

Cellulase assays for gut samples of abalone that were fed various diets were performed by measuring the formation of glucose concentrations with the use of the dinitrosalicylic acid method. The standard curve of reducing sugar concentration against absorbance was created using a concentration range (0.2 to 1.0 mg. mL^{-1}) of glucose ($R^2 = 0.9967$) as standard. Cellulase assay reaction conditions were the same as those used during the production of the standard curve.

2.2.9 Statistical analysis

Statistica 13 (Dell Software, Aliso Viejo, California) was used to calculate normality and homogeneity of variances. This was done using the Shapiro-Wilk test and Levene's test, respectively. Factorial analysis of variance (ANOVA) were performed to determine significant interactions between the independent variables: diet, sex, and sampling date. To compare specific enzyme activity levels between different sampling dates, and the other diets, one-way ANOVAs and Kruskal-Wallis ANOVAs were performed for when no significant interactions were observed. To compare specific enzyme activities between males and females for each treatment, t-tests and Mann-Whitney U tests were performed for when no significant interactions were observed.

2.3 RESULTS

Table 2.2: Proximate analysis of the treatment diets: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal only (SBM) (Wu 2020).

Treatment	Protein (%)	Moisture (%)	Lipid (%)	Ash (%)
FM	27.72	11.73	3.37	5.42
FM-SBM	28.61	11.41	3.01	4.89
FM-SFM	26.18	10.48	3.57	5.12
SBM	30.91	10.96	3.43	3.21

2.3.1 CELLULASE

Significant interactions were present between the variables: Diet and Sampling Date, Diet and Sex, and Diet, Sampling Date and Sex (Table 2.3). No significant interactions were found between the variables: Sampling Date and Sex (Table 2.3).

Abalone fed the FM diet exhibited significantly higher cellulase activity levels than those fed FM-SBM, FM-SFM and SBM on sampling days 182 (ANOVA; df = 3; F = 3.91; P = 0.02; Figure 2.1). No significant differences in cellulase activity was observed between treatment diets on sampling day 91 (ANOVA; df = 3; F = 0.09; P = 0.96), 273 (ANOVA; df = 3; F = 1.02; P = 0.96) and 357 (ANOVA; df = 3; F = 2.25; P = 0.96).

Diet and the sex of abalone had a significant interaction on the cellulase specific activity levels of the abalone (Table 2.3). For abalone fed the FM diet, females displayed significantly higher cellulase activity levels than males on sampling day 357 (T-test; df = 5; t = -5.42; P = 0.003; Figure 2.1). Male abalone fed the FM-SBM diet exhibited significantly higher cellulase activity levels than

females on sampling day 357 (T-test; df = 6; t = 7.12; P = 0.0004; Figure 2.2). Male abalone fed the FM-SFM diet exhibited higher cellulase activity levels than females on sampling day 182 (T-test; df = 6; t = 2.86; P = 0.03; Figure 2.2) and 357 (T-test; df = 6; t = 9.75; P = 0.00007; Figure 2.1). When fed the SBM diet, the cellulase activity levels of female abalone was significantly higher than males on sampling day 357 (T-test; df = 6; t = -5.54; P = 0.0015; Figure 2.1).

Table 2.3: The univariate results of a factorial ANOVA for each independent variable concerning cellulase specific activity levels.

Effect	Degrees of Freedom	F-value	P-value
Diet	3	0.45	0.71
Sampling Date	3	25.31	0.0000001*
Sex	1	2.47	0.12
Diet-Sampling Date	9	2.86	0.0052*
Diet-Sex	3	7.98	0.00009*
Sampling Date-Sex	3	1.94	0.13
Diet-Sampling Date-Sex	9	4.30	0.00012*

The cellulase activity level of males fed either of the diets was significantly higher than females on sampling day 182 (ANOVA; df = 30; F = 1.50; P = 0.017; Figure 2.2). There were no significant differences between the cellulase activity levels of males and females for sampling days 91 (ANOVA; df = 24; F = 1.63; P = 0.71), 273 (ANOVA; df = 30; F = 1.93; P = 0.45), and 357 (ANOVA; df = 30; F = 5.27; P = 0.38; Figure 2.1).

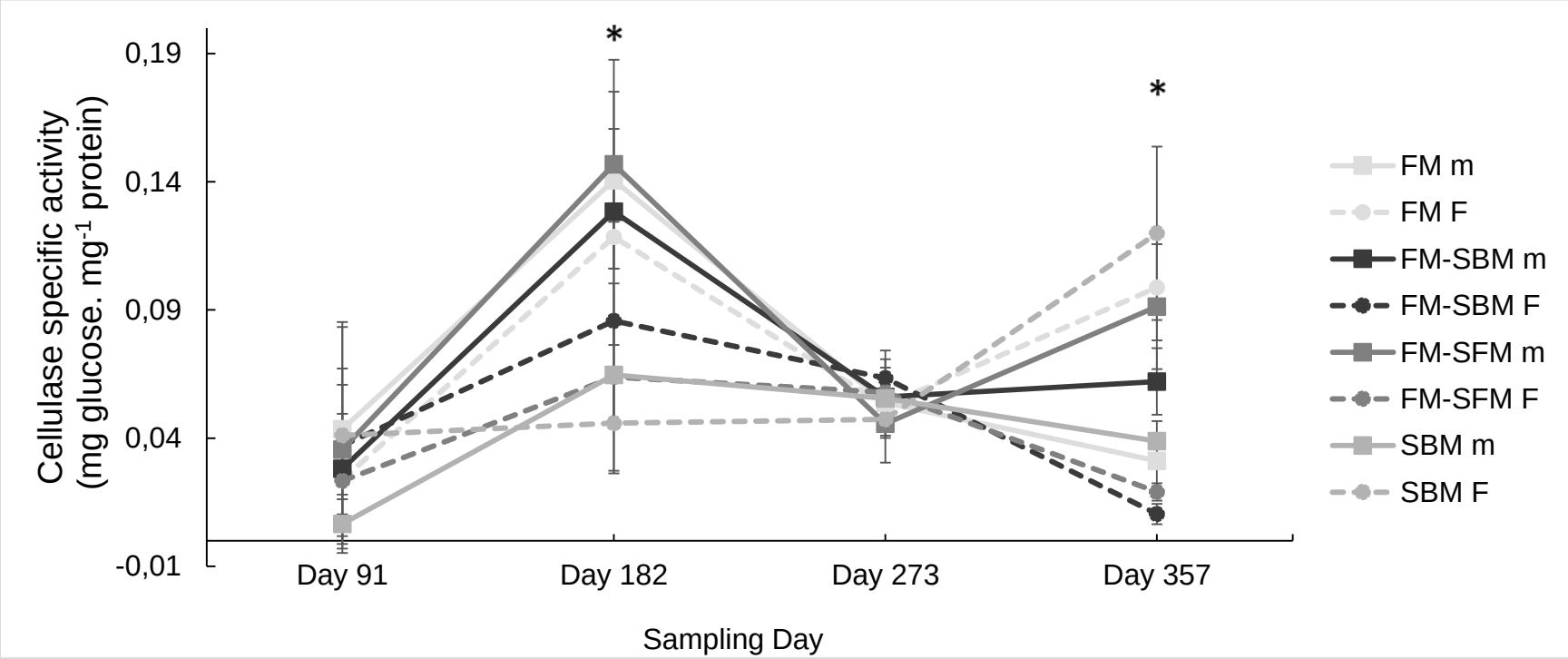

Figure 2.1: The mean cellulase specific enzyme activity (A410nm/min *1000*volume of the reaction mixture)/8800*mg protein) from the digestive tract extracts of male and female *H. midae* abalone fed: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM) over four sampling periods. “m” represents males, and “f” females. The symbol “*” signifies statistically significant differences.

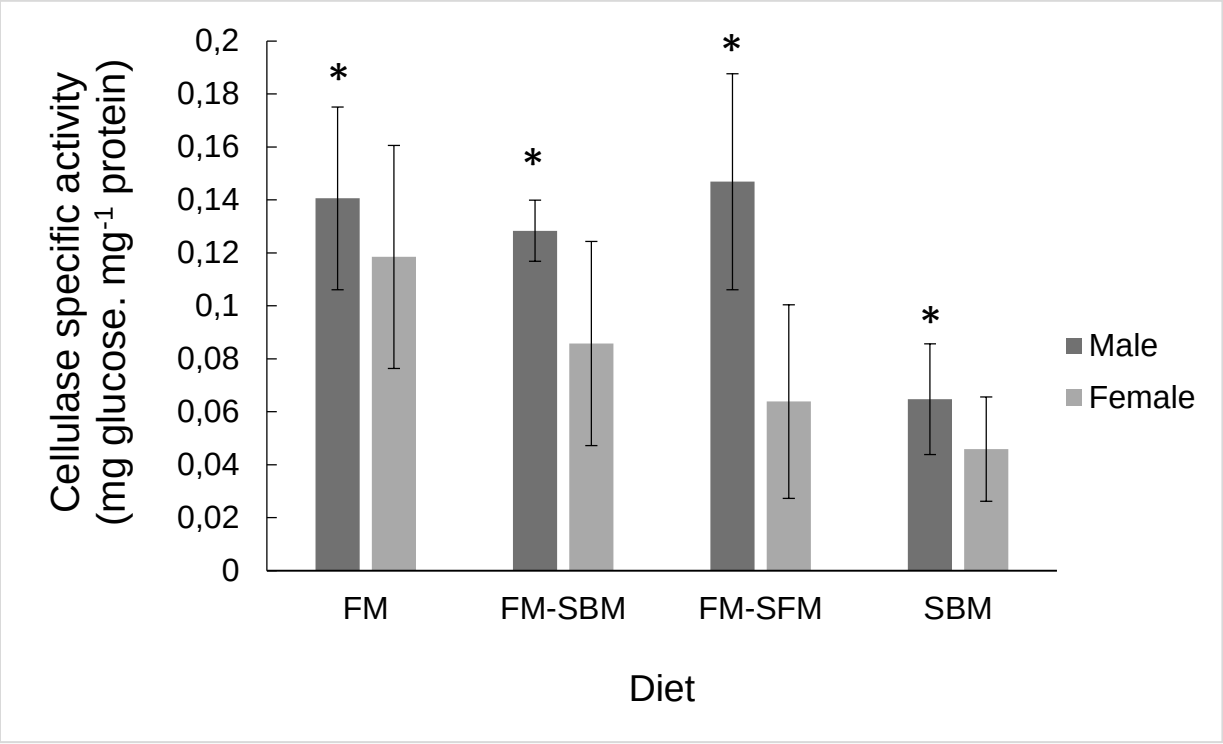

Figure 2.2: The means (± standard deviation) for cellulase specific enzyme activity (A410nm/min*1000*volume of the reaction mixture)/8800*mg protein) from the digestive tract extracts of male and female *H. midae* abalone fed four diets on sampling day 182 (N = 32): fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM). The symbol "*" signifies statistically significant differences.

2.3.2 CHYMOTRYPSIN

Significant interactions were only displayed between the variables Diet and Sampling Date (Table 2.4). No significant interactions were seen between: Diet and Sex, Sampling Date and Sex, and Diet, Sampling Date and Sex (Table 2.4).

A significant difference between the chymotrypsin specific activity levels of abalone fed different diets over time was observed (Table 2.4; Figure 2.4). Abalone fed the diet SBM were observed to have the highest chymotrypsin activities compared to the other three diets on sampling day 91. Abalone fed the FM diet had significantly higher chymotrypsin activity levels than those fed FM-SBM, FM-SFM and SBM throughout sampling days 182, 273 and 357.

Table 2.4: The univariate results of a factorial ANOVA for each independent variable concerning chymotrypsin specific activity levels.

Effect	Degrees of Freedom	F-value	P-value
Diet	3	2.89	0.04*
Sampling Date	3	58.51	0.0000001*
Sex	1	14.46	0.00025*
Diet-Sampling Date	9	2.46	0.015*
Diet-Sex	3	0.45	0.72
Sampling Date-Sex	3	1.19	0.32
Diet-Sampling Date-Sex	9	1.19	0.31

Since there was no significant interaction between Diet and Sex, t-tests were performed comparing the chymotrypsin activity levels between abalone sexes for each diet and sampling date (Figure 2.3). Female abalone fed FM-SBM diet displayed significantly higher activity levels than males on sampling day 91 (T-test; $df = 6$; $t = -2.66$; $P = 0.038$). No significant differences in chymotrypsin activity levels were found between males and females for abalone fed FM (T-test; $df = 6$; $t = -1.86$; $P = 0.14$), FM-

SFM (T-test; df = 6; t = 1.50; P = 0.31), and SBM (T-test; df = 10; t = 0.35; P = 0.73). Male abalone fed FM-SFM had significantly higher chymotrypsin activity levels than females on sampling day 182 (T-test; df = 6; t = 2.86; P = 0.029), though no significant differences in activities were present between males and female abalone fed FM (T-test; df = 10; t = 0.35; P = 0.77), FM-SBM (T-test; df = 6; t = 1.64; P = 0.15), and SBM (T-test; df = 6; t = 1.30; P = 0.24). No significant differences in chymotrypsin activity levels between male and female abalone fed FM (T-test; df = 6; t = 0.14; P = 0.89), FM-SBM (T-test; df = 6; t = -1.15; P = 0.23), FM-SFM (T-test; df = 6; t = -1.33; P = 0.23), or SBM (T-test; df = 6; t = 0.82; P = 0.44) were present on sampling day 273. Female abalone exhibited higher chymotrypsin activity levels than males in every treatment on sampling day 357: FM (T-test; df = 5; t = -5.42; P = 0.0029), FM-SBM (T-test; df = 6; t = 7.12; P = 0.00039), FM-SFM (T-test; df = 6; t = 9.75; P = 0.0000067), SBM (T-test; df = 6; t = -5.54; P = 0.0015).

There was a significant difference between the chymotrypsin activity levels of male and female abalone for sampling day 91, with females having higher levels for all diets except SBM, where males displayed higher levels (T-test; df = 30; t = -2.07; P = 0.047; Figure 2.3). Female abalone had significantly higher chymotrypsin activity levels than males for sampling day 357 (T-test; df = 30; t = -2.24; P = 0.033; Figure 2.3). No significant differences between male and female abalone chymotrypsin activity levels were found on sampling days 182 (T-test; df = 30 t = -1.95; P = 0.061) and 273 (T-test; df = 30; t = -0.57; P = 0.57).

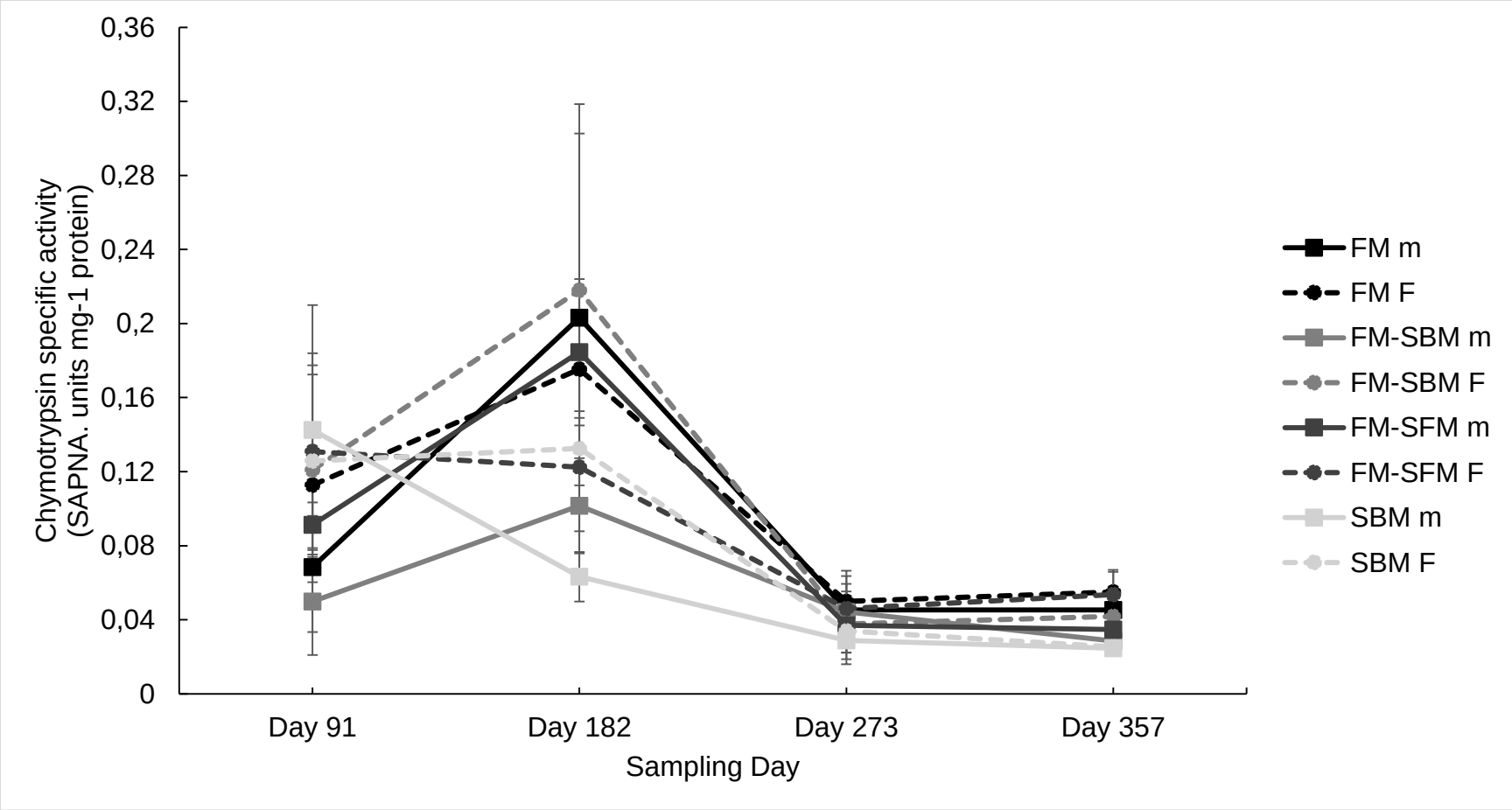

Figure 2.3: The mean chymotrypsin specific enzyme activity (A410nm/min*1000* volume of the reaction mixture)/8800*mg protein) from the digestive tract extracts of male and female *H. midae* abalone fed fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM) over four sampling periods. “m” represents males, and “f” females.

Water parameters

There were no significant differences in water temperature, dissolved oxygen (% and mg. L^{-1}) and pH between treatments over the trial period of 12 months (RM-ANOVA; $p > 0.05$; Table 2.5; Wu 2020)

Table 2.5: Average (± standard deviation) seawater temperature, oxygen (O_2) and pH of the tanks containing abalone fed the different treatments are displayed: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM) over 12 months (RM-ANOVA, $p > 0.05$).

	Treatment	Mean	Minimum	Maximum
Temperature (°C)	FM	15.08 ± 1.54	11.80	18.70
	FM-SBM	15.09 ± 1.54	11.80	18.70
	FM-SFM	15.08 ± 1.54	11.70	18.70
	SBM	15.08 ± 1.53	11.70	18.70
O_2 (mg. L^{-1})				
	FM	7.98 ± 0.41	6.70	9.00
	FM-SBM	7.98 ± 0.40	6.82	8.99
	FM-SFM	7.97 ± 0.39	6.92	8.97
	SBM	7.97 ± 0.39	6.91	8.99
O_2 saturation (%)				
	FM	97.58 ± 3.19	86.20	104.00
	FM-SBM	97.50 ± 2.96	88.80	103.90
	FM-SFM	97.34 ± 2.93	88.10	103.60
	SBM	97.54 ± 2.91	89.80	103.80
pH				
	FM	7.71	6.90	7.94
	FM-SBM	7.71	6.93	7.96
	FM-SFM	7.71	6.93	7.97
	SBM	7.71	6.94	7.96

Table 2.6: Average (± standard deviation), minimum and maximum seasonal seawater temperatures measured from the header tank of Aqunion Whalerock farm.

		Seawater temperature (°C)		
Season	Sampling day	Mean (±SD)	Minimum	Maximum
Spring	91	16.31 ± 1.55	12.20	19.82
Summer	182	15.45 ± 2.07	10.78	22.95
Autumn	273	15.94 ± 1.88	11.23	20.34
Winter	357	16.65 ± 0.32	13.98	18.03

2.4 DISCUSSION

The different terrestrial plant-based protein meals in formulated abalone feed in the current study were expected to influence the activity levels of the digestive protease chymotrypsin in *H. midae*. There were no significant differences between diets until the end of the trial, where the chymotrypsin activities were highest in FM only and FM-SFM diets (Figure 2.3). The activities of abalone fed SBM treatment were consistently lower than the other treatments while the others had similar levels to one another within each sampling date. Higher levels of ANFs, specifically protease inhibitors may have caused lower chymotrypsin activities (Krogdahl *et al*. 1994; Olli *et al*. 1994; Anderson 1995; Takii *et al*. 1998; Refstie *et al*. 2006; Lazzari *et al*. 2010). It is already well-known that legumes, such as soybeans, and other terrestrial plant materials contain protease inhibitors that bind with proteases like chymotrypsin, making them inactive and ineffective (Silva *et al*. 1995; Shipton 1999). A study by Burgess *et al.* (1994) showed that levels of protease inhibitors as low as 0.1% in diets can cause mortality in black field crickets, *Teleogryllus commodus* (W. 1869). Mortality in abalone due to protease inhibitors in their feed is unlikely, as they may compensate for lower protease efficacy by producing more proteases. This process observed by Olli *et al.* (1994) where Atlantic salmon, *Salmo salar* (L. 1758) were fed diets containing five different levels of trypsin inhibitor from soybeans. Feeding salmon diets with inhibitor levels of 5 mg per gram of feed or less resulted in negligible effects on the trypsin activities, showing that there is some capacity for enzyme compensation (Olli *et al.* 1994).

As mentioned before, the main method to reduce the effects of protease inhibitors is to heat-treat plant material to inactivate them. Hsu *et al.* (1977) found that by undergoing heat treatment, the digestibility coefficient of soybean meal improved by 45.1 % and sunflower meal by 7 % compared to a cold mechanic pressing method. Such large differences in digestibility

improvements illustrate how difficult it is to compare different plant meals to one another as they contain protease inhibitors of differing amounts, with various structures and actions (Silva *et al*. 1995; Shipton 1999). The soybean and sunflower meal used in the current study for the diets were also heat-treated, yet the process does not fully negate the effects of the inhibitors and may explain why abalone fed the SBM diet exhibited such differing chymotrypsin activities to the other treatments.

The diets used for the study all had the same percentage composition of protein, as the aim of the trial was to compare the effect that different plant-based proteins had on the enzyme activities of *H. midae* abalone. It was expected that there would be differences in chymotrypsin activity between treatments, especially between combination and single protein source treatments. The results of the current study have shown that combination protein diets exhibit higher protease activities than the SBM only treatment. Interestingly, the FM only treatment had significantly higher chymotrypsin activities than of the other treatments used at the end of the trial. For the first two sampling days, however, there was a high variation in chymotrypsin activities in all the treatments. The last two sampling dates showed that the variation reduced considerably, possibly indicating some level of adaptation to the introduced diets or due to physiological changes during maturation.

Similar to the findings of the present study, the results by García-Carreño *et al.* (2003) showed some variation of serine proteases when *H. fulgens* abalone were introduced to a feeding regime of four different seaweeds. Juvenile abalone fed the seaweed with the highest protein content also exhibited the highest chymotrypsin activity levels and García-Carreño *et al.* (2003) conjectured that the secretion and combination of digestive enzymes were regulated by an adaptation to diet, similar to that observed in Atlantic salmon when responding to protease

inhibitors in feed of poor protein quality (Olli *et al.* 1994). Knauer *et al.* (1997) also found that a high protein level formulated diet fed to juvenile *H. midae* equated to higher protease activities compared to those fed macroalgae or diatoms. While macroalgae may have consistently low protein content, diatoms have a protein composition range of up to 47 % (Ansell *et al.* 1964; Darley 1977; Knauer *et al.* 1997). This may explain why abalone can digest food with high protein levels and have adequate amounts of digestive proteases (Knauer *et al.* 1997). Edwards and Condon (2001) researched whether a high protein diet influenced the protease activities of *H. rubra*. The two diets used were Rhodophyta red seaweed and Makara® casein-based formulated feed. The results showed that the protease activities of abalone fed either treatment were similar and showed no response to the high protein content of the formulated diet (Edwards and Condon 2001).

The effect formulated diets have on the digestive carbohydrases of abalone have not been as intensively researched since it is already known that they graze on carbohydrate-rich foods in the wild (Barkai and Griffiths 1986). In the current study, all treatments had the same protein level, but since the proteins were of different sources and consistencies, different levels of starches and fibres had to be added for the leaflet pellets to keep their shape. The FM only diet had the highest level of starch, the combination diets FM-SBM and FM-SFM were roughly the same, while the SBM only diet had the lowest amount of starch.

Monje and Viana (1998) compared the cellulolytic activity of *H. fulgens* abalone fed either a 20 % sodium alginate or 19 % cellulose supplemented formulated diet. The cellulolytic activity in the stomach of abalone fed the cellulose supplemented diet was higher than those fed the sodium alginate supplemented diet (Monje and Viana 1998). Increased enzymatic activity may seem to be a digestive advantage, as cellulose in the diet stimulated the cellulase production within the

digestive tract of the abalone which are known to produce endogenous cellulases, among other carbohydrases (Stone and Morton 1958; Leighton 1968; Livingston and de Zwaan 1983; Monje and Viana 1998). As mentioned by Erasmus (1996) though, bacteria found in abalone digestive tracts also may play a role in cellulose degradation by producing cellulases and being involved in plant cellulose fermentation.

Garcia-Esquivel and Felbeck (2006) analysed the enzyme activity levels of *H. rufescens* abalone fed a natural diet and two formulated diets, with the natural diet being kelp, and the formulated diets were 25 and 38 % protein composition feeds. The protein sources were fishmeal and soybean meal, with both diets also including kelp meal. Garcia-Esquivel and Felbeck (2006) observed low protease activity for abalone fed the diets, while cellulase exhibited high activities in abalone fed the two formulated feed treatments. This may have been due to a higher amount fibre present in the two formulated treatment diets, as increased fibre content was linked to cellulase activity stimulation in *Oreochromis mossambicus* (P. 1852; Manju and Dhevendaran 2002; Garcia-Esquivel and Felbeck 2006).

Garica-Esquivel and Felbeck (2006) found that abalone fed the lower protein diet had higher protease activity levels than those fed the higher protein diet. The starches and fibre content included in the formulated feeds of the current study may have stimulated endogenous and exogenous carbohydrase secretion. The hydrolysed feed increases the substrate surface area within the gut and promote colonisation of bacterial endosymbionts (Erasmus *et al.* 1997; Garcia-Esquivel and Felbeck 2006). Both studies show an abalone's ability to modulate enzyme activity levels to efficiently utilise available nutrients of dietary substrates in the formulated feed (Erasmus *et al.* 1997; Garcia-Esquivel and Felbeck 2006).

The differences in starch and fibre content may have had a role to play in the variability in the cellulase activity and why as all but SBM treatment showed highly similar activities for most of the sampling period. The diets FM, FM-SBM, and FM-SFM had at least 49 % starch composition, while SBM had only 33 % starch composition, which is why SBM cellulase and chymotrypsin activities were low for most of the trial and displayed a different pattern in activity over time. Yet the SBM treatment should have been the diet that stimulated the production of cellulase the most as the soybean meal is known to have a high fibre content (Liener 1994; Francis *et al*. 2001; Manju and Dhevendaran 2002; Krogdahl *et al*. 2003; Garcia-Esquivel and Felbeck 2006; Heikkinen *et al*. 2006; Murashita *et al*. 2015). The diets FM-SBM, FM-SFM, and SBM all should have theoretically had similar cellulase activities with the FM diet exhibiting a different activity pattern over time. This is supported by the fact that studies have shown that high levels of complex polysaccharides, such as cellulose fibres, in diets are able extend fermentation time and therefore influence the growth of microbiota, including exogenous carbohydrase-producing bacteria (Tugwell and Branch 1989; Day and Cook 1995; Dolara *et al.* 2005; Lee *et al.* 2006; Nel *et al.* 2016).

The crude fibre content between treatment diets was different. The FM-SFM and SBM-only diets had the highest levels present with 1.34 and 1.37 % respectively. This may have affected activity levels of cellulase found in the current study. It has been observed in previous literature that diets with a higher fibre content than those with none can stimulate digestive enzyme activity levels, however this was not the case with the SBM treatment, as cellulase activity was surprisingly low for most of the sampling period. Unlike the pattern seen from the other treatments, the cellulase activity of SBM fed abalone gradually increased over time until it exhibited the highest activity at the end of the sampling period (Fagbenro 1990; Das and Tripathi 1991; Szlaminska *et al*. 1991;

Chakrabarti *et al*. 1995; Saha and Ray 1998; Gatesoupe 2008; Bagheri *et al.* 2008; Mazouk *et al.* 2008; Salnur *et al.* 2009; Merrifield *et al.* 2010; Maity *et al.* 2011; Ray *et al.* 2012; Nel 2016). Due to the higher crude fibre presence in the FM-SBM, FM-SFM and SBM-only treatment diets, the substrate analysed was already degraded in the assay, and possibly causing an overestimation for the treatment diet with less fibre. This would explain the activity levels observed for SBM, however the FM-SFM treatment diet had very similar levels of crude fibre and followed a similar pattern to the treatments containing little or no crude fibre.

Water temperature may have had an influence in the enzyme activity levels of the abalone in the current study as it is the most prominent environmental variable affecting aquatic invertebrate metabolic and growth rate (Kikuchi and Uki, 1974a, 1974b; Schaeffer *et al.* 2013; Kang *et al.* 2019; Wu 2020). No differences in water temperature between treatments was observed, though seasonal water temperature changes occurred throughout the trial period. Additionally, differences in water temperature variability within each season was noted (Table 2.6). Winter (Day 357) sea water temperatures were the warmest and most stable, summer (Day 182) conversely, had the coldest and most variable temperatures. Even though the water temperatures were lower in summer, the cellulase and chymotrypsin activity levels were highest on day 182. Temperature fluctuations may then have also had an influence on the enzyme activities. Schaeffer *et al.* (2013) found that lower water temperatures resulted in lowered enzyme activity and the reduction in absorptive capacity of the digestive tract in *Haliotis laevigata*. This triggered surface area growth of the stomach epithelial tissue to increase the efficacy of nutrient absorption and enzyme excretions (Schaeffer *et al.* 2013). This trigger to improve digestive ability in colder waters may have occurred in abalone of the current study during summer.

Bansemer *et al.* (2016) found that increased water temperature did not influence the protease trypsin activity significantly in *H. laevigata*.

The abalone used in the current study came from the animals used in the sister study by Wu (2020), which concentrated on the effects of dietary protein sources had on *H. midae* growth and reproductive development. The treatment diets FM-SBM and FM-SFM performed better than the single protein diets FM and SBM in terms of growth over time and weight gain. Of the two combination diets, FM-SBM was better utilised when analysing FCR and stable isotope signatures, due to more balanced amino acid profiles (Sales and Britz 2001a; Banaszkiewicz 2011; Wu 2020). Furthermore, the average feed conversion ratio (FCR) of the abalone fed the SBM treatment diet was considerably poorer than the other treatments. Temporal changes in the utilisation of combination treatment diets were observed. The FM treatment diet was notably utilised in somatic and reproductive tissue and growth, increasing as the need for reproductive tissue development increased as well. This observation correlates to the noticeable increase in chymotrypsin activity in treatment diets containing fishmeal on sampling day 182, and is potentially why the activity levels of the abalone fed the SBM treatment were lower.

The allergenic properties of soybean meal cannot be ruled out as an influence on feed utilisation and gut morphology. Soya as a raw material, is identified as one of the eight major allergenic foods according to the World Health Organisation (WHO) and Food and Agriculture Organisation (FAO) (Yang *et al.* 2018). Major allergenic proteins such as β-conglycinin and glycinin in soybean meal has been observed to cause allergic reactions such as intestinal villus atrophy in new born animals (Yang *et al.* 2018). Allergenic proteins are poorly digested by digestive enzymes and induce intestinal inflammation in *Sus scrofa domesticus* (E. 1777) piglets, which can result in the destruction of proteins in the intestinal wall, diarrhoea and a reduced

utilisation of nutrients (Dreau *et al.* 1994; Fu *et al.* 2007; Chen *et al.* 2011; Zhao *et al.* 2015). *Oncorhynchus mykiss* (W. 1792) fed feed with toasted soybean meal as the main protein source, Rumsey *et al.* (1994) observed lesions, blunted mucosal folds and epithelial necrosis of the proximal and distal intestine. This effect on the digestive tract in *O. mykiss* led to a reduced nutrient absorptive capacity and decreased function, resulting in poor growth and protein utilisation. Abalone, unlike salmonids, are predominantly herbivorous and have a ruminant-like digestive tract, therefore these allergenic effects of ingested soybean meal may not have been as severe (Knauer *et al.* 1996; Sawabe 2006). Heat treatment is usually applied to soy to inactivate ANFs in the meal, though β-conglycinin and glycinin are not temperature sensitive and can in fact increase the severity of their allergenic effects (Banaszkiewicz 2011), yet the severity of these effects on the abalone used in the study are unknown. Considering though that abalone fed the SBM treatment diet exhibited lower cellulase and chymotrypsin activity for most of the trial and a low feed utilisation, it is possible that the allergenic properties of soy reduced the synthesis of digestive enzymes through an inflammatory response.

It has been mentioned previously that abalone, while naturally herbivorous, can utilise various foodstuffs by modifying the levels of proteases and carbohydrases to the required amount (Knauer *et al.* 1996). Garcia-Esquivel and Felbeck (2006) observed high protease activities in *H. rufescens* abalone when fed a low protein diet, and low cellulase activities when fed a high carbohydrate diet. The opposite was seen in the current study, where protein-rich and high levels of starch and fibres in the diets fed to *H. midae* resulted in high activities of both cellulase and chymotrypsin in certain sampling periods. The contrasts may be due to the different abalone used in the current trial, and the time in which they were fed the diets. Younger abalone exhibit very different, flexible digestive enzyme behaviour than older ones, which can be supported by

Bansemer *et al.* (2016) in that protease activity levels of one-year-old *H. laevigata* abalone were found to be higher than that of two-year-old abalone in response to a diet change. Additionally, since abalone are able to adapt to different diets in a relatively short period, it is reasonable to assume that some level of adaptation might have occurred fairly quickly after being fed the same diet for a year (Edwards and Condon 2001). Yet the time needed for physiological adaptation for endogenous enzyme production is not known in sub-adult *H. midae* (Nel *et al.* 2016).

Nel *et al.* (2016) studied the influence diet has on abalone enzyme activities, with sub-adult *H. midae* being fed either Abfeed®- K26 as the control, or a kelp-supplemented formulated feed. Neither carbohydrase, nor serine protease activity levels showed significant differences between the two treatments and there were high levels of variability in carbohydrase activities (Nel *et al.* 2016). A possible reason for those results was that the two experimental diets were too similar in composition (Nel *et al.* 2016). For all the diets in the current study, excluding SBM, there was a similarity in the activity levels as well as a similar pattern over time which may be due to the similarity in composition as well. Additionally, for the first two sampling dates, the activities also had high variability. This can be hypothesised to be due to abalone still developing the physiological adaptations to produce endogenous digestive enzymes for effective utilisation (Nel *et al.* 2016). The variability seen may have, therefore, signified that formulated diets influenced the gut environment.

The sex of abalone as a factor for digestive enzyme activity in diet trials has not been well represented in literature, yet it should not be overlooked. *Haliotis midae* have sexually dimorphic coloured gonads which are easy to identify, cream-colour in males and green-blue in females (Newman 1967; Ayres 2013; Wu 2015). In the current study, sexes were compared between each treatment for each sampling day. The gender of the abalone had a significant effect on all

treatments for both chymotrypsin and cellulase, but there was no distinct link between sex and enzyme activities due to significant differences being observed for both sexes at different sampling days and treatments. When comparing the protease activities of various sites within *H. fulgens* digestive tracts, Serviere-Zaragoza *et al.* (1997) also divided the samples up by sex for comparison and higher levels of trypsin activity were found in females. While the chymotrypsin activity levels of both sexes were similar, females had higher levels in intestine and males had higher levels in the rectum (Serviere-Zaragoza *et al.* 1997). Comparable results on enzyme activity levels between sexes at the time were currently lacking, therefore the reasons for these results cannot be accurately assessed, only hypothesised. The importance of it is not diminished as it may still provide valuable information for the industry.

Lower levels of chymotrypsin activity observed in the current study compared to *H. fulgens* Serviere-Zaragoza *et al.* (1997) may be due to the current study using whole gut samples, while the authors had the digestive tract sectioned. The digestive gland is a large organ that may have diluted the concentrations of proteases in the sample. The enzyme assay method used for the current study can be further modified by sectioning the abalone digestive tract to obtain a better overview of enzyme activity for each distinctive sites. However, adding to the characterisation of the abalone digestive enzymes to better understand their adaptive ability to utilise the protein content in macroalgae and formulated diets is important.

Conclusions

- For cellulase activity, there were significant interactions between the diet, sampling date and sex of the abalone used in the study.
- With regards to chymotrypsin activity, there were significant interactions between the diets fed and the sampling dates.

- The general pattern for both enzymes over time showed a rise and fall in activity throughout the study period, which could be due to fluctuations in seawater temperature, with the peak present on sampling day 182 which in summer (fluctuations in temperature), and the decline on sampling day 272 in April to sampling day 357 in winter (stable water temperatures).
- The reason for significant differences in enzyme activity between the sexes of abalone and diets fed needs to be further investigated.
- The levels of starch and fibres in the diets were the only components that differed between treatments. Starch levels seem to have affected enzyme activity levels, specifically cellulase. There was much less starch in the SBM diet, but there were more fibres present which may have attributed to the differing cellulase activity levels seen with abalone fed the SBM diet. Abalone fed the SBM treatment displayed the lowest activities in cellulase for most of the trial until the last sampling day, while abalone fed the other treatments followed the initial general trend of an increase and decrease in activity from day 91 to 273.

CHAPTER 3

3 THE EFFECT OF PLANT-DERIVED DIETARY PROTEINS IN FORMULATED FEED ON THE GUT-BACTERIAL COMMUNITY OF CULTURED SOUTH AFRICAN ABALONE, *HALIOTIS MIDAE*

3.1 INTRODUCTION

Bacteria in the gastrointestinal tract of abalone play an important role in nutrient absorption and digestion (Harris 1993; Erasmus 1997; Sawabe 2006; Cicala *et al.* 2018; Tanaka *et al.* 2016). For example, enteric bacteria have been observed degrading polysaccharides into smaller units, which are easier for the abalone hosts to assimilate (Erasmus 1997; Tanaka *et al.* 2016). This may be the reason for the presence of these bacteria within the gut since brown macroalgae is a part of an abalone's diet and is a significant source of complex polysaccharides (Honya *et al.* 1993; Tanaka *et al.* 2016). The presence, abundance, and diversity of gut bacterial communities within marine organisms are influenced by various factors, including diet, pH, ontogeny and the surrounding aquatic environment (Hansen and Olafsen 1999; Ingerslev *et al.* 2014; Nel *et al.* 2018). The gastrointestinal tract is a favourable site for bacterial colonisation due to it being a nutrient-rich environment, having sites of specific pH along the tract (Enger *et al.* 1990; Plante and Jumars 1992; Harris 1993).

The predominant role of gut-microbiota in abalone is that of digestion and fermentation of polysaccharides found in macroalgae (Takami *et al.* 1998; Nel *et al.* 2017b; Gobet *et al.* 2018). Due to strong selective pressures within the gastrointestinal tracts of abalone such as the varying pH levels, surface areas (ciliated and non-ciliated) and anaerobic nature, there is a restricted range of micro-organisms present, known as the gut-microflora (Harris 1993; Erasmus *et al.* 1997;

Harris *et al.* 1998). The activities of these bacteria usually benefit the aquatic invertebrate host in several ways, including food-preconditioning, increased host resistance to pathogens, cellulose breakdown and nitrogen fixation (Harris 1993; Harris *et al.* 1998). The relationship between microbes and invertebrates is not as well understood as compared to that of vertebrate hosts, yet the presence and function of cellulolytic microbiota in invertebrate hosts have been extensively studied (Harris *et al.* 1998). Substrates such as algal carbohydrates have been used to study the role microbes play in the digestive physiology of aquatic herbivorous invertebrates (Harris *et al.* 1998). The activities of cellulase, b-1.4 glucanase, and alginate lyase are several examples that have been examined (Galli and Giese 1959; Vitalis *et al.* 1988; Harris 1993; Sawabe *et al.* 1995; Erasmus *et al*. 1997; Harris *et al.* 1998). Other enzymatic activities such as proteases have been observed by some bacterial strains from marine invertebrate digestive tracts as well (Harris 1993).

The digestive tracts of invertebrates are an anaerobic environment. Facultative aerobic bacteria activities reduce oxygen in the tract, therefore allowing the proliferation of obligate anaerobes (Galli and Giese 1959; Prim and Lawrence 1975; Musgrove 1988; Harris *et al.* 1998). Bacteria are found in all parts of abalone digestive tracts as there is plenty of surface area for bacterial colonisation due to morphological complexity (Campbell 1965; Harris 1993; Harris *et al.* 1998). The crop, stomach and style sacs of abalone tend to have lower levels of bacterial diversity due to the relatively acidic environment of those sites (Harris 1993). Several studies have identified gut microbiota by isolating strains from the digestive tracts of cultured abalone. Tanaka *et al.* (2003) discovered that a majority of bacteria from homogenised whole guts of *Haliotis discus hannai* (I. 1953) were identified as facultative anaerobes. The bacteria were further identified to be either fermenters, such as the genus *Vibrio*, or non-motile fermenters. *Vibrio* bacteria are very prominent symbionts of abalone that are fed on brown macroalgae and formulated feeds with alginate

inclusion (Sawabe 2006; Tanaka *et al.* 2016). *Vibrio* bacteria benefit abalone hosts by providing an energy source in the form of acetic acid which is produced after the breakdown of alginate polysaccharides (Sawabe 2006; Nel 2016; Tanaka *et al.* 2016).

There have been multiple studies researching the effect natural and formulated diets have on the gut-bacterial communities of abalone species (Harris *et al.* 1998; Tanaka *et al.* 2004; Huang *et al.* 2010; Zhao *et al.* 2012; Iehata *et al.* 2014; Madigan *et al.* 2015; Lee *et al.* 2016; Nel *et al.* 2017b; Cicala *et al.* 2018; Gobet *et al.* 2018; Nam *et al*. 2018; Nel *et al.* 2018). *Haliotis laevigata* abalone fed a mixture of several types of macroalgae and the majority of the bacteria identified from the whole gut samples of the abalone belonged to the family Enterobacteriaceae (Gammaproteobacteria) (Harris *et al*. 1998), which is a group known to utilise various carbohydrates as energy sources (Madigan *et al.* 2015). The bacterial association of abalone fed only one kind of macroalgae and those fed various macroalgae are distinctly different (Harris *et al.* 1998; Madigan *et al.* 2015).

In whole-gut samples of *H. discus hannai*, Proteobacteria and Tenericutes were most dominant (Tanaka *et al.* 2004). Tanaka *et al.* (2004) compared the influence diet had on gut-bacterial communities of three groups of abalone: one group being fed formulated feed, one being fed brown macroalgae, and the last group being starved. The dominant bacterial groups fed formulated feed were in descending order, Alpha-, Gamma-, Epsilonproteobacteria and Mollicutes (Tanaka *et al.* 2004). The most prominent groups of gut-bacteria for abalone fed brown macroalgae were Mollicutes, with Alphaproteobacteria at a slightly lower abundance (Tanaka *et al.* 2004). Fusobacteria was the dominant group of bacteria in abalone that were starved, with Mollicutes, Alpha- and Gammaproteobacteria present to a lesser extent (Tanaka *et al.* 2004).

Tanaka *et al.* (2004) also found that *H. discus hannai* abalone with the highest bacterial diversity were those fed formulated feed compared to those that were either starved or fed brown macroalgae. There were no specific dominant species present in the guts of abalone fed formulated feed, while those that were fed brown macroalgae or starved exhibited some degree of dominance of particular species (Tanaka *et al.* 2004). Zhao *et al.* (2012) observed contrasting results, as *Haliotis diversicolor* (R. 1846) abalone fed formulated feed had considerably lower levels of gut-bacterial diversity in comparison to abalone fed seaweed. Interestingly, the hypothesis brought forward as to why there were lower bacterial diversity in juvenile abalone fed formulated food was that they were weaned off diatoms, which further suggests that dietary changes may disrupt gut bacterial communities in abalone (Zhao *et al.* 2012).

Studies have also been conducted to determine the effect diet has on the gut-bacterial communities of *Haliotis midae* (Nel *et al*. 2017b; Nel *et al*. 2018). When fed with formulated or kelp-supplemented formulated feed, abalone from both treatments had Proteobacteria and Tenericutes as the dominant gut-bacteria (Nel *et al.* 2017b). While there was little overall difference in the gut-bacterial composition, there was a marked difference in the relative abundance of certain *Mycoplasma* strains (Nel *et al.* 2017b). Higher abundances of *Mycoplasma* were observed in abalone fed the kelp-supplemented formulated feed (Nel *et al.* 2017b). It was postulated that the higher *Mycoplasma* abundance may have been due to the increased levels of nutrients present in the feed from the kelp supplementation (Pereyre *et al.* 2009; Nel *et al.* 2017b).

Age may be another factor that affects gut microbiota composition. When *H. midae* were weaned onto either a formulated feed, a kelp-supplemented formulated feed, or fresh kelp, anaerobic *Clostridia* bacteria were the most predominant group in the digestive tracts of *H. midae* abalone fed fresh kelp (Nel *et al.* 2017b), which may have been due to the higher levels of

fermentable polysaccharides from the digested kelp (Lahaye and Kaeffer 1997; O' Sullivan *et al*. 2010; Huesemann *et al.* 2012; Nel *et al.* 2017b). The dominance of *Clostridia* bacteria was also linked to lower gut-bacteria diversity compared to the abalone fed the kelp-supplemented and formulated feeds (Nel *et al.* 2018). *Clostridia* dominance waned as the abalone grew to the grow-out phase. The changes in gut-bacterial communities during abalone developmental stages can be due to the maturation of the digestive tract (Nel *et al.* 2017b). The change in the gut environment of abalone at a grow-out developmental phase would also change the selective pressures within the tract, which favour the proliferation of anaerobic bacteria such as Mollicutes and Fusobacteria, and outcompete previously dominant groups within the digestive tract (Nel *et al.* 2017b).

It is clear that being a host to a gut bacterial community can be advantageous, what is unknown though is the extent of its necessity to an organism (Reese and Dunn 2018). In some cases, it can be better to limit diversity as not all bacterial groups are beneficial, either by being pathogenic or using up available nutrients that would otherwise have been utilised by the host (Foster *et al*. 2015; Nel 2016; Reese and Dunn 2018). This preference can result in the gut bacterial community being made up of a few dominant groups (Nel 2016; Nel *et al.* 2017). Increasing diversity can potentially destabilise a gut bacterial community (Coyte *et al*. 2015; Reese and Dunn 2018). Reese and Dunn (2018) propose that in many species, hosts prefer keeping gut bacterial communities the way humans farm: "low diversity, but function systems". Diversity metrics are often used in amplicon sequencing-based assessments of communities, and variation in diversity are calculated between treatments (Reese and Dunn 2018). According to Reese and Dunn (2018) though, "without a theory of diversity applicable to microbiomes, however, it is hard, if not impossible, to interpret these results". This should be taken into account

when analysing the results of the current chapter. The diversity indices used act as a tool to allow for the comparison of other relevant literature.

The aim of Chapter 3 was to investigate the effect of plant-protein inclusion on the gut-microbiota of cultured abalone. To achieve this, the following objective was addressed: to compare the gut-bacterial community profiles between farmed abalone fed one of four diets over time: fishmeal-only (FM), soybean meal only (SBM), fishmeal and soybean meal (FM-SBM), and fishmeal and sunflower meal (FM-SFM), through the use of metagenomic sequencing.

3.2 METHODS

3.2.1 SAMPLE COLLECTION

The *H. midae* abalone used for the feeding trial in the previous chapter were also used for the analysis of the gut-bacteria and were sampled on day 357, the last day of the trial. The abalone of each treatment were fed for 12 months and purged for one day before sampling. A total of 60 abalone were collected: 15 abalone from each of the four treatments (Table 2.1; Chapter 2). They were collected at random from all tanks. The sampled abalone were then placed in a freezer and euthanised at -20 °C for an hour. The animals were submerged whole in 70 % ethanol for one minute, removing any surface-associated bacteria, and dissected aseptically on ice (Sawabe *et al.* 1995). With each sample, the dissected digestive tract was cut into small pieces with sterilised scissors and homogenised with a mortar and pestle, and kept chilled. Samples were placed into 10 mL falcon tubes and kept at -20 °C in a freezer before with deoxyribonucleic acid (DNA) extraction procedure.

3.2.2 DNA EXTRACTION PROTOCOL

The cetyltrimethylammonium bromide (CTAB) DNA extraction protocol was used for the DNA extraction of the abalone samples. A volume of 25 µl of each homogenised sample was placed into individual 1.5 mL Eppendorf tubes. Keeping the lids open, the homogenised samples were placed into a heat block at 56 °C for six minutes. This was to allow for excess 70 % ethanol from the previous disinfecting step to evaporate from the samples. The CTAB digestion buffer reagent volumes per sample consisted of 12.5 mg of cetyltrimethylammonium bromide; 360 µl of sterile water, 62.5 µl of 1 M Tris buffer (pH 8); 175 µl of 5M NaCl; 25 µl of 0.5 M Ethylenediaminetetraacetic acid (EDTA); and 1.25 µl of β-mercaptoethanol. The reagents were mixed together inside a 50 mL falcon tube and placed into a rotating incubator at 65 °C until completely dissolved. Each sample then had 40 µl of Proteinase K (at 10 mg/mL) and 600 µl of CTAB buffer added to it and was mixed by vortexing. All samples were then placed into a rotating incubator for two to three hours at 65 °C. The samples were vortexed intermittently during this step.

After protein digestion was completed, samples were left at room temperature for 10 min. Afterwards, 15 µl of RNase (10 mg/mL) was added to each sample and the samples were placed on a heat block for 15 min at 37 °C. The samples were then centrifuged for seven min at 13000 revolutions per minute (rpm), and 550 µl of supernatant from each sample was placed into an empty Eppendorf tube to which 550 µl of chloroform was added. The tubes were then vortexed until they had a milky white colour, and then centrifuged at 13000 rpm for 15 min. Afterwards, the upper phase of each tube was pipetted and transferred into new Eppendorf tubes to which 550 µl of chloroform was again added, and the samples were vortexed and centrifuged at 13000 rpm for 15 min. The upper phases were then transferred to a new set of Eppendorf tubes

containing 750 µL of chilled isopropanol and were left in the freezer overnight. The following day, the samples were centrifuged at 13000 rpm for 30 min and the supernatant was aspirated and discarded, leaving the pelleted DNA behind. An amount of 200 µl of cold 75 % ethanol was added, and each tube was gently inverted for a few times to rinse the pellet. The samples were then centrifuged for five min at 7000 rpm, the supernatant was aspirated afterward, and the tubes were placed in a heat block for 10 min at 60 °C to dry off excess ethanol. The DNA was eluted in 50 µl of Tris-Ethylenediaminetetraacetic acid (TE)/elution buffer.

3.2.3 Agarose gel preparation for DNA viewing

To assess the concentration and quality of the genomic DNA in each sample, a Qubit fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a NanoDrop Nucleic Acid Quantification (Thermo Fisher Scientific, Waltham, Massachusetts, USA) device were used, and agarose gel electrophoreses were performed using 1.0 % agarose gels with five µl of one mg/mL ethidium bromide (EtBr). The Qubit protocol was used for analysing the concentration of the genomic DNA of each sample, and a Nanodrop was utilised to assess the genomic DNA quality of each sample. The polymerase chain reaction (PCR) amplified DNA product was also analysed using gel electrophoresis to establish the size of the amplified DNA in the number of base pairs and to see the reaction specificity of the PCR targeted for 16s rRNA products.

3.2.4 Polymerase Chain Reaction protocol

The extracted DNA from whole gut samples were amplified in a 25 µl PCR reaction, having used one µl of Ampliqon AccuPOL™ DNA Polymerase and 2.5 µl of Ampliqon AccuPOL™ Ammonium Buffer (Ampliqon A/S, Odense, Denmark), 0.7 µl of dNTP's, 0.7 µl for both the reverse and forward primers (MiSeq 16s), 14.4 µl of PCR-grade water, and five µl of sample DNA

template. The primer pair used were 515F-Y (5′-GTGYCAGCMGCCGCGGTAA-3′) and 926R (5′-CCGYCAATTYMTTTRAGTTT-3′) which encompasses the V4 and V5 hypervariable regions and was able to produce accurate community abundance estimates (Quince *et al.* 2011; Parada *et al.* 2016). A modified version of a standard PCR cycling protocol was used (Table 3.1).

Table 3.1: Thermal cycling protocol for MiSeq 16s DNA amplification for *H. midae* gut-extracts.

Initial Denaturation	98 °C	5 min	1 cycle
Denaturation	98 °C	45 s	
Annealing	45 °C	30 s	8 cycles
Extension	72 °C	1 min	
Denaturation	98 °C	45 s	
Annealing	45 °C	30 s	28 cycles
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	1 cycle

3.2.5 AGAROSE GEL ELECTROPHORESIS PROTOCOL

A volume of 25 µl of each PCR product was added to one µl of loading dye. Each sample was then placed into two individual gel wells. five µl of KAPA Universal Ladder (Kapa Biosystems, Wilmington, Massachusetts, USA) was added to the last well of each lane in order to confirm that the correct base pairs were amplified during PCR (600 bp). The electrophoresis tank was filled with 1 x TBE and the gel was electrophoresed at 100 V for 30 min. After electrophoresis, the gel was placed onto a cleaned slide-out trans-illuminator with the FIREReader software interface for visualisation. If the samples were at an optimal concentration, they were prepared for gel purification.

3.2.6 DNA PURIFICATION

For the extraction of DNA fragments from an agarose gel to isolate pure DNA, the Gel Extraction Protocol was performed, using the FavorPrep™ Gel/PCR Purification Kit. The gel was placed on a UVP® Ultraviolet Transilluminator (Analytik Jena US LLC), and the appropriate samples were excised using sterile scalpel blades and placed into 1.5 mL Eppendorf tubes. Each sample was added to 500 µl of solubilisation and binding buffer that had a pH indicator. The samples were then placed into a heat block at 55 °C for approximately ten minutes and vortexed every two to three minutes until the agarose gels were dissolved. Once the gels were dissolved, the samples were cooled to room temperature. Filter columns were placed into collections tubes and 800 µl of each sample mixture was added to the columns. The collection tubes and filter columns were centrifuged for 30 s at 11000 relative centrifugal force (RCF), and the flow-through was discarded. Each filter column had 750 µl of wash buffer deposited and the samples were again centrifuged for 30 seconds at 11000 RCF with the flow-through being discarded. The columns were then dried by centrifuging for four minutes at 18000 RCF in order to avoid residual liquid inhibiting subsequent enzymatic reactions. The filter columns were placed into new Eppendorf tubes and 40 µl of elution buffer was added. The columns were left to stand for two minutes before being centrifuged for one minute at 18000 RCF to elute the DNA. The columns were subsequently removed, and the purified DNA samples were placed into a freezer to be stored at -20 °C.

3.2.7 DNA CURATION AND STATISTICAL ANALYSIS

Nextera XT Indices (using the recommended manufacturer specifications, Part no. 15044223 Rev. B) were used to multiplex the purified PCR products. They were then pooled and sequenced utilising

the MiSeq Reagent kit v3 (600 cycles) with a 10 % PhiX spike on the MiSeq platform (SY-410-1003, Illumina Inc.) (Matcher *et al.* 2011).

Mothur (Schloss *et al.* 2009) was used for 16S microbial analysis and: to filter out and remove sequences with ambiguous bases and reads with base pairs less than 350 and more than 650, to perform de novo clustering of sequences into operational taxonomic unit (OTUs) at a 97 % sequence similarity, to assign and align sequences into taxonomies using Silva (release 132) database, to search and remove chimeras and undesirable sequences form the assigned OTUs, and to produce alpha-rarefaction files. Shannon diversity and Simpson diversity indices, non-metric multidimensional scaling (n-MDS) and a one-way analysis of similarities (ANOSIM) using Bray-Curtis similarity measures were performed using R on RStudio. The total number of shared OTUs was displayed using a Venn diagram using InteractiVenn (Herbele *et al.* 2015).

Shapiro-Wilk and Levene's tests were performed to calculate normality and homogeneity of variances of the OTU relative abundances between samples, respectively. The phyla, class, and genera with relative abundances of above 1 % were used for statistical analyses. one-way analysis of variance (ANOVA) and Kruskal-Wallis ANOVAs were performed to compare the relative abundances of gut-bacterial phyla between abalone fed the diets: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM). The statistical analyses were performed using Statistica 13.

3.3 RESULTS

3.3.1 RAREFACTION, DIVERSITY INDICES AND STATISTICAL ANALYSIS

The rarefaction curves of observed species for each sample was produced and showed that all of the *H. midae* abalone samples of each treatment reached a proper plateau, and therefore were sampled to saturation of the community coverage (Figure 3.1). Shannon and Simpson diversity indices and the Good's coverage values showed that each treatment had high diversity levels, all with at least 94.7 % coverage (Table 3.2).

Analysis of variance (ANOVA) showed that no significant differences in Shannon diversity (ANOVA; df = 3; F = 2.12; P = 0.12), Simpson diversity (ANOVA; df = 3; F = 2.61; P = 0.07), observed species (ANOVA; df = 3; F = 2.055; P = 0.13), number of reads (ANOVA; df = 3; F = 2.53; P = 0.077) or Good's coverage (ANOVA; df = 3; F = 0.93; P = 0.44) were observed between treatments. The FM-only treatment had relatively high values, with a Shannon index of 3.9 and 0.9 for the Simpson diversity index. The two protein combination diets exhibited very similar index values and coverage.

Table 3.2: Means (± standard deviation) for the number of sequences read, observed species detected and the diversity indices and Good's coverage displayed for the *H. midae* abalone samples fed: FM (fishmeal-only), FM-SBM (fishmeal and soybean meal), FM-SFM (fishmeal and sunflower meal) and SBM (soybean meal-only) diets.

Diet	FM	FM-SBM	FM-SFM	SBM
# of reads	26046.8	11149.0	20892.2	19150.0
Observed species	193.8 ± 5.9	131.8 ± 18.6	141.1 ± 16.3	127.8 ± 7.9
Shannon diversity index	3.9 ± 0.4	3.7 ± 0.5	3.7 ± 0.3	2.9 ± 0.3
Simpson diversity index	0.9 ± 0.04	0.9 ± 0.04	0.9 ± 0.03	0.8 ± 0.03
Good's coverage (%)	96.7 ± 0.9	94.7 ± 1.8	95.4 ± 1.1	96.8 ± 0.6

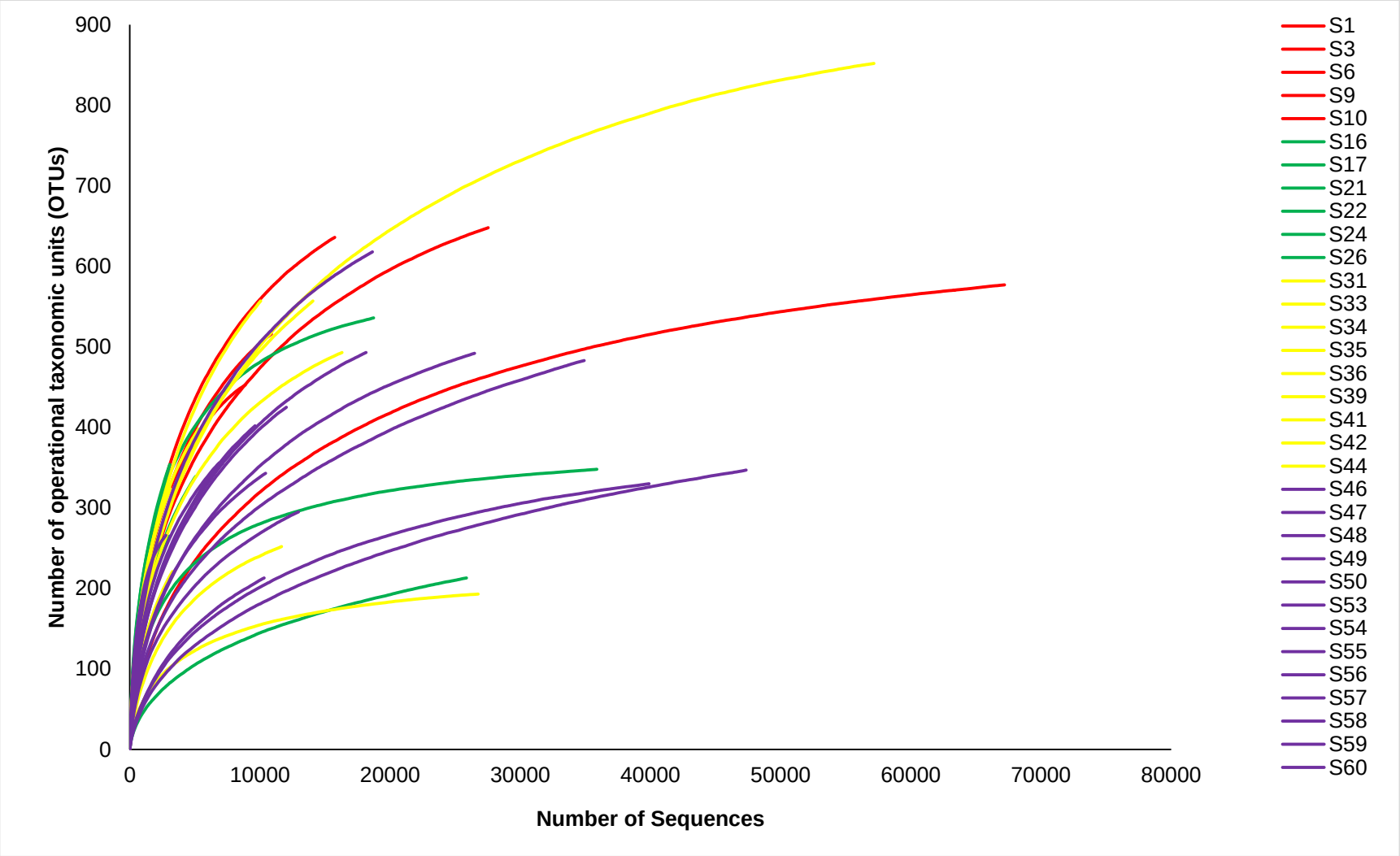

Figure 3.1: Rarefaction curves of the observed species per sample against the number of sequences per *H. midae* abalone sample for each diet: fishmeal-only (FM; S1-S10), fishmeal and soybean meal (FM-SBM; S16-S26), fishmeal and sunflower meal (FM-SFM; S31-S44) and soybean meal-only (SBM; S46-S60). "S" represents sample.

3.3.2 Bacterial composition of abalone fed four different formulated diets

The most dominant bacteria phyla found in the abalone digestive tracts of all diets made up roughly 85 % of the total abundance: Proteobacteria, Planctomycetes, Verrucomicrobia, Tenericutes, and Bacteroidetes (Figure 3.2). Abalone fed SBM had a significantly higher relative abundance of Verrucomicrobia at 12.6 % (ANOVA; df = 3; F = 4.087; p = 0.016).

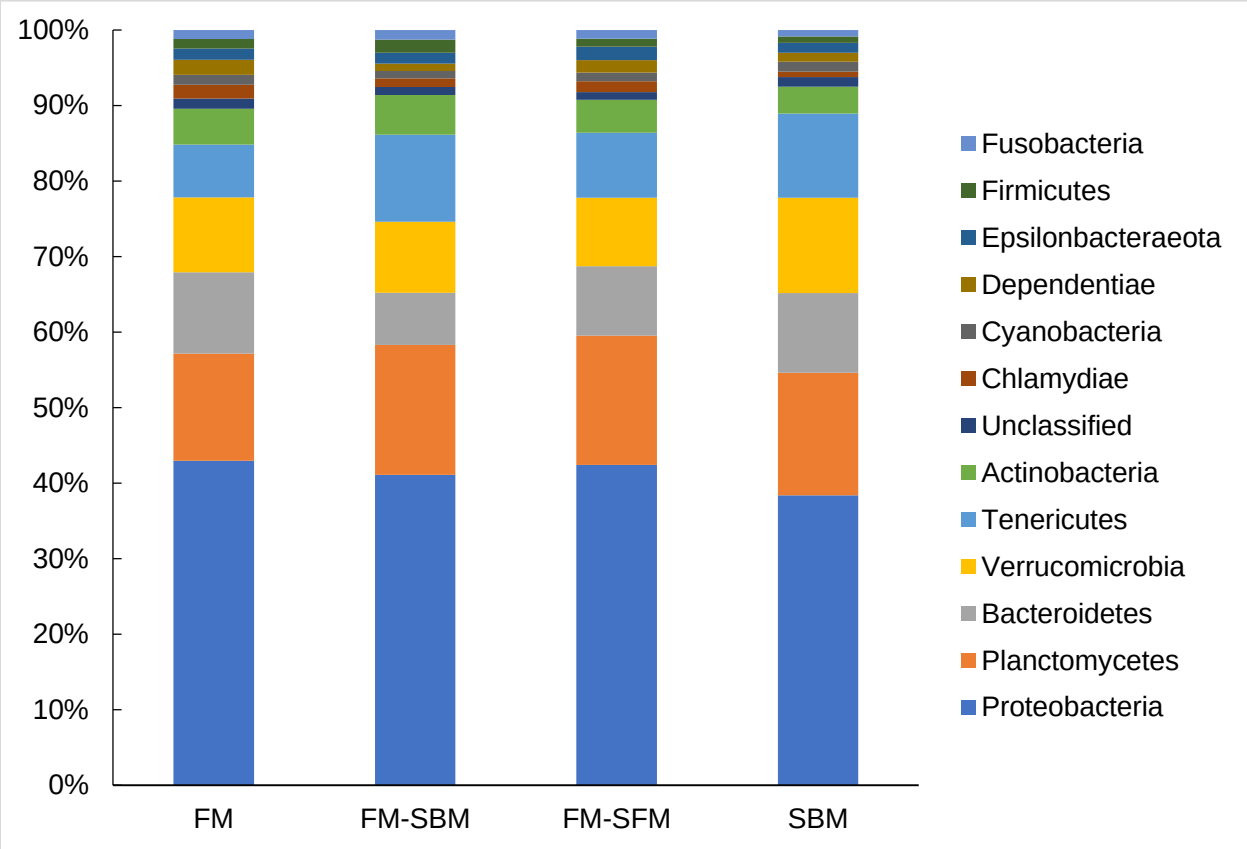

Figure 3.2: The mean relative abundances (%) of different phyla present in *H. midae* abalone gastrointestinal tracts fed the diets: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM).

The dominant classes of bacteria consisted of Gammaproteobacteria, Planctomycetacia, Alphaproteobacteria, Verrucomicrobiae, Mollicutes, and Bacteroidia (Figure 3.3). Verrucomicrobiae was the only class to have displayed a significant difference, with abalone fed the SBM (12.9 %) treatment having the highest relative abundance (ANOVA; df = 3; F = 4.23; p = 0.013).

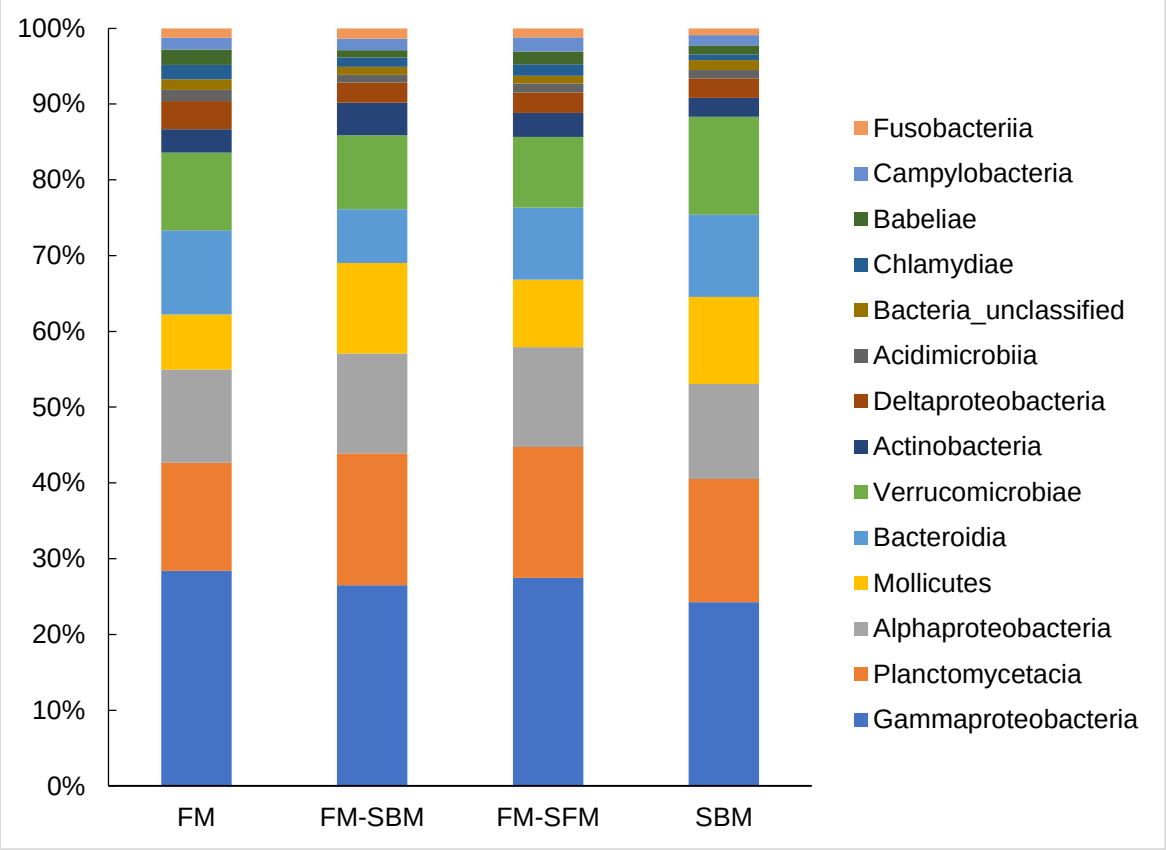

Figure 3.3: The mean relative abundances (%) of different classes present in *H. midae* abalone gastrointestinal tracts fed the diets: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM).

The dominant genera of bacteria found in the abalone gastrointestinal tracts of all four treatments were *Blastopirellula*, *Mycoplasma*, and Unclassified Gammaproteobacteria (Figure 3.4). The relative abundance of *Rubritalea* was observed to be significantly higher in abalone fed the SBM treatment than those fed the other diets with 5.77 % (ANOVA; F = 4.49; p = 0.01).

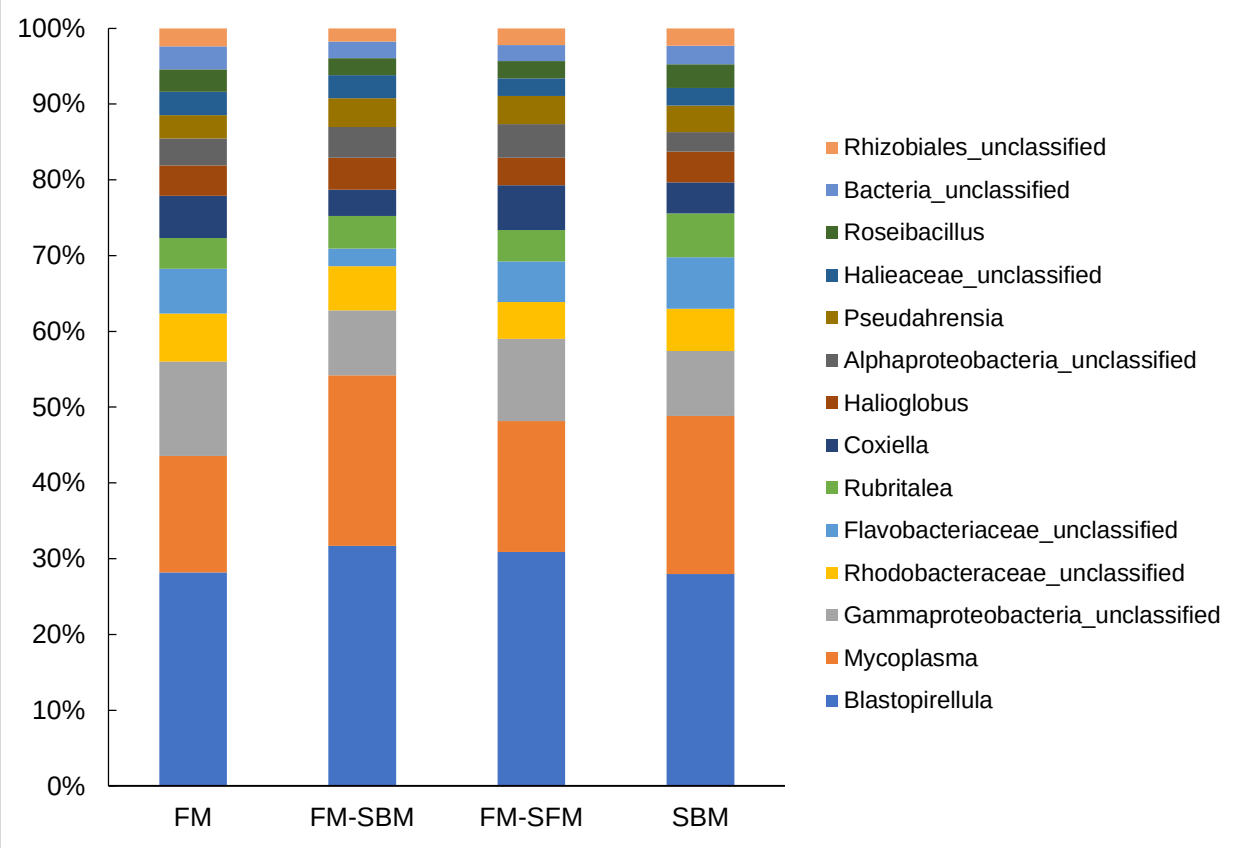

Figure 3.4: The mean relative abundances (%) of different genera present in *H. midae* abalone gastrointestinal tracts fed the diets: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM).

A one-way analysis of similarity ANOSIM (R-value = 0.258, p = 0.017) and a non-metric multi-dimensional scaling (NMDS) plot using a Bray-Curtis dissimilarity index established that at an OTU level, there was a significant interspecific differentiation and that diet had a slight impact on the gut bacterial community. The n-MDS plot displays some clustering of the FM-only, FM-SBM and FM-SFM diets, with a few outliers from samples of the two protein combination diets. The SBM-only treatment showed a slightly separate cluster, with some samples overlapping with the cluster, made up of the other three treatments (Figure 3.5).

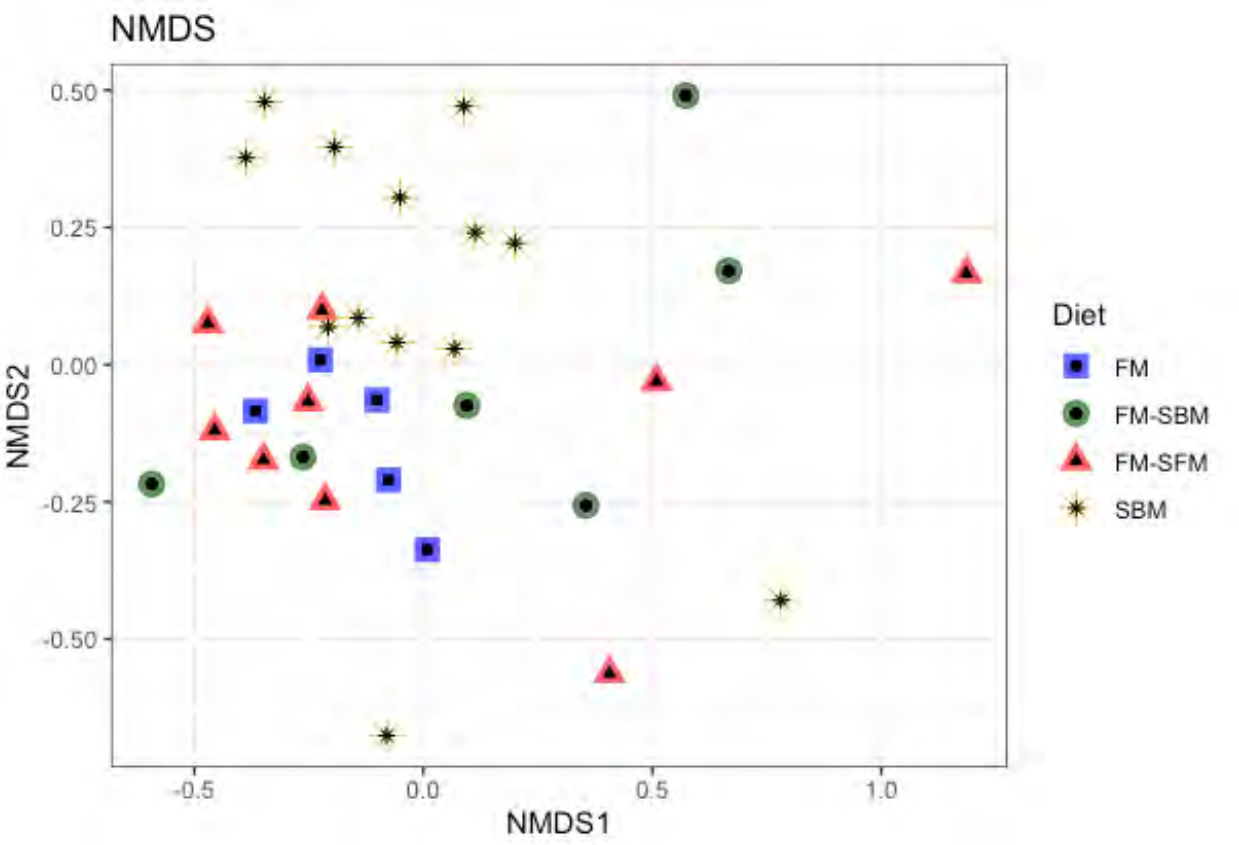

Figure 3.5: A non-metric multi-dimensional scaling (NMDS) plot (stress-value = 0.17) of gut-extract samples of the *H. midae* abalone fed the diets: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM) using a Bray-Curtis measurement of similarity.

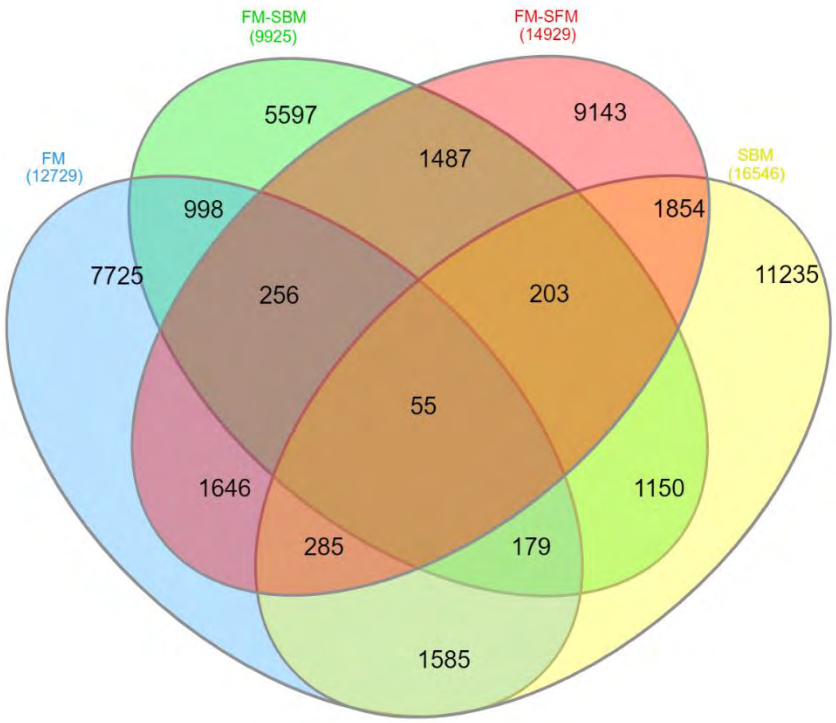

Figure 3.6: A Venn diagram (Herbele *et al.* 2015) of the total OTUs shared among the *H. midae* abalone fed the diets: fishmeal-only (FM), fishmeal and soybean meal (FM-SBM), fishmeal and sunflower meal (FM-SFM), and soybean meal-only (SBM) from gut-extract samples.

The SBM treatment diet had the highest number of total and unique OTUs of 11235 compared to the FM, FM-SBM and FM-SFM with 7725, 5597, and 9143 OTUs respectively (Figure 3.6). SBM having more unique OTUs is further reinforced by the slight separate cluster seen in Figure 3.5.

3.4 DISCUSSION

The diversity of the *H. midae* gut-bacterial community in the study were high compared to those found in *Haliotis gigantea* (G. 1791) by Iehata *et al.* (2014) and in *H. midae* by Nel (2016), but no significant differences between treatments were observed. These values were within the same range as those for *H. diversicolor* abalone fed formulated feed or probiotic-supplemented formulated feed (Zhao *et al.* 2018). The indices of the current study were higher than what was found in *H. gigantea* (G. 1791) abalone fed either formulated feed or probiotic-supplemented formulated feed (Iehata *et al.* 2014), *H. discus hannai* abalone fed either formulated feed, macroalgae or starved (Tanaka *et al.* 2004). The ranges of both Simpson and Shannon diversity indices were similar to those of grow-out stage *H. midae* abalone weaned onto either kelp, formulated feed or kelp-supplemented formulated feed (Nel *et al.* 2018). All treatments exhibited high Good's coverage values and therefore the majority of the total species in the samples were represented. The coverage values of the current study were within a similar range of values to those found in the gut bacterial communities of *H. discus hannai* (Tanaka *et al.* 2004; Lee *et al.* 2016) and *H. diversicolor* (Zhao *et al.* 2018).

The gut bacterial composition of *H. midae* abalone fed either of the diets in the current study was similar, with the dominant group of core microbiota present making up 85 % of the relative abundance. Proteobacteria was the most dominant phyla, followed by Planctomycetes, Bacteroidetes, Verrucomicrobia, and Tenericutes. The phyla Verrucomicrobia, class Verrucomicrobiae and the genus *Rubritalea*, which are classified under Verrucomicrobia, showed significant differences in relative abundance, with the SBM treatment displaying the highest abundances in all three instances.

Abalone fed the diet SBM had significantly higher relative abundances of bacteria from the phyla Verrucomicrobia, class Verrucomicrobiae and the genera within the phyla and

class, *Rubritalea*, than abalone fed the FM, FM-SBM or FM-SFM diet. Verrucomicrobia bacteria are not usually prominent in the gut-bacterial community of abalone, however, they have been known to be dominant in cultured *H. midae* abalone during the post-settlement development phase when they feed on diatoms (Nel *et al.* 2018). Members of the Verrucomicrobiae class are known to be symbionts of marine plants and animals (Petroni *et al.* 2000; Vandekerckhove *et al.* 2000; Weidner *et al.* 2000; Alain *et al.* 2002; Bowman and Nowak 2004). Species of *Rubritalea* have been identified as having either an aerobic and chemo-organotrophic or a facultatively anaerobic metabolism (Scheuermayer *et al*. 2006; Yoon *et al*. 2007). When isolated from the water column and sea hares, Yoon *et al.* (2007), found that *Rubritalea* bacteria were able to grow in liquid cultures with a temperature range of 15 to 37° C, a pH range of 6.5 to 8.5 and using glucose, xylose, pectin or cellobiose as an energy source. This may explain the presence of *Rubritalea* within *H. midae* digestive retracts, and why those fed the SBM diet exhibited the highest relative abundance of that genera. The temperature and pH range of *H. midae* digestive tracts fall within the range for successful *Rubritalea* growth. Furthermore, abalone fed the SBM diet exhibited higher cellulase activity levels on sampling day 357 than the other treatment diets, which was in winter (Table 2.6), due to higher seawater temperatures. Therefore, higher amounts of hydrolysed cellulose such as cellobiose would have been present, due to increased enzymatic action. More available cellobiose may have allowed for the digestive tract of SBM fed abalone to have potentially been a site for successful *Rubritalea* proliferation. The FM-SFM treatment diet also had a similar crude fibre content to that of the SBM diet, yet it resulted in having the lowest relative abundance of the phyla Verrucomicrobia and class Verrucomicrobiae, and one of the lowest in *Rubritalea*. This may be attributed differing antioxidants, bioactive compounds, complex polysaccharides between the two plant proteins, or prebiotic effects of certain phytates, tannins or

fibres found predominantly in soybean meal (Hayes *et al.* 1977; Erasmus *et al.* 1997; Shipton 1999; Yi-You 2004; Johnston *et al.* 2005; Garcia-Esquivel and Felbeck 2006; Dhanaraj *et al*. 2010; O'Sullivan *et al*. 2010; Banaskiewicz 2011; Rijavec and Zupin 2011; Bolanho and Beléia 2012; Desai *et al*. 2012; Khan and Ghosh 2012; Askarian *et al*. 2013; Nel 2016; Kemp 2017).

Bacteroidetes was one of the dominant phyla found in the gut of the *H. midae* abalone in the study. The relative abundance of Bacteroidetes bacteria was found to be higher in the gut samples of abalone fed the FM diet than the other diets. Most Bacteroidetes groups are known for their ability to ferment and degrade complex organic molecules such as cellulose, chitin, and pectin (Holdeman *et al.* 1975; Salyers *et al.* 1977; Reichenbach 2006; Nel 2016). Bacteroidetes obtain carbon and energy by fermenting polysaccharides that would usually be deemed as inert and indigestible, such as cellulose fibres and are usually in association with the dissolved organic matter within the marine environment (Salyers *et al.* 1977; Kirchman 2002; Reichenbach 2006; Nel *et al.* 2016). It would be expected that abalone fed a diet high in cellulose fibres and plant material would have a higher relative abundance of Bacteroidetes bacteria. While usually associated with saccharolytic activities, some strains of Bacteroidetes are proteolytic and are therefore found in high abundance in some organisms with high protein content diets such as salmonids (Krieg 2011; Zarkasi *et al.* 2016). Protein from fishmeal is more readily digested by abalone than many plant proteins (Sales and Britz 2001a), and this might explain why animals fed diets, with more digestible fishmeal in the current study, had higher relative abundance of Bacteroidetes bacteria (Krieg 2011; Zarkasi *et al.* 2016).

When investigating the development of gut-bacterial communities of juvenile *Oncorhynchus mykiss* (W. 1792) after the first feeding, Ingerslev *et al.* (2014) discovered than

trout fed two commercial diets, containing fishmeal as the protein source, had higher abundances of Bacteroidetes bacteria than trout fed diets containing pea meal and rapeseed oil as the main protein source. Michl *et al.* (2017) stated that animal proteins in formulated feeds promote the Bacteroidetes bacteria in the guts of *O. mykiss* when switching from a predominantly pea meal protein source diet to a 97 % animal protein diet consisting of fish, shrimp, mussel and blood meal. The results from the current study are therefore in line with those from Ingerslev *et al.* (2014) and Michl *et al.* (2017) that animal proteins in diets promote the growth of Bacteroidetes bacteria in gut-bacterial communities in *H. midae* abalone.

Tenericutes was another prominent phylum found within the gut microbiome of the study animals. Mollicutes, a class under the phylum Tenericutes, have been described as commensals and saprophytic (Holt *et al.* 1994). Miles (1992) and Thompson *et al.* (2004) have described *Mycoplasma*, a genus under the class Mollicutes, as facultative anaerobes. Their presence seems to be associated with coastal waters and macroalgae (Teeling *et al.* 2014; Nel *et al.* 2017b). Nel *et al.* (2017) found that *Mycoplasma* were more abundant in the gut of *H. midae* abalone fed a diet of kelp-supplemented formulated feed than those fed a non-supplemented formulated feed. Similarly, higher levels of fibres in the supplemented diet may have influenced the higher relative abundances of *Mycoplasma* bacteria in the current study.

Wang *et al.* (2016) performed a genomic characterisation of symbiotic *Mycoplasma* from *Bathynomus* isopod stomachs and established that *Mycoplasma* were involved in the degradation of oligosaccharides, amino sugars, and glycans and therefore assisted in supplying the host with amino sugars and simple carbohydrates. Cicala *et al.* (2018) linked this kind of symbiosis with the mutualistic association between *Haliotis fulgens* and *Haliotis corrugata* (W. 1828) abalone and the *Mycoplasma* identified in their digestive tracts, as they seem to have played a similar role in

metabolic functions as those in *Bathynomus* isopods. *Spiroplasma* bacteria (Class: Mollicutes) have a symbiotic relationship with *Acyrthosiphon* pea aphids that synthesise amino acids for their hosts (Douglas 1998; Fukatsu *et al.* 2001). It may be reasonable to assume that *Mycoplasma* could play a similar role in abalone hosts. Therefore, the higher levels of plant fibres from the soybean meal in the diets SBM and FM-SBM may have influenced the relative abundance of Tenericutes and the dominance of *Mycoplasma* within the gastrointestinal tract of abalone fed those diets in the current study.

Grow-out developmental stage *H. midae* abalone fed either kelp, formulated feed or kelp-supplemented formulated feed also showed to have Proteobacteria as the dominant phyla in the gut-bacterial community (Nel *et al.* 2017b; Nel *et al.* 2018). The digestive tracts of *H. discus hannai* (Tanaka *et al.* 2004; Lee *et al*. 2016; Nam *et al.* 2018), *H. diversicolor* (Huang *et al.* 2010), and *Haliotis tuberculata* (Gobet *et al.* 2018) abalone species also have Proteobacteria as the dominant phyla. Proteobacteria is a large phylum, with many of the groups being free-living in the marine environment, and being the most dominant group in the gut microbiome of the study animals. The abalone in the study were most likely introduced to the phyla from the seawater that is pumped from the shore onto the farm as it is a flow-through system (Eilers *et al.* 2000; Tanaka *et al.* 2004; Lee *et al.* 2016). The dominance of Proteobacteria in the gut of *H. midae* abalone in the current study can be attributed to certain groups colonising the gut and competitively replicating due to the high levels of energy sources from formulated feed present in the gastrointestinal tract (Enger *et al.* 1990; Tanaka *et al.* 2004; Madagan *et al.* 2015; Nel *et al.* 2018).

Planctomycetes was the second most abundant phylum found in all treatments of the current study, though when observing the relative abundances of genera present, the

genus *Blastopirellula* is seen to be the most dominant. In most of the literature, Planctomycetes is not a prominent group in the gut-bacterial community of wild or cultured abalone species and Nel *et al.* (2018) found that they were only very abundant and dominant in the gut of post-settlement *H. midae* abalone during the diatom-feeding stage, with the dominance waning significantly as they were weaned onto formulated diets and macroalgae. The reason for the continued dominance in the current study may be due to their constant exposure to the abalone. Planctomycetes are also a free-living group that has been known to attach to diatoms and marine detritus particles (DeLong *et al.* 1993; Fuerst 1995; Crump *et al.* 1999; Morris *et al.* 2006; Woebken *et al.* 2007) and therefore their introduction may be the same as that of Proteobacteria, by originating from pumped seawater. Diatom growth is common on top of feeder plates and is known to be grazed upon by the animals during the night. Coupled with the high nutrient load from formulated diets, Planctomycetes can proliferate within the digestive tracts of abalone.

There were numerous bacterial groups found in the current study that made up less than two percent (%) of the total relative abundance of bacteria within the gut of the abalone fed either of the four diets. Nel *et al.* (2018) too found this in their study when weaning juvenile *H. midae* onto either kelp, kelp-supplemented feed or formulated feed. The authors proposed that these bacteria are transient as well, originating from the aquatic environment like the other more dominant groups. This may explain why the gut bacterial composition and abundance of all four treatments in the current study were similar. Gobet *et al.* (2018) suggest that the physiochemical and biochemical conditions of abalone gut morphology, as well as diet composition, may be responsible for the current gut microbiota structure. The abalone digestive tract is a stable, anaerobic environment, with the crop and digestive gland being relatively acidic, therefore the type of bacteria present are specifically adapted for the environment (Gomez-Pinchetti *et al.* 1993;

Harris *et al.* 1998). Russell *et al.* (1996) have shown that a combination of low pH and volatile fatty acids can be toxic for some microbiota, which is why there are only a few core microbiota groups present. The presence of rare, less abundant bacteria may be due to them surviving the selective pressures of the gut and therefore participate in the digestive activities of the abalone digestive tract (Lorenz and Wackernagel 1994). The method of DNA extraction used in the current study may have also influenced the bacterial composition results, as a bead-beating step was not included. This is known to cause some level of bias against gram-positive bacteria, however, since *Mycoplasma* is a gram-positive bacterium and was the second most prominent genera in all treatments, it can be concluded that both groups of bacteria have been well represented (Yuan *et al.* 2012; Gill *et al.* 2016; Cicala *et al.* 2018).

Conclusions

- The diversity indices for each treatment were high, though no significant differences between each diet was observed.
- Proteobacteria, Planctomycetes, Verrucomicrobia, Tenericutes, and Bacteroidetes were the most dominant phyla observed in the gut samples of *H. midae* abalone.
- Gammaproteobacteria, Planctomycetacia, Alphaproteobacteria, Mollicutes, Bacteroidia, and Verrucomicrobiae were the most dominant classes observed in each treatment.
- The phyla and class, Verrucomicrobia and Verrucomicrobiae respectively, displayed significant differences between diets. Abalone fed the SBM treatment diet had the highest relative abundances of Verrucomicrobia and Verrucomicrobiae bacteria.
- Blastospirellula, *Mycoplasma*, and unclassified Gammaproteobacteria were the most dominant genera, yet significant differences between treatments were only observed

between the relative abundance of *Rubritalea* bacteria. Abalone fed the SBM diet had the highest relative abundance among the treatments.

CHAPTER 4

4 CONCLUDING DISCUSSION

The research of the current study has shown that the inclusion of plant-derived protein sources in formulated feeds have a significant effect on the enzyme activity and gut-microbiota of cultured *Haliotis midae* abalone.

The literature review discussed the development and use of formulated feeds in abalone aquaculture and the effects plant proteins such as soybean meal (SBM) and sunflower meal (SFM) had on the overall health and growth on abalone. Substituting fishmeal with alternative proteins seems to be the general trend followed, with reasons ranging from sustainability issues to saving costs, and growth rates alone are not the only aspect considered. Anti-nutritional factors (ANFs) found in plant protein meals are still a significant concern and therefore the present research consisted of comparing the effects soybean meal and sunflower meal had on the digestive enzyme activities and the gut-bacterial community of *H. midae* abalone.

Cellulase (polysaccharidase) and chymotrypsin (protease) activity levels in the guts of abalone were analysed and compared among those fed one of the four diets: FM-only, SBM-only, FM-SBM, and FM-SFM. The aim was to determine whether diets with differing protein sources had an effect on the enzyme activity levels of the abalone.

The cellulase activity levels were compared between each treatment on every sampling day and a significant difference was only observed on day 182, where abalone fed the FM-only diet had the highest cellulase activity The cellulase activities of male and female abalone of each treatment and sampling date were also compared. Female abalone fed the FM and SBM diets, and male abalone fed the FM-SBM diet were observed to have the highest activities on sampling day 357, respectively. Male abalone fed the FM-SFM diet had higher activity levels in sampling day

182. The results show that diet had an effect on the carbohydrase activity in the gut of male and female farmed abalone.

The chymotrypsin activity levels exhibited significant difference between each diet over time. Abalone fed the SBM diet had significantly higher activities on sampling day 91, whereas those fed the FM treatments were significantly higher on sampling days 182, 273 and 357. The chymotrypsin activity levels between males and females of each treatment and sampling date were compared. Females had significantly higher activities than males on sampling day 91 when fed the FM-SFM diet. Males had significantly higher activities than females on sampling day 182 when fed the FM-SBM diet. In all four treatments, females were observed to have significantly higher activities than males on sampling date 357. Therefore, diet had an effect on the protease activity in the gut of male and female farmed abalone.

No differences in water parameters were found between treatments, however there was variability when comparing seasonal temperatures. Summer seawater temperatures were the lowest and most variable, while winter temperatures were the highest and the most stable.

Thus, formulated diets have a direct effect on the digestive enzyme activity, depending on the ingredient sources used. Different levels of starch, and plant materials, such as fibres, in the diets may have played a role in the higher cellulase activities of abalone fed the FM treatment and the dissimilarity in activities demonstrated in abalone fed the SBM. The FM formulated feed had higher levels of starch included compared to the other treatments, for better binding ability as fishmeal contains more oils. Cellulase does not, however, hydrolyse starch and therefore does not explain the high activities seen in abalone fed the FM-only treatment. Performing amylase assays would be able to shed light on the effect the higher starch would have on activity levels.

The observed cellulase activity levels from the study may have been influenced by the crude fibre content of the treatment diets. Two of the treatments that were fed to the abalone, FM-SFM and SBM, had similar crude fibre contents though differing cellulase activities were observed. Abalone fed the SBM treatment diet exhibited low cellulase activities in most of the sampling periods even though it was the diet containing the most crude fibre. Interestingly, it was the treatment that caused the highest activity on the last sampling day. These results are unlike what has been observed in previous literature (Fagbenro 1990; Das and Tripathi 1991; Szlaminska *et al*. 1991; Chakrabarti *et al*. 1995; Saha and Ray 1998; Gatesoupe 2008; Bagheri *et al.* 2008; Mazouk *et al.* 2008; Salnur *et al.* 2009; Merrifield *et al.* 2010; Maity *et al.* 2011; Ray *et al.* 2012; Nel 2016). the substrate analysed was already degraded in the assay, and possibly causing an overestimation for the treatment diet with less fibre. This would explain the observed activities of the SBM treatment, but fails to explain why the activities of FM-SFM treatment samples exhibited were similar to those found in FM and FM-SBM, which in fact had less or no crude fibre present.

Concerning the influence of chymotrypsin activity levels, the presence of protease inhibitors may be a factor. Especially considering that protease inhibitors are found in plant protein sources, chiefly soybean meal (Silva *et al*. 1995; Shipton 1999). The plant proteins used in the formulation of the diets used in the study where heat-treated, which denatures the protease inhibitors and reduces their negative effects. They are not all completely inactive, though, and still might be able to affect the efficacy of proteases in the gut. Diatoms within the farm system may play a role as well. Since all water on the farm is pumped straight from the sea, diatoms tend to settle in the tanks within which the abalone are kept. Having very variable levels of protein composition, abalone grazing on the diatoms may stimulate the digestive tract (Knauer *et al.* 1996).

The notable increase in chymotrypsin activity on sampling day 182, of the abalone fed FM-only, FM-SBM and FM-SFM could be attributed to the temporal changes in utilisation (Wu 2020). During that sampling period it was summer, which tends to be the time that the farmed abalone spawn (Wu 2020). It was noted that abalone fed the treatment diets containing fishmeal also developed their reproductive tissues (Wu 2020). This can explain low chymotrypsin activities in abalone fed SBM-only.

The low utilisation of the SBM-only treatment diet may be partially attributed to the presence of allergenic properties. The proteins β-conglycinin and glycinin found in soybean meal are known to be poorly digested and induce inflammation, resulting in the deterioration of proteins in intestinal walls, greatly reducing nutrient utilisation and absorptive capacity leading to poor somatic growth. (Dreau *et al.* 1994; Rumsey *et al.* 1994; Fu e*t al.* 2007; Chen *et al.* 2011; Zhao *et al.* 2015). Compounding the issue is that the severity of the allergenic effects of β-conglycinin and glycinin increases when heat-treated, a common procedure done to soybean meal (Banaszkiewicz 2011). Though abalone have ruminant-like digestive systems, it is likely that the animals used in the current study that were fed the SBM treatment diet were affected by the allergenic compounds present, evident by the low cellulase and chymotrypsin activities and feed utilisation observed (Knauer *et al*. 1996; Sawabe 2006; Wu 2020).

Since abalone are ectotherms, the water temperatures they are exposed to may have affected their digestive enzyme activities. Some species such as *H. rubra* have demonstrated higher protease activity levels and growth rates with animals that are kept at higher water temperatures (Edwards and Condon 2001). Water temperature fluctuations, not average temperature, may have had an impact of the enzyme activities observed in the current study. Both cellulase and chymotrypsin activity levels were highest on sampling days 182, which was during

summer. The average summer water temperatures was low during the trial period, but had the highest variability of the four seasons. This has also been observed Schaeffer *et al.* (2013) in *H. laevigata*, where drops in water temperature lowered enzyme activity and triggered a growth in digestive epithelial tissue to allow for uncreased nutrient absorption and enzyme interactions. This mechanism allows abalone to improve digestive function when exposed to low water temperatures and therefore may explain the high enzyme activity observed in the current study from the samples collected in summer.

The size of the abalone used in the study at the start were roughly 40 to 50 g. The growth rates of younger abalone tend to be fast, slowing done over time as they reach larger sizes. During this period of fast development, abalone are better at adapting to fluctuating environments and diets, exhibiting very different and flexible digestive enzyme behaviours than older abalone (Bansemer *et al.* 2016). Once adapted to the diets, the abalone were able to better utilise the diets presented, which is exhibited by the peak in digestive enzyme activity.

The gut bacterial diversities of abalone fed all the diets were high. Additionally, a rarefaction curve displayed that all the abalone gut samples reached a plateau, meaning that they were sampled to saturation of the community coverage. Diet did not influence the overall diversity of gut bacterial communities of abalone fed any of the diets as there was no significant difference in the diversity indices between treatments. Having analysed the relative abundances of bacterial groups present in the abalone gut samples, it was discovered that the community structure was very similar between all four treatments, yet the diet compositions were different. Since abalone have unique physiochemical and biochemical conditions, bacteria present would be specifically adapted to the stable, anaerobic and acidic conditions (Gomez-Pinchetti *et al.* 1993; Harris *et al.* 1998; Gobet *et al.* 2018).

Proteobacteria, Planctomycetes, Bacteroidetes, Verrucomicrobia, and Tenericutes were the most dominant phyla. Planctomycetacia, Gamma- and Alphaproteobacteria, Mollicutes, Bacteroidia, and Verrucomicrobiae were the most dominant classes observed. The most prominent genera were *Blastopirellula*, *Mycoplasma*, and unclassified Gammaproteobacteria. Abalone fed the SBM diet had significantly higher relative abundance of the phylum Verrucomicrobia, class Verrucomicrobiae and genus *Rubritalea*. This shows that the diet did affect the composition of gut-microbiota in abalone, specifically the SBM treatment diet. To further support this, a Bray-Curtis similarity measure was performed and displayed in the form of a n-MDS plot which concluded that treatments FM, FM-SBM, and FM-SFM treatments shared a high level of similarity, with SBM showing dissimilarity due to clustering separately from the other treatments. Additionally, a Venn diagram of the shared OTUs between treatments was produced to confirm the conclusion.

The relative abundance of the bacteria phyla Verrucomicrobia, class Verrucomicrobiae, and genus *Rubritalea* were highest in the treatment containing soybean meal as the sole protein source: SBM. These bacterial groups were influenced by the plant material from soybean meal, as hydrolysed cellulose was broken down by endo- and exogenous cellulase allowed for the proliferation of *Rubritalea* bacteria due to them using cellobiose as an energy source.

Higher relative abundances in Tenericutes bacteria in abalone fed the FM-SBM treatment was likely due to the exposure of higher plant materials and fibres from the soybean meal as some *Mycoplasma* (Phyla: Tenericutes; Class: Mollicutes) species are known to degrade oligosaccharides amino sugars from those sources. Therefore, exposure to formulated feeds with high levels of these plant materials would allow for higher proliferation than if they were when

exposed to formulated feeds higher in animal proteins. This may be why the relative abundances of Tenericutes in abalone fed SBM were also high.

The reason for Bacteroidetes dominance was due to the phyla having bacterial species that can breakdown proteins and species that can break down carbohydrates. It seems that the animal protein present in the composition of the FM diet had a positive influence on the proliferation of proteolytic Bacteroidetes bacteria. It is believed that the high levels of cellulose from the plant fibres in the soybean meal-only diet (SBM), promoted the growth of saccharolytic Bacteroidetes bacteria.

The dominance of the phylum Proteobacteria, the class Gammaproteobacteria and the genus *Mycoplasma* are groups found within the gut of several abalone species, however, the presence of Planctomycetes and *Blastopirellula* are groups not prominent in the literature. *Vibrio* and *Shewanella* are usually found in high relative abundances in the guts of abalone, nevertheless, abalone in the current study only had those genera in small numbers. Several of the bacterial groups found within the gut of abalone are opportunists that originate from the water column and colonise along the digestive tract when ingested. Certain groups display dominance by outcompeting others due to being better equipped to the exposure of high nutrient loads. Planctomycetes do not typically fall into this category and the reason for their predominance can be attributed to the diatoms that are grazed upon by the abalone in the study. Abalone on the farm tend to move along the feeder plate at night and eat the diatoms that have settled on top of the plate. The bacteria that are a part of the Planctomycetes phylum are usually a free-living group, though, are known to attach themselves to diatoms and marine detritus particles.

There were several aspects of the current study's methodology that can be improved on to allow for more thorough and conclusive results for the inclusion of various protein sources in

formulated feeds and the effects they may have on the enzyme activity and gut-bacterial community of *H. midae* abalone. For instance, diets with less than 30 % protein levels may have ensured a more similar composition, as the diets used in the current study had differing starch levels that may have interfered with comparisons and influenced the enzyme activities and gut bacterial community. Additionally, more diets with lower levels of each protein source could have been included in the study to further investigate the influence they have on the digestive tract of abalone. A test to confirm the presence and concentration of enzyme inhibitors in the SBM treatment diet could have been performed so as to not rely on speculation. This could have been done by using pure chymotrypsin in the presence of differing soybean meal levels in an enzyme reaction. After heat treating the soybean meal, one can confirm if inhibition is still present. In order to confirm the influence temperature had on enzyme activities in the study, the gene expression, for example, of each enzyme tested could have been measured using reverse transcription polymerase chain reaction (RT-PCR). Accompanying this, more proteases, carbohydrases, and lipases should be studied by performing more assays. This would allow for a bigger picture of the influence diet has on the digestive enzyme activity of abalone.

Analysis of the gut bacterial community is an important aspect when studying the digestive tract of abalone and the effect various diets may have on it. Since abalone have distinct levels of adaptability within different life stages, it should be important to track any changes within the gut as well. The abalone used in the current study were weaned off Abfeed®- S34 onto one of the four diets. To be able to achieve an improved long-term view of the abalone gut-bacterial community, more frequent sampling of gut extracts should have taken place. This would have allowed for the ability to identify shifts in the core-microbiota groups within the gut and better compare each diet's influence. In addition, the method of DNA extracted could have been slightly different as well.

Including a bead-beating step in the DNA extraction method to break up the cells in the samples would improve the amount of bacterial DNA released and allow for more accurate community analysis (Tanaka *et al*. 2016; Nel *et al*. 2017b; Cicala *et al*. 2018; Nam *et al*. 2018; Nel *et al*. 2018).

The literature and results from the current research have shown that studying the digestive physiology and function of abalone can provide information that may help obtain a better understanding of how abalone function in a cultured environment and how they react and adapt to formulated diets with plant protein sources. Further research should be done on the bacterial groups present in the digestive tracts of abalone, and to discover the specific roles they play. This can be complemented by studying more abalone digestive enzymes and their response to plant protein source inclusion in the formulated feed. These aspects may contribute to the development of more optimised, digestible diets that are also less reliant on fishmeal protein and do not result in the reduction if abalone health and growth rates in a farming environment.

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