Organic acids as potential growth promoters in abalone culture

by

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Thesis submitted in fulfilment
of the requirements for the Degree

of

Master of Science in Engineering
(Chemical Engineering)

in the Department of Process Engineering
at the University of Stellenbosch

Supervised by

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Stellenbosch

December 2007

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Signature   Date

LIST OF ABBREVIATIONS

Abbreviation	Meaning	Units
SGR	Specific growth rate	d^{-1}
FCR	Feed conversion ratio	-
AGRL	Apparent growth rate based on length	μm/day
AGRW	Apparent growth rate based on weight	mg/day
IC	Incidence Cost	R/ton abalone
Fulton CF	Fulton condition factor	-
FI	Feed intake	g
CoF	Cost of feed	R/ton feed
W	Weight	g
L	Length	mm
t	Time	days

ABSTRACT

The first successful captive spawning of the South African abalone *Haliotis midae* occurred in the 1980's and subsequently the commercial abalone industry in South Africa has developed, with an estimated investment of US$ 12 million and annual output of 500 to 800 tons by 2001, making South Africa the biggest abalone producer outside of Asia. Natural kelp is currently the major feed and the development of a suitable substitute, and improved disease management in abalone culture are seen as the primary factors limiting expansion of the industry in South Africa. Further, abalone growth rates are very slow and improvements in growth rate will lead to shortened production times with benefits to producers. Diseases in aquaculture have traditionally been combated using antibiotics as treatment (therapeutic usage) and preventative measure (prophylactic usage). In terrestrial livestock management, antibiotics are also used as growth promoters. The use of antibiotics in aquaculture has recently sparked concerns about the development of antibiotic resistance in pathogens of humans and aquaculture organisms, and alternative strategies to using antibiotics mainly focus on manipulating the microbial composition in the host organism, in order to establish a beneficial   microbial population to prevent disease.

The role that organic acids and their salts can play as growth promoters in the South African abalone *Haliotis midae*, and as manipulators of the gut microflora of this species of abalone was investigated and compared to the effects of antibiotics. Three different treatments were tested against a negative control and a positive control containing 30ppm avilamycin, a commercial antibiotic growth promoter (AGP) used in the pig and poultry industry. The 3 treatments consisted of 1% acetic and 1% formic acid (treatment AF), 1% sodium benzoate and 1% potassium sorbate (treatment SBPS), and 1% benzoic and 1% sorbic acid (treatment BS). Three different experiments were conducted to test the effects of the different acids and salts. The first experiment was under controlled optimum water temperature conditions (16.5ºC), another at elevated water temperature (20.5ºC) in order to test response during temperature stress conditions, and the final trial was conducted under uncontrolled practical production conditions. In an attempt to establish the mechanism by which the treatments have their effects (if any), the composition of the gut microflora of the abalone was monitored.

It was found that the organic acids and salts investigated can enhance the growth rate of *Haliotis midae* in the size class 23 mm to 33 mm mean length significantly when compared to both control treatments. It was further found that the tested AGP had no effect on growth rate. None of the treatments had a significant effect on feed conversion ratio (FCR), Incidence cost (IC) or feed intake. It could also not be shown that the treatments affected the intestinal microflora of the abalone, although this might be due to inadequate microbiological methods. The mechanism by which the acids and salts have their effects could not be established.

It was found that the animals in the controlled system underwent an initial adaptation period, which led to improvement in specific growth rate (SGR), FCR and IC as the experiment progressed during the controlled optimal conditions experiment. Large differences in FCR and IC was seen for controlled optimal conditions and production conditions which means that there is still a large scope for developing methods to improve practical on-farm feed utilisation by abalone.

SGR, FCR and IC were negatively influenced by raising water temperature from 16.5ºC to 20.5ºC. The composition of the gut microflora of the abalone also changed significantly after the water temperature was raised. It appears that animal weight gain and shell growth respond differently to changing water temperatures, which is reflected in a change in Fulton condition factor.

A relationship between the length and weight of abalone between 15 mm and 47 mm was established and it was found that *Haliotis midae* does not follow an isometric growth relationship. This relation can be used as a tool to improve farm management and therefore also profitability.

Various micro-organisms were isolated from *Haliotis midae* during the trial, but their relationship and interaction with abalone is not clear. Clear dominance by specific species of bacteria was observed during certain periods.

The current research has clearly showed the potential of organic acids and their salts to act as growth promoters in the South African abalone *Haliotis midae*, with application in both the local aquaculture and feed manufacturing industries. The possibility further exists that some aspects of the current research can be adapted to be applicable in other abalone species and even in other aquaculture species.

OPSOMMING

Die eerste suksesvolle aanteel van die Suid-Afrikaanse perlemoen *Haliotis midae* in gevangeskap is in die 1980's gerapporteer, waarna 'n suksesvolle akwakultuur industrie ontwikkel het met 'n geskatte produksievermoë van 500 tot 800 ton en kapitaalbelegging van US$ 12 miljoen in 2001. Suid-Afrika is tans die grootste perlemoen-produserende land wat buite Asië geleë is. Die ontwikkeling van 'n geskikte alternatiewe voedselbron vir natuurlike kelp (tans die algemeenste voedselbron wat gebruik word in die kweek van perlemoen), sowel as verbeterde siektebestryding word tans gesien as die hooffaktore wat verdere uitbreiding in die Suid-Afrikaanse industrie beperk. Perlemoen het verder baie stadige groeitempo's en enige verbetering in hierdie verband sal produksietye verkort en dus produsente bevoordeel. Siektes in akwakultuur word tradisioneel bestry deur gebruik te maak van antibiotiese behandeling (terapeutiese bestryding) of van voorkomende behandeling (profilaktiese bestryding). In gewone diereproduksie-sisteme (bv. varke en hoenders) word antibiotika ook gebruik as groeistimulante. Die gebruik van antibiotika in akwakultuur het onlangs die bekommernis laat ontstaan dat sekere menslike en diere-patogene weerstand kan ontwikkel teen sommige middels, wat die behoefte laat ontstaan het om siektebestryding sonder die gebruik van antibiotika te ontwikkel. Alternatiewe strategieë fokus grootliks daarop om die samestelling van die mikrobiese bevolking van die gasheer te manipuleer en sodoende 'n voordelige bevolking in die gasheer te vestig, wat dan siektes voorkom.

Daar is ondersoek ingestel na die rol van organiese sure en hul soute as groeistimulante en manipuleerders van die mikrobiese bevolking in die Suid-Afrikaanse perlemoen *Haliotis midae*. Drie verskillende behandelings is getoets en vergelyk met beide 'n negatiewe- en positiewe kontrole (wat 30 dele per miljoen van 'n kommersiële antibiotiese groeistimulant bevat het). Die drie formulasies het onderskeidelik bestaan uit 'n mengsel van 1% etanoë- en 1% metanoësuur (behandeling AF), 1% bensoë- en 1% sorbiensuur (behandeling BS) en 1% natriumbensoaat en 1% kaliumsorbaat (behandeling SBPS). Om die effekte van hierdie formulasies te toets, is daar 3 proewe gedoen. Een proef is gedoen onder temperatuur-beheerde toestande teen 'n optimum watertemperatuur van 16.5ºC terwyl 'n ander gedoen is onder onbeheerde, praktiese produksie-omstandighede. 'n Verdere beheerde proef is gedoen teen 'n watertemperatuur van 20.5ºC om die effek van die verskillende formulasies te toets wanneer die diere aan temperatuur-spanning blootgestel word. Die samestelling van die mikrobiese bevolking in die dunderm van die perlemoen is deurentyd gemonitor in 'n

poging om die meganisme vas te stel waarvolgens die sure en soute hul effek het, indien daar enige effek waargeneem word.

Daar is gevind dat die onderskeie sure en suursoute die groeitempo van *Haliotis midae* met 'n gemiddelde lengte van 23 mm tot 33 mm beduidend kan verhoog in vergelyking met die groeitempo's van beide kontroles. Daar is gevind dat die antibiotiese groeistimulant geen effek het op die groei van die diere nie en dat geen behandelings 'n beduidende effek op voeromsetting, voerkoste of voerinname gehad het nie. Daar kon nie bewys word dat enige van die formulasies of die antibiotika 'n effek gehad het op die mikrobes in die spysverteringskanaal van die perlemoene in die sisteem nie, alhoewel die gebrek aan 'n effek moontlik toegeskryf kan word aan die onakkurate en onvoldoende mikrobiologiese metodes wat gebruik is tydens die studie. Die meganisme waarvolgens die sure werk kon nie vasgestel word nie.

Daar is verder gevind dat die diere in die temperatuur-beheerde eksperiment aanvaklik deur 'n aanpassingsperiode gegaan het, wat tot gevolg gehad het dat die spesifieke groeitempo, voeromsetting en voerkoste verbeter het met die verloop van die eksperiment. Daar is groot verskille gevind in die voeromsetting van beheerde optimale toestande en onbeheerde produksietoestande, wat impliseer dat daar nog baie ruimte en geleenthede is om metodes te ontwikkel wat beter voeromsetting bewerkstellig tydens perlemoenproduksie.

Spesifieke groeitempo, voeromsetting en voerkoste is nadelig beïnvloed toe die watertemperatuur verhoog is vanaf 16.5ºC na 20.5ºC. Die samestelling van die mikrobiese bevolking in die spysverteringskanaal van die perlemoen het ook beduidende veranderinge ondergaan tydens hierdie temperatuur verhoging. Dit wil voorkom asof die lengtegroei van die dop en die toename in massa verskillend reageer op 'n verandering in watertemperatuur en hierdie effek word weerspieël in die verandering in Fulton-kondisiefaktor.

'n Verwantskap tussen totale doplengte en totale gewig van *Haliotis midae* kon vasgestel word vir diere tussen 15 mm en 47 mm en daar is gewys dat *H. midae* nie 'n isometriese groeipatroon volg nie. Hierdie verwantskap kan aangewend word tydens produksiebestuur om produksie te verbeter en daardeur ook winsgewendheid te verhoog.

Verskeie mikrobes is tydens die verloop van die proef geïsoleer, maar die rol van en interaksie tussen hierdie mikrobes en die Suid-Afrikaanse perlemoen is nie duidelik nie. Sekere bakterieë het die mikrobiese bevolking in die spysverteringskanaal van die perlemoen in hierdie proef oorheers tydens sekere groeiperiodes.

Die huidige navorsing het duidelik aangetoon dat organiese sure en hul soute as groeistimulante kan optree in die Suid-Afrikaanse perlemoen *Haliotis midae*, met toepassings in die plaaslike akwakultuur- en voervervaardigins-industrieë. Dit beskik verder oor die potensiaal om aangepas te word sodat dit toepaslik is in ander perlemoenspesies en selfs ander akwakultuur organismes.

ACKNOWLEDGEMENTS

There is a host of people that I need to thank who all made an immeasurable contribution to this project.

I would like to thank my supervisors Dr. Johann Görgens, Dr. Lourens de Wet and Prof. Chris Aldrich, for support, guidance and inputs throughout the whole project. It was a great learning experience working under your supervision.

My thanks to Irvin & Johnson for kindly providing facilities where the investigation could be conducted, and to all the people who helped me in so many ways during my project. Thanks, Lize for your time and inputs in the project, and also to Obert who had the unenviable task to clean and feed the animals used in the trials.

I gratefully acknowledge the personal and research funding received from the National Research Fund, THRIP and the Department of Process Engineering at the University of Stellenbosch, without which this project would not have been possible.

Many thanks to Dr. Hafizah Chenia from the Department of Microbiology, University of Stellenbosch for performing the PCR reactions and 16S identification of the micro-organisms and for training, advice and guidance that I received from her in order to complete my microbiological studies. Also to the other students of the Biolab (Leonhard, Aingy B, Remmy Charl and Isa): thank you for many great hours, it was great working with you guys. Thank you for many insightful conversations not concerning microbiology. Further my thanks to Resia Swart from the Department of Animal Science for analysis of the feed.

Thank you to all the people who helped me weigh and measure the thousands of animals used in the project: Lourens, Wiehan, Schalk, the late Alvin Arnold, Wynand, Ruben and Johnno (in order of appearance). I appreciate your time and efforts.

Thank you to Tiaan, Faf, Lourens, Gus, SJ and all my other dear friends for moral support, encouragement and reminding me of the lighter side of things when the going got tough. I appreciate the role you played in the success of this project.

Finally, I would like to thank my Lord and Saviour, Jesus Christ for the ability and the strength to finish this project.

DEDICATION

I dedicate this thesis to my family in remembrance of the role that they played in my life. To my parents, Jurgens and Neranzè who helped me to find my passion in life and allowed me to pursue it, and for their guidance throughout my life. To my sister Dominique who always had some encouraging words for me and to my brother Carl who was always prepared to help with random aspects of the project and other things in the res.

Thank you all for your help and encouragement throughout this thesis. I love you all.

1.   INTRODUCTION

In 1965, 2280 tons of South African abalone *Haliotis midae* was harvested from the South African coastline. It was realised that this utilisation of the natural resource was not sustainable and strict conservation measures were implemented in an attempt to prevent overexploitation of the fishery. Supply could not keep up with demand and commercial industry showed interest in the culture of the species, especially since by that time Japan developed the technology to successfully produce juvenile abalone. The first successful spawning in captivity of *Haliotis midae* occurred in the early 1980's which paved the way for commercial production (Genade *et al.*, 1988). Since then a number of commercial ventures have been established and it was estimated that by 2001, US $12 million had been invested in the industry with an estimated output of 500 to 800 tons per annum (Sales and Britz, 2001), making South Africa the biggest producer of abalone outside of Asia (FAO, 2004). Expansion in production has been driven by high market prices for abalone and further developments are expected. In 2004 the South African abalone production industry employed approximately 1390 people. Due to its labour intensive nature the industry provides permanent employment to especially poor coastal communities, making it a key industry in alleviating poverty in the country. In order to continue industry expansion it is necessary to develop efficient alternative feeds as sustainable limits are being approached for kelp harvest in many areas, especially since kelp is the primary feed used in the culture of abalone in South Africa (Troell *et al.*, 2006). Other macroalgae are also being fed to abalone, although only in low quantities due to very low occurrence naturally and erratic supply (Troell *et al.*, 2006), thus further increasing the need to develop reliable alternative abalone feeds. Except for eliminating the difficulties associated with collection and culture of natural macroalgae, formulated diets offer other advantages e.g. the opportunity for a feed manufacturer to formulate diets that yield better survival and optimal growth rates (Spencer, 2002).

Improved feed utilisation or improved animal performance due to feed optimisation will have tangible benefits for aquaculture producers. This is especially true in the case of abalone culture where the production period is 3 to 4 years (Spencer, 2002). In order to continually improve profitability, the aquaculture industry always strives to improve feed consumption, feed conversion efficiency and growth rate (Alanärä, 1996; Britz *et al.*, 1996) as these have direct economic implications for ventures. Many of the production costs in aquaculture are time dependant and a reduction in production time resulting from increased growth rates

would result in reduced expenditures, as well as reduce exposure to risks like disease and adverse environmental conditions that could result in economic losses (Cook *et al.*, 2000). Research is done in many areas to address problems related to aquaculture production, including genetics, animal science and husbandry, feed science etc. One of the most important factors in aquaculture is efficient utilisation of feeds by animals (Alanärä, 1996; Fleming, 1995) as feed expenditure represents the single biggest operating cost. Feed wastage resulting from overfeeding as well as reduction in growth rates resulting from underfeeding will result in unnecessary economic losses (Ang and Petrell, 1997; Britz *et al.*, 1996; Britz *et al.*, 1994). For many years, the limiting factor in expanding aquaculture was water quality, but due to progress in this area in recent times, nutrition has become the new key limitation for increasing production (Staykov *et al.*, 2005), therefore it is critical to develop feeds that are utilised optimally by animals. A further benefit of improved feed utilisation is the reduction of nutrient release into the environment and a better utilisation of natural resources that are under pressure (e.g. fish meal, a main component in many aquaculture feeds, including abalone feed (Pinto and Furci, 2006)), thereby contributing to environmentally friendly aquaculture. Few other ventures have the benefit of simultaneously improving economic performance and reducing environmental impact (Alanärä, 1996).

Alternatives to antibiotics are sought continually as demand for environmentally friendly aquaculture practices increase (Macey and Coyne, 2005) and the emphasis shifts from disease treatment to disease prevention, which is likely to be a more cost effective way of combating disease (Verschuere *et al.*, 2000). One of the major issues surrounding sustainability is that of antibiotic use in aquaculture and its possible effects on human health, either due to development of resistance by pathogens or because of antibiotic residues found in food products (Balcázar *et al.*, 2006; FAO, 2004; Reilly and Käferstein, 1997). Antibiotics have traditionally been used to combat and/or prevent certain bacterial diseases in aquaculture (Li and Gatlin, 2005). Combating disease in aquaculture, however, is impaired by open production systems and the intimate relationship that exists between hosts animals and pathogens (Olafsen, 2001). The whole environment surrounding the aquaculture organism supports pathogens independently of the host and concentrations of pathogens can become high (Moriarty, 1998). Also, the indigenous flora of the cultured organism is altered during intensive production, which could cause increased susceptibility to disease or a decrease in feed utilisation (Olafsen, 2001). In order to combat disease and retain high productivity, it might be necessary to selectively manipulate the interaction of the cultured animals and microbes, which is not achievable with antibiotics (Verschuere *et al.*, 2000). Due to the disadvantages and environmental impact of antibiotics in aquaculture, considerable interest has been shown in alternative substances that can be used in disease

prevention and combat, including vaccines and dietary supplements in the form of immunostimulants, probiotics and prebiotics.

This study is an attempt to address a number of issues within aquaculture with specific application to the South African abalone industry. It is investigated whether organic acids can act as viable alternatives to antibiotics in aquaculture. The aquatic environment creates very different circumstances and challenges in animal production compared to terrestrial conditions, which means disease control is not as simple in aquatic environments, thus requiring novel solutions. Organic acids are known to have antimicrobial effects, therefore there is logic to investigating them as substitutes for antibiotics. Sorbic, benzoic and propionic acids have definite antimicrobial properties when used as food additives and/or preservatives. Sorbic acid is used as a broad spectrum antimicrobial and exhibits inhibition against yeasts, molds and some bacteria, benzoic acid is effective against yeasts and molds and propionic acid inhibits molds but not yeasts or bacteria (Liebrand and Liewen, 1992).

Further, the effects that organic acids have on the growth rate, feed utilisation and intestinal microbial community of the South African abalone *Haliotis midae* are investigated and compared to the effect of a commercial antibiotic growth promoter used regularly in the pig and poultry industries. It is also investigated whether the effects of the organic acids, if any, are due to a simultaneous effect on the intestinal microflora of the abalone and whether this affects the efficiency of feed utilisation. The advantages of better growth rates and improved feed utilisation are obvious, yet the benefits that could be gained from altering the gut microflora of the abalone are not clear. It is possible that digestion could be improved or that pathogens could be eliminated from the intestinal tract of the animals and these possibilities are investigated. As is the case with much of the research done on abalone culture in South Africa, this study has been fuelled and funded by private industry and the results will prove useful even if a full fundamental understanding of the mechanisms involved in the results is lacking.

Mariculture is a relatively new industry in South Africa and the culture of abalone is seen as the pioneering industry (Sales and Britz, 2001). It has a favourable outlook due to high prices obtained for abalone products when exported, especially in the Far East (Oakes and Ponte, 1996). This project is an attempt to make a contribution to the South African abalone industry by adding to the current knowledge regarding abalone nutrition and production.

2.   LITERATURE SURVEY

2.1   Antibiotics in animal production

It has been shown that antibiotic growth promoters (AGP's) consistently increase growth rate and feed efficiency in food animals (Doyle, 2001). As an example: In more than 1000 experiments conducted between 1950 and 1985, an improvement in growth rate and FCR was observed for all stages of pig production (Cromwell, 2002). Other animals in which AGP's have been used regularly include poultry and ruminants (Anonymous, 1999; Edwards *et al.*, 2005). In order to improve profit, antibiotics have been used regularly in animal production since the 1950's (Hardy, 2002). The antibiotics are used in 3 different ways: to treat animals with clinical symtoms (therapeutic use), as pre-emptive treatment (prophylactic use) to prevent outbreak of disease when animals are subjected to certain conditions [Conditions during production of food animals frequently include high densities, large groups, frequent movement, mingling and relatively young animals, which are conducive to the outbreak and spread of disease and it is frequently necessary to use antibiotics to combat this (Wegener, 2003)] and finally to increase growth rate and feed efficiency when incorporated as feed additives (Gunal *et al.*, 2006; Wegener, 2003). .

There are several proposed mechanisms for the effect of sub-therapeutic levels of antibiotics in animal feeds: (1) inhibition of infections not showing clinical symptoms, (2) a reduction of the amount of microbes and therefore growth inhibiting metabolites from microbes, (3) a reduction of microbial use of nutrients in the intestines, thus rendering more nutrients available to the animal and finally (4) enhanced uptake and use of nutrients due to a thinner intestinal wall in antibiotic fed animals, yet the exact mechanism of action is still not clear (Collett and Dawson, 2002). It has been suggested that the location where ingested antimicrobials have their effect is in the gut, as many of the AGP's used are not absorbed by the animals (Dibner and Richards, 2005; Feighner and Dashkevicz, 1987). A number of physiological, nutritional and metabolic effects have been reported upon the use of AGP's (Gaskins *et al.*, 2002). All of the proposed mechanisms share the assumption that certain microbes depress animal growth through their metabolic activities (Gaskins *et al.*, 2002). This is supported by results obtained by Coates *et. al* who showed that penicillin significantly enhanced the growth of normal chickens, but that it had no effect on the growth of germ-free chickens (Coates *et al.*, 1963). There are other reports that contradict these findings, showing that AGP's have no significant effects on growth (Engberg *et al.*, 2000; Gunal *et al.*, 2006) even though the antibiotics influenced the microflora. A possible explanation is that

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AGP's have a more marked effect under conditions of poor hygiene, but when animals are produced under favourable husbandry practices and nutrition, the effects are minimal (Gunal *et al.*, 2006). It has also been observed that although AGP's do enhance growth, the main effects are normally enhanced utilisation of feed (Dibner and Richards, 2005). Even though there remain many questions surrounding the working of AGP's, these substances have been used as feed additives in low concentrations (Hardy, 2002) for many years in commercial animal production in order to enhance growth and improve feed utilisation. Inclusion levels are in the parts per million (ppm) range. Various studies used concentrations from 4ppm to 200ppm (Feighner and Dashkevicz, 1987), 1000ppm (Gunal *et al.*, 2006), 20ppm and 60ppm (Engberg *et al.*, 2000), 40ppm (Manzanilla *et al.*, 2004) and 13.6ppm (Butaye *et al.*, 2005).

The continuous use of high amounts of antibiotics in low doses and over long periods in animal production has sparked concerns about the development of antibiotic resistance, especially resistance to therapeutic drugs used in the treatment of human cases (Hardy, 2002). AGP's have been used in animal production for more than 30 years in Europe and it is estimated that more than half of all antibiotics are used as growth promoters (Wegener *et al.*, 1999). A study done on chickens and pigs showed that there is a large association between the use of the AGP Avoparcin and the development of highly resistant *Enterococcus faecium* against the drug vancomycin (Bager *et al.*, 1997). This is a troublesome result due to the fact that *Enterococci* bacteria were responsible for the third most cases of nosocomial blood stream infections in a study in the USA (Jones *et al.*, 1997), thereby indicating the danger associated with development of drug resistance by bacteria. In an effort to curb the development of antimicrobial resistance the European Union (EU) has banned all antibiotics used for growth promotion purposes in animal production (Anadón *et al.*, 2005), effective since 2006.

There are reports of antibiotic resistance in aquatic bacteria due to injudicial use of antibiotics in aquaculture. Resistance to antibiotics has been reported among Gram-negative bacteria isolated from farmed catfish in Vietnam, where antibiotics are commonly used (Sarter *et al.*, 2007) and in *Aeromonas hydrophila* [a known human pathogen (Janda and Abbott, 1998)] isolated from cultured tilapia (Son *et al.*, 1997). 90% of bacteria isolated from a freshwater prawn hatchery where antibiotics are used prophylactically showed resistance to antibiotics (Hameed *et al.*, 2003), while bacteria isolated from Australian trout farms also displayed resistance to various antibiotics (Akinbowale *et al.*, 2007). Antibiotics have traditionally been used in combatting disease in aquaculture (Defoirdt *et al.*, 2007), either as therapeutic or prophylactic treatment. The use of these substances in aquaculture pose a risk to human

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health due to the development of drug resistance in certain organisms that are known human pathogens (Daskalov, 2006), and that could cause infection (Alderman and Hastings, 1998). Factors that contribute to this are the prophylactic and therapeutic use of antibiotics in aquaculture, the use of agents also used in human health and the persistent and toxic nature of these substances (Holmström *et al.*, 2003) in the environment. It is therefore imperative that the use of antibiotics in aquaculture should be reduced (Cabello, 2006) and to search for suitable alternatives.

Antibiotics do not seem to have the same consistent beneficial effect on growth in aquaculture as in terrestrial animals. Some investigators reported that antibiotics have no growth promoting effect and that animal performance tended to decrease instead (Rawles *et al.*, 1997; Toften and Jobling, 1997a, b). Antibiotics did increase digestibility of some nutrients in rainbow trout, although the effect this had on growth was not reported (Choubert *et al.*, 1991). Other reports indicated that AGP's do improve growth rate of carp and tilapia (Viola and Arieli, 1987; Viola *et al.*, 1990) and rainbow trout (De Wet, 2005). From this evidence it is clear that the use of AGP's in aquaculture is not as simple as in terrestrial animals.

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2.2   Substitutes for antibiotics

Following the ban of antibiotic growth promoters (AGP's) by the European Union in 2006, a large scale search has started to find substances to replace AGP's in animal production systems (Gunal *et al.*, 2006). Promising substances under investigation as candidates to replace AGP's include organic acids, probiotics, prebiotics and natural products (e.g. plant extracts). These substances differ in effect, mechanism and inclusion levels.

2.2.1 Organic acids and their salts

Various organic acids have shown promise as growth promoters in a variety of food animals, including pigs, poultry and fish (De Wet, 2005; Gauthier, 2005; Øverland *et al.*, 2000) and as substances that could regulate rumenal fermentation (Castillo *et al.*, 2004; Khampa and Wanapat, 2007) with implications on animal health and productivity. Many studies regarding use of organic acids have been done on swine, although the effects of acids and salts are not limited to pigs. In a study done on piglets, growth, average daily feed intake and FCR was improved and post-weaning oedema disease was reduced compared to a negative control. The experimenters concluded that the acids tested (lactic and citric acids) should be used as substitutes for antibiotics as feed additives whenever antibiotics are not permitted (Tsiloyiannis *et al.*, 2001a). In a similar experiment six different acids (propionic, lactic, formic, malic, citric and fumaric acids) led to significantly improved feed intake over a negative control diet in piglets during an outbreak of post-weaning diarrhoea. Of all treatments, lactic acid consistently performed best in this particular study (Tsiloyiannis *et al.*, 2001b). Although the benefits of organic acids seem to more pronounced in piglets, it has been shown that acids enhances growth during both grower and finisher periods (Partanen *et al.*, 2002). It was also found that the addition of sorbate enhanced the efficacy of formic acid to act as a growth promoter during finisher periods, while there was no statistical difference in growth between the formic acid-sorbate blend and pure formic acid during the grow out period (Partanen *et al.*, 2002).

The effects of organic acids have not been studied to any great extent in aquaculture and literature is scarce, but it has been shown that these substances can have growth promoting effects in fish, although some results are contradictory. A study done on rainbow trout (*Onchorynchus mykiss*) fed a commercial aquaculture acid supplement containing a mixture

of formic acid, ammonium formate and sodium formate on diatomaceous earth carrier and coated with sorbic acid (GrowHow, 2007), at a supplement level of 1.5%, resulted in significant improvement in growth rate when compared to a negative control, while no statistically significant difference was found between the treatment and a positive control containing AGP's (De Wet, 2005). Studies done on Atlantic salmon (*Salmo salar*) found that dietary supplementation of organic acids had no effect on growth (Bjerkeng *et al.*, 1999; Gislason *et al.*, 1994). No growth effects were seen upon dietary addition of citric acid to diets of rainbow trout, but the acid improved nutrient availability to the fish (Vielma *et al.*, 1999).

The question as to the mechanism by which organic acids and their salts are able to enhance growth in various animals has not been answered yet (Knarreborg *et al.*, 2002; Partanen and Mroz, 1999; Schöner). Various hypotheses have been put forth for the working. These include purely bacteriocidal activity, where the acids/salts are toxic to microbes, a pH effect which in turn has more possible methods of working, or the acids can act as an energy source to the host. It was shown that benzoic acid and to a lesser extent fumaric acid both have a clear bacteriocidal effect towards lactic acid bacteria. Benzoic acid was found to be toxic to coliform bacteria as well, and superior in this regard to a number of other acids (propionic, formic, butyric, lactic and fumaric acid) tested in this experiment. It was further found that pH significantly influenced the amount of viable coliform bacteria (Knarreborg *et al.*, 2002). pH can affect intestinal bacteria and animals in different ways. Firstly, the acids may decrease the gut pH to conditions unfavourable to most bacteria [which in turn means either selective colonisation (Knarreborg *et al.*, 2002) or overall lower levels (Øverland *et al.*, 2007; Tsiloyiannis *et al.*, 2001b) of colonisation of the gut of animals], or leads to better nutrient digestion, absorption and utilisation by the animal (Schöner). An alternative mechanism associated with the property of organic acids to stay undissociated at lower pH levels (depending on the pKa of the specific acid) has also been proposed. In undissociated form, organic acids are generally lipophylic and can easily diffuse across cell membranes into the cytoplasm of micro-organisms, where it can accumulate and/or dissociate (depending on the pH of the cytoplasm and the pKa of the specific acid), causing disruption of cell enzyme systems and nutrient transport (Farhi *et al.*, 2006; Partanen and Mroz, 1999). One question not answered satisfactorily is why under certain conditions the acid salts seem to improve results when mixed with acids (Partanen *et al.*, 2002). The mechanism of the working of the salts cannot be a lowering of pH. On the contrary, depending on the pKa of the particular acid and the pH of the medium in which the molecule is suspended, the salt may cause an increase in pH due to thermodynamic equilibrium considerations (Chang, 2002). A possible explanation for this could be that the acid salts

dissociate into their respective ions, the anions acquire protons, diffuse across cell membranes and then act as described above, lending some credibility to this mode of action. Finally, it has also been proposed that the organic acids can act as an additional energy source for the host animal, which may lead to improved growth if the acids are present in sufficient amounts (Partanen and Mroz, 1999; Sawabe *et al.*, 2003). It has been shown that certain short chain fatty acids (SCFA) can play an important role in the colon of humans and other mammals as substances that make a contribution in the health of the colon, as energy sources for the colonic mucosa (Royall *et al.*, 1990; Scheppach, 1994) and as substances that are important in nutrition (Roediger, 1980; Scheppach, 1994). Butyrate has been found to be an important fuel for colonocytes in the human colon (Roediger, 1980), and has also been used as successful treatments for colitis in humans (Scheppach *et al.*, 1992). Acetic, propionic and butyric acid are viewed as the acids that are most important to the human colon, as these are the products of bacterial fermentation (Wong *et al.*, 2006). There is a large body of literature dealing with the role of SCFA's in the human and mammalian colon, but no similar literature could be found for aquaculture species in general and abalone in particular.

Mixed acids generally yield better results than single acids due to substance specific dissociation properties which leads to action throughout the different regions in the gut (Hardy, 2002). Evidence of this was found in pigs (Partanen *et al.*, 2002). There is also some evidence that the efficacy of acids could be enhanced when combined with other products. In an experiment where plant extracts (comprising of 5% carvacrol, 3% cinnamaldehyde and 2% capsicum oleoresin extracted from oregano, cinnamon and Mexican pepper respectively) and formic acid were added to a diet for piglets and tested against a control where only formic acid was used, it was found that the effects of formic acid and plant extracts were additive and yielded better results than the control diet (Manzanilla *et al.*, 2004), while another study concluded that plant extracts from *Rutaceae* and various organic acids (citric, formic, lactic, propionic acids) are synergistic in their effects against microorganisms (Calvo *et al.*, 2006).

Inclusion levels of organic acids and their salts are generally much higher than for antibiotics. Levels ranging from 0.5% to 1.8% have been used in several studies done on pigs (Canibe *et al.*, 2001; Manzanilla *et al.*, 2004; Øverland *et al.*, 2000), 1% for use in turkeys (Çelik *et al.*, 2003) and up to 1.5% in rainbow trout (De Wet, 2005). A commercial feed acid manufacturer recommends inclusion levels ranging from 0.2% to 1.2% (Kemira, 2007).

2.2.2 Probiotics

Probiotics have been reported to have various beneficial effects in a wide variety of host organisms (Macey and Coyne, 2005), including improved feed utilisation, contribution to enzymatic digestion, inhibition of pathogenic organisms, anticarcinogenic and antimutagenic effects, increased immune response and improvement in growth rate (Verschuere *et al.*, 2000). Probiotics have been defined as: "Microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health" (Gatesoupe, 1999) and as "Living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" (Ouwehand *et al.*, 2002).

Aquaculture systems seem to benefit greatly from probiotic treatment. Improved SGR, disease resistance and an immunostimulatory effect (leading to enhanced survival after infection with *Vibrio anguillarum*) was observed in the South African abalone *Haliotis midae* when the diet was supplemented with a mixture of three putative probionts, consisting of one bacteria, *Vibrio midae*, and two yeasts *Cryptococcus* sp. and *Debaryomyces hansenii*. The SGR of animals with a mean length of 20 mm was enhanced by 8%, while the SGR of animals with a mean length of 67 mm improved by 34% due to the probiotic treatment in this trial (Macey and Coyne, 2005; Macey and Coyne, 2006). A probiotic treatment consisting of equal amounts of *Bacillus* species and photosynthetic bacteria improved the growth performance of the commercially important shrimp *Penaeus vannamei,* with the best treatment leading to a 20.2% improvement in growth when compared to a negative control (Wang, 2007). Another study found that a *Bacillus* species added to the diet of Indian white shrimp *Fenneropenaeus indicus* significantly reduced mortality and possibly played a role in the observed improvement in SGR by 2.9% and FCR by 12.6% (Ziaei-Nejad *et al.*, 2006). Mortality due to vibriosis was significantly reduced in rainbow trout (*Onchorynchus mykiss*) by the use of a strain of *Pseudomonas fluorescens* as a probiotic (Gram *et al.*, 1999). In humans, reduction of atopic disease has been demonstrated in infants (Kalliomäki *et al.*, 2001; Ouwehand *et al.*, 2002) and probiotics have been used to treat various gastro intestinal diseases (Ouwehand *et al.*, 2002).

There is still a large amount of uncertainty as to the mechanisms by which probiotics achieve their effects (Verschuere *et al.*, 2000). Various possibilities have been proposed, including prevention of pathogen colonisation, stimulation of the immune response, health benefits to the host due to release of substances by the probiotics, antagonism toward pathogens

(Olafsen, 2001; Vijayan *et al.*, 2006) and improved feed utilisation due to improved enzymatic activity (Macey and Coyne, 2005). Efficacy of probiotics in aquaculture has been attributed to two possible effects: direct improvement of animal health (by the various mechanisms mentioned above) or the improvement of water quality parameters, but yet again the exact modes of action remain largely unknown (Irianto and Austin, 2002). In one investigation, five different strains of *Bacillus* improved survival (after infectious outbreak of *Edwardsiella ictaluri*), net production per hectare and FCR of channel catfish (Queiroz and Boyd, 1998), while *Bacillus* was also shown in another study to protect shrimp from infection with *Vibrio* species and significantly improve survival (Moriarty, 1998). In the catfish study the effects of the added probiotics on the water quality was investigated and although the treatments succeeded in improving production performance, this success could not be attributed to improved water quality parameters. In the second study the success was attributed to the inhibition of *Vibrio* species by the added *Bacillus* and not to water quality enhancement. Much research is still necessary in this regard.

The range of currently known probiotics is large and includes various micro organisms. In aquaculture, organisms that are examined as potential probiotics include bacteria [both Gram positive (*Bacillus, Carnobacterium, Lactobacillus, Lactococcus*) and Gram negative (*Aeromonas, Pseudoalteromonas, Pseudomonas, Roseobacter* and *Vibrio*) organisms (Gatesoupe, 1999)], bacteriophages, micro algae and yeasts (Irianto and Austin, 2002). Mostly, practical probiotics in aquaculture are either lactic acid bacteria, *Vibrio*, *Bacillus* or *Pseudomonas*, although there are other genera that are also used (Verschuere *et al.*, 2000). In humans, the species include bacteria (*Lactobacillus, Bifidobacterium Propionibacterium, Bacillus, Escherichia, Enterococcus* spp.) and yeast e.g. *Saccharomyces* sp. (Ouwehand *et al.*, 2002).

It is generally assumed that organisms already showing dominance in a host or living in close association with the host are good candidates for probiotics, as they are already well adapted in the host and thereby will be able to exclude pathogens by competition (Verschuere *et al.*, 2000). There is some evidence that this assumption is valid. Two yeasts and a bacteria isolated from the digestive tract of the South African abalone *Haliotis midae* were demonstrated to have beneficial effects on growth and disease resistance (Macey, 2005; Macey and Coyne, 2005), while Maeda *et. al* stated that bacteria that improve growth rate of prawns usually live in close association with the host (Maeda *et al.*, 1997). Improved growth and survival was seen in shrimp after probiotic treatment with bacteria isolated from shrimp ponds (Rengpipat *et al.*, 1998), while growth performance was significantly enhanced in common carp by a bacteria isolated from carp ponds (Wang and Zirong, 2006)

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2.2.3 Prebiotics

Prebiotics, defined as "A nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health", (Gibson and Roberfroid, 1995) are substances that can be added to the diet of animals, including aquaculture organisms. Information about the effects of prebiotics in aquaculture is very limited as the whole concept of functional feeds (formulating diets that provide more to the animal than its basic nutritional needs) is rather novel in this industry (Li and Gatlin, 2004). A limited number of very recent aquaculture trials have demonstrated the potential for these substances to enhance performance and health of aquatic animals. Supplementation of mannan oligosaccharides (MOS, derived from the outer cell walls of the yeast *Saccharomyces serevisiae*) at 2% and 4% significantly improved growth rate and feed intake of European sea bass (*Dicentrarchus labrax*), while simultaneously activating the immune system and resistance to intestinal bacterial infection (Torrecillas *et al.*, 2007). In another trial done on hybrid sea bass (*Morone chrysops × M. saxatilis*), feed efficiency, immune response and resistance to bacterial infection was improved significantly by addition of a commercial prebiotic (Grobiotic[TM] AE) to fish diet (Li and Gatlin, 2004). Addition of 3 g/kg MOS to Tiger shrimp (*Penaeus semisulcatus*) diet resulted in significantly higher body mass and increased survival after a growth trial lasting 48 days (Genc *et al.*, 2007). Bio-Mos®, a commercial prebiotic, supplemented at 2g/kg significantly improved final weight, FCR and immune capacity of rainbow trout (*Salmo gairdneri irideus* G.) and common carp (*Cyprinus carpio* L..) (Staykov *et al.*, 2005). These studies all point to the potential off these substances to be used as feed additives in the aquaculture industry, although no studies have been conducted on other culture organisms than fish.

Prebiotics have their effect by reaching the intestine without being digested by the host, where it is selectively fermented (mainly to organic acids) by beneficial endogenous microbes and not by potential pathogens, which leads to a microbial gut composition beneficial to the host (Gibson and Roberfroid, 1995). Substances that are able to act as prebiotics include oligosaccharides (although not all non-digestible oligosaccharides show prebiotic action (Macfarlane and Cummings, 1999)), resistant starch, non-starch polysaccharides or dietary fibre and proteins and amino acids. Most investigations into prebiotics focus on oligasaccharides (low molecular weight carbohydrates (Mussatto and Mancilha, 2007)). The main end products of the fermentation of carbohydrates are volatile

short chain fatty acids (VSCFA), mainly butyric, propionic and acetic acids which can be metabolised further by the host in order to obtain energy (Cummings *et al.*, 2001; Manning and Gibson, 2004; Scheppach, 1994). The most commonly evaluated prebiotics are normally those that stimulate growth of lactic acid bacteria, mainly *Lactobacillus, Enterococcus* and *Bifidobacterium* species (Reid *et al.*, 2003a; Weese, 2002). It has been shown in animal and human trials that some oligosaccharides promote the growth of *Bifidobacteria* and that oligosaccharides have the potential to significantly alter the microbial composition of the gut (Gibson and Roberfroid, 1995; Kolida *et al.*, 2002; Reid *et al.*, 2003a). Oyarzabal *et. al* showed that *Salmonella*, a pathogen spread through poultry products, cannot ferment fructooligosaccharide (FOS, a potential prebiotic), while some lactic acid bacteria were able to utilise it as sole carbon source, producing lactic acid in the fermentation process. The authors concluded that FOS could act as a fermentative substrate that could lead to the exclusion of *Salmonella* due to the establishment of unfavourable growth conditions for the pathogen (Oyarzabal *et al.*, 1995). In pigs, an increase in *Bifidobacteria* coupled with an increase in production of VSCFA's was observed after the addition of galactooligosaccharide to their diet. (Tzortzis *et al.*, 2005). Another study reported essentially the same result: galactooligosaccharides significantly increased the numbers of *Bifidobacteria* and *Lactobacilli* and increased production of VSCFA's (Smiricky-Tjardes *et al.*, 2003). Research on the effects of prebiotics is increasing and should be done in conjunction with research on organic acids due to the fact that the main fermentation products of prebiotics are VSCFA's. Prebiotic research could provide valuable information as to the mode of action of VSCFA's in the gastro-intestinal tract of animals.

2.2.4 Natural plant extracts

Natural plant extract have shown potential as substances that could enhance growth and general animal performance in some animals, including fish. Triterpenoid saponins improved FCR and had a growth promoting effect in carp and tilapia at 150ppm and 300ppm respectively (Francis *et al.*, 2005). Trials done on chickens demonstrated that the natural plant alkaloids sanguinarine and chelerythrine led to better growth and meat yield when compared to a control group fed a diet with 10 ppm flavomycin, while alkaloids improved FCR and water consumption when compared to flavomycin (Butler, 2005). It was shown that addition of 400 mg/kg diet Anise oil led to significantly improved weight gain and FCR when compared to 10 mg/kg avilamycin as AGP (Ciftci *et al.*, 2005). Investigation of various essential oils as compounds that can affect rumenal fermentation have shown some promise, but results are variable and more research is needed in this area (Benchaar *et al.*, 2007)

2.3   *Microflora of abalone*

The endogenous microflora found in the digestive tract of the South African abalone *Haliotis midae* is known to be very diverse (Mouton, personal communication), which is confirmed by the study of Erasmus (Erasmus, 1996). The microflora consists of bacteria (Gram positive and Gram negative) and yeasts (Erasmus, 1996; Macey, 2005). Eleven different genera of bacteria were isolated from the gastro-intestinal tract of the South African abalone *Haliotis midae* (Erasmus, 1996), although no mention is made whether any yeasts were isolated. Macey (Macey, 2005) only studied the effects of three specific organisms (one bacteria and two yeasts), but gave no indication of the biodiversity of the microbial community in the abalone gut. It is generally known that the microflora associated with marine molluscs includes a wide variety of organisms. These animals are unique accumulators of specific microbes, leading to unique associations between certain animals and microbes (Romanenko *et al.*, 2006). A study done on bacteria isolated from *Anadara broughtoni*, a marine ark shell, yielded a total of 149 strains of bacteria from the genera *Bacillus*, *Paenibacillus, Saccharothrix, Sphingomonas, Aeromonas,* and *Saccharothrix* (Romanenko *et al.*, 2006). There is a need for studying the microbial diversity of marine organisms in order to understand the role they play in their host and to determine what effects an alteration of the microflora will have on the host animal, especially since it has been proposed that certain

bacterial species can be used in the biological control of aquaculture (Olafsen, 2001; Romanenko *et al.*, 2006).

 It has been shown that certain microorganisms can play a role in the digestion and general health of abalone (Erasmus, 1996; Macey, 2005; Sawabe *et al.*, 2003). Erasmus *et. al* concluded that digestion of complex polysaccharides by *Haliotis midae* may be improved by bacteria resident in the digestive tract due to excretion of exogenous enzymes in the gut. *Vibrio* and *Pseudomonas* species were generally the best at hydrolysing the tested polysaccharides (laminarin, carboxymethylcellulose (CMC), alginate, agarose and carrageenan) that are found in macroalgae (Erasmus *et al.*, 1997). Macey and Coyne demonstrated that microorganisms found in the gut of abalone can improve growth rate and disease resistance. Another study found that *Vibrio halioticoli* isolated from the gut of various abalones (*Haliotis. discus hannai, H. discus discus, H. diversicolor aquatilis, H. diversicolor diversicolor* and *H. midae*) could play a significant role in the digestion of the natural food of the abalone by fermenting alginate to produce acetic and/or formic acid. It was suggested that the fermentation products could contribute significantly to the energy metabolism of the host and that the bacteria could aid with digestion of alginate, which is found in the natural food of abalone (Sawabe *et al.*, 2003). The bacteria are able to produce acetic and formic acid from alginate under laboratory conditions and the authors concluded that it is possible for the bacteria to ferment alginate to produce the same products in the gut of abalone, due to prevalent conditions in the gut.   Another study also stated that *Vibrio halioticoli* may be a significant symbiotic partner in digestion of alginate into volatile short chain fatty acids that abalone could utilise as an energy source (Sawabe *et al.*, 2002) and a symbiotic association between *Vibrio gallicus* (isolated from the gut) and the abalone *Haliotis tuberculata* was hypothesized (Sawabe *et al.*, 2004b). *Vibrio* bacteria are commonly associated with abalone.   Although these bacteria are known to be pathogens of abalone under certain circumstances, other investigations show that these organisms can have a positive influence on the health of host abalone. *Vibrio midae* was confirmed as a probiotic organism in *Haliotis midae* (Macey and Coyne, 2005) and that the organism readily colonises the digestive tract of the host (Macey and Coyne, 2006).

Various bacteria have been known to cause disease in abalone, but some evidence exists that some of these organisms are opportunistic pathogens and that virulence can be increased by certain environmental factors.   *Vibrio alginolyticus* and *Clostridium lituseberense* are known pathogens of the South African abalone *Haliotis midae* (Dixon *et al.*, 1991).   Various *Vibrio* species have been implicated in diseases of a number of other abalone species. *Vibrio parahaematolyticus* was confirmed as a pathogen to *Haliotis*

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diversicolor supertexta postlarvae (Cai *et al.*, 2006a), *Vibrio charchariae* was established as a pathogen of the European abalone *Haliotis tuberculata* (Nicolas *et al.*, 2002) and *Vibrio parahaematolyticus* was found to be pathogenic to *Haliotis diversicolor supertexta*, but the authors noted that it seemed to be an opportunistic pathogen (Liu *et al.*, 2000). In another study it was demonstrated that both *Vibrio parahaematolyticus* and *Vibrio alginolyticus* are pathogenic to *Haliotis diversicolor supertexta* and that pathogenicity increased as the water temperature increased to temperatures warmer than the optimum for the abalone (Lee *et al.*, 2001). Both these studies indicate that disease outbreak could be triggered by sub-optimal production conditions. Other bacterial pathogens reported in abalone include *Klebsiella oxytoca* and *Shewanella alga* in *Haliotis diversicolor supertexta* (Cai *et al.*, 2006a; Cai *et al.*, 2007) *Candidatus Xenohaliotis californiensis* in various American abalone species (Friedman, 2002), as well as in European abalone *Haliotis tuberculata* (Balseiro *et al.*, 2006).

It seems that *Vibrio* bacteria are commonly associated with abalone and a number of other sea organisms, but that this relationship is very complex. It is important to study these associations between hosts and micro-organisms in order to better comprehend the interaction of the two, as this understanding can be very important in predicting under what circumstances certain bacteria will become virulent, and how to prevent this from happening. *Vibrio* bacteria are often isolated from abalone and other marine animals and new species are continuously being identified in a variety of host animals. Examples of recent newly identified organisms include *Vibrio midae* from the South African abalone *Haliotis midae,* (Macey, 2005) *Vibrio neonatus* sp. nov. and *Vibrio ezurae* sp. nov. form Japanese abalones *Haliotis discus discus, H. diversicolor diversicolor* and *H. diversicolor aquatilis* (Sawabe *et al.*, 2004a), *Vibrio gallicus* sp. nov. from the French abalone *Haliotis tuberculata* (Sawabe *et al.*, 2004b) and *Vibrio inusitatus* sp. nov., *Vibrio rarus* sp. nov., and *Vibrio comitans* sp. nov. from the abalones *H. rufescens, Haliotis discus discus, H. gigantea* and *H. madaka* (Sawabe *et al.*, 2007), *Vibrio gigantis* sp. nov. from the haemolymph of oysters (*Crassostrea gigas*) (Le Roux *et al.*, 2005) and *Vibrio coralliilyticus* sp. nov. from the coral *Pocillopora damicornis* (Ben-Haim *et al.*, 2003). Other examples of Vibrio association with sea animals include *Vibrio alginolyticus, Vibrio parahaemolyticus, Vibrio cholerae, Vibrio vulnificus* and *Vibrio harveyi* with blue mussels *Mytilus edulis* (Lhafi and Kühne, 2007), *Vibrio fischeri* that colonises the light organ of the bobtail squid *Euprymna scolopes* (McFall-Ngai and Montgomery, 1990; Ruby and McFall-Ngai, 1999), while *Vibrio tapestis* has been isolated from Atlantic halibut *Hippoglossus hippoglossus* (Reid *et al.*, 2003b) and *Vibrio vulnificus* was found in marine and brackishwater fish (Thampuran and Surendran, 1998).

2.4 Conclusions

From the literature surveyed it is possible to make the following observations and conclusions:

Antibiotic growth promoters (AGP's) have been used as feed additives to enhance animal performance for a number of years and have proved to be very effective, even though the precise mechanism(s) by which these substances work have not been established. The development of antibiotic resistance in microbes known to be human pathogens resulted in legislation banning the use of AGP's in many countries, including the whole European Union, due to the fears that these bacteria may develop resistance to drugs used in human treatment. In order to retain profitability and productivity, it is necessary to find replacements for AGP's in animal production systems. Various substances have shown promise as candidates for replacing AGP's e.g. organic acids and acid salts, probiotics, prebiotics and natural plant extracts. These substances have various advantages over AGP's: they have the same effect as AGP's, yet they do not lead to antibiotic resistance to therapeutic drugs and they do not leave unwanted residues in animal products.

Antibiotic resistance has also been reported in bacteria (including known human pathogens) isolated from aquaculture systems in which antibiotics have been used as therapeutic and prophylactic treatments. Conflicting reports regarding the efficacy of antibiotics as growth promoters in aquaculture also create doubt as to whether their use is justified in this capacity. Both these two factors strengthen the need to find substitutes to antibiotics, especially in an aquaculture context.

Organic acids are able to enhance the performance in some animal production systems when incorporated as feed additives, comparable to that of AGP's. Although most literature on the effect of organic acids are on swine, it is clear that organic acids can have performance enhancing effects in poultry, ruminants and fish too. There are a number of proposed mechanisms for the working of organic acids, but the exact mode of action is yet to be established. The effects of acids have not been studied extensively on aquaculture organisms, but the potential clearly exists to make a significant impact on aquaculture feed technology. It seems that there is a synergistic effect when more than one acid/acid salt is used in a treatment, or when acids are combined with some natural products. The inclusion levels of organic acids are generally much higher than that of AGP's.

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Probiotics have proved to be beneficial in a number of ways in a variety of organisms. These organisms can improve growth, feed utilisation and general health and disease resistance in the host. Reports indicate that various aquaculture organisms can benefit from the application of probiotics e.g. abalone, shrimp and fish, but like in the case of both AGP's and organic acids, there are still many remaining questions regarding the mechanism(s) of the probiotic organisms. The range of potential probiotic organisms is large but practical probiotics in aquaculture normally include *Bacillus*, *Vibrio* and *Pseudomonas*. It is generally assumed that dominant microorganisms already associated with a host species are good candidates for probiotics as they are already well adapted to conditions.

Prebiotics added to feeds have shown improved growth performance and improved immunity in a few fish species, but no aquatic organisms except fish have been investigated thus far. These substances work by remaining undigested until it reaches the intestine of the host, where they are fermented to volatile short chain fatty acids that inhibit pathogens, lead to favourable intestinal microbial composition and/or can be utilised as energy source by the host.

The endogenous microflora of the abalone and marine molluscs in general is known to be very diverse. It is necessary to study the interactions and associations of the host and microflora in order to comprehend what benefits can be gained by altering the composition of the microbial community in the gut. Certain microbes can contribute to the digestion and general health of abalone, yet under certain conditions bacteria commonly associated with the host organism can act as opportunistic pathogens and cause disease. Bacteria from the genera *Vibrio* seem to commonly associate with abalone and can act as pathogens and beneficial organisms in abalone and a number of other aquatic organisms, depending on conditions. The relationship between aquatic animals and microbes is very complex and the interaction is poorly understood at this stage, therefore warranting further investigation.

The following hypotheses are being put forward from the literature survey:

1. Organic acids can act as growth promoters in abalone.
2. Organic acids can equal the performance of AGP's in abalone culture.
3. Organic acids and their salts are equally effective at promoting growth in abalone.
4. The mechanism by which organic acids work is microbial in nature.
5. Organic acids alter the intestinal microbiology of abalone.

3. PROBLEM STATEMENT

The current production time of South African abalone is 3 to 4 years which is very long compared to most other intensively reared aquatic animals. The production technology has been established, but there are still many areas in which the industry seeks to improve on, including maximising growth rates in order to cut production time or cultivate larger animals for the market. Nutrition science is one of the fields that is still developing in abalone culture, seeking to optimise production rates by improving feeding regimes, feed utilisation, growth rates and general animal health. One of the ways in which this can be achieved is by the addition of feed additives that enhance animal performance by a variety of mechanisms which are not always fully understood. One of the feed additives most used to enhance animal production is in-feed antibiotics. Antibiotics have become very unpopular, especially in developed countries due to evidence that the use of these substances cause drug resistance in many species of bacteria, including human pathogens. Because of the risk this poses to human health, the European Union (EU) has banned the use of all antibiotics in animal feeds since 2006, causing a large scale search for alternative substances to enhance growth and improve animal health. One group of substances is receiving a lot of attention as possible substitutes of AGP's: organic acids and their salts. It has been shown in various animals that organic acids and their salts can have certain health benefits and growth promoting effects when used as feed additives but it has not been investigated whether the same effects can be achieved with the addition of organic acids and their salt to abalone feed.

This investigation is an effort to determine the effects that organic acids may have on abalone when administered as feed additives. The important questions are:

1.   What is the effect of organic acids on the production parameters of abalone?

2.   How do the organic acids compare to a commercial antibiotic growth promoter (AGP)?

3.   If these substances affect production, what are the mechanisms involved in the working?

4.   What is the significance of these effects (if any) on the abalone aquaculture industry?

The AGP was chosen on the basis that it is often used in the poultry and pig industries and was readily available. The other treatments were chosen in a specific way to attempt to establish a mechanism of working of the organic acids. It was decided not to use single acids in order to be able to test a higher number of different acids simultaneously, as there is a large variety of acids that are reported to have beneficial effects, further to test one combination of acids and to compare those results with a treatment containing the salts of the same acids in order to establish whether the mechanism is linked to the acid *per se*, to the anion of the acid or to neither, and lastly to choose substances that are known to have antimicrobial activity, in order to establish whether the modes of action might be linked to antimicrobial effects of these molecules. Microbiological monitoring of the intestinal microflora of the abalone was done in order to determine whether the effects were microbial or not. In an attempt to determine whether the mechanism is linked to an energy effect the last treatment was chosen as a mixture of acetic and formic acid, as literature suggested that these acids could act as energy sources to abalone. Finally, the inclusion levels of all substances were set at levels that have shown good results in other organisms in order to ensure that if these substances do have any effects on the abalone, it will be detectable. In a fundamentally scientific study it would be more correct to determine at which levels the substances do have an effect etc., but due to time constraints this could not be investigated in this study.

It is further important to recognize that the effects obtained with feed additives during optimal production conditions may differ when compared to stressful conditions (refer to Section 2.1). It is therefore important to determine whether performance of abalone can be enhanced during sub-optimal production conditions by the different treatments used in this study. In order to accurately determine this, it is necessary to cause controlled, sub-optimal culturing conditions in the system used for the study. The only way in which this could be done at the specific laboratory facility was to manipulate water temperature, as it is known that abalone have a preferred optimal water temperature range, and if animals are subjected to water temperatures outside of this range, they experience stress and production performance suffers as a result. The effects of the different treatments were tested by subjecting the abalone to sub-optimal, raised water temperatures.

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If there are any benefits in adding organic acids and salts to abalone feed, it is necessary to investigate whether these effects can be realised under practical production conditions. Practical on-farm production is subjected to the same temperature and water quality variations experienced on the coast, which may affect the performance of these additives. A large part of the current investigation was conducted under controlled laboratory conditions, but these conditions cannot be used for commercial culture of *H. midae* as the costs would be prohibitive. In order to investigate whether the different feed additives would be effective under non-controlled practical conditions, a separate trial was done.

The main research questions to be answered in this study are stated as follows:

1. Do organic acids and their salts have a growth promoting and/or microbial effect in the South African abalone?

2. If the abovementioned substances do have any effects, how large are these effects?

3. If the substances have any effects, what are the mechanism(s) of these effects?

4. MATERIALS AND METHODS

4.1 Acidification of feed and leaching experiment

The effect that different acids have on the pH of the abalone feed used in the trials was investigated by adding various acids to the feed during preparation, and measuring the resulting pH. Three different acids were tested: tartaric acid, sulphuric acid and hydrochloric acid. The effect of seawater on the pH of the feed was determined by immersing the prepared feed in seawater and measuring the pH at different time intervals.

Abalone feed was prepared as a gel by mixing 1 part of (feed + acid) with 5 parts of boiling water and allowing it to set in metal containers. The acid was added so that the concentration was 1% of the (feed + acid). The pH of the different preparations was measured with a pH probe, after which the preparations were immersed in seawater. The pH of these immersed preparations was measured at various time intervals to determine any changes in pH.

4.2 Experimental setup

Trials were conducted at Irvin & Johnson Abalone farm in Gansbaai, South Africa over a period of 5 months, from 18 December 2006 to 21 May 2007. Two different systems were used for the trials, one in which water temperature could be controlled and one in which water temperature could not be controlled.

The system where water temperature could be controlled consisted of 40 containers of 20 litres each inside the farm research laboratory. Containers were continuously supplied with water and aeration. Incoming water was filtered to 25 µm and water temperature was kept constant. Shelter was provided for the animals by halved pvc pipes in the containers and containers were covered with nets to prevent animals from escaping (Figure 1).

Figure 1 Container setup for controlled conditions experiment. Containers were covered with nets to prevent animals from escaping, while continuously supplied with water and aeration.

The uncontrolled production system consisted of 29 containers where conditions were identical to those in the commercial production section of the facility. Containers were continuously supplied with normal unfiltered seawater and aeration. Shelter was provided by modified halved pvc pipes (Figure 2).

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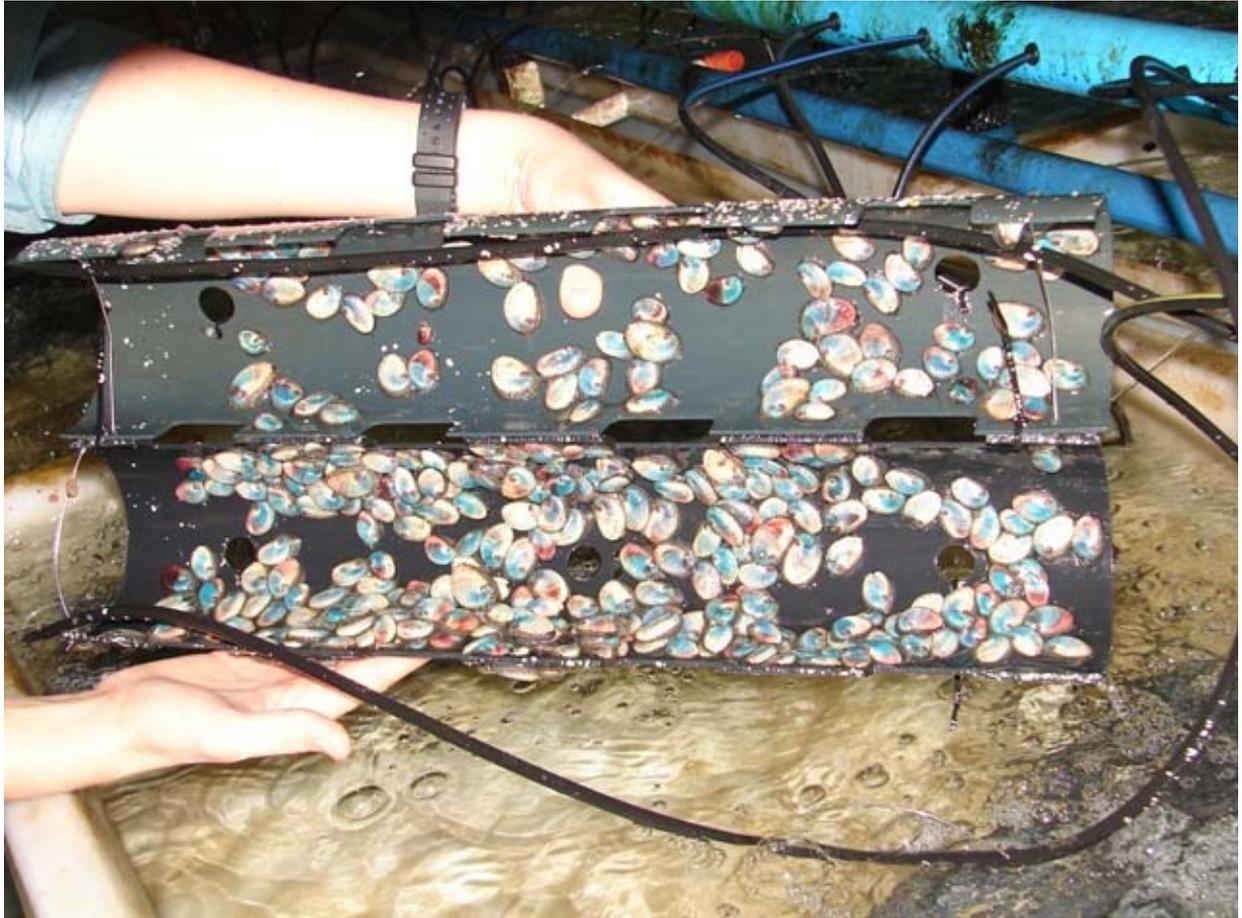
Figure 2 Shelter for animals in production conditions was provided by modified pvc pipes.

4.3 Feed preparation

The basal feed used in all trials was kindly sponsored by Aquanutro, South Africa. Acids and acid salts were added to this feed to have a final concentration of 2% by weight. Glacial acetic acid (99.8% pure) and formic acid with purity of 98% - 100% was obtained from Merck. Food grade benzoic acid was obtained from Warren Chem Specialities, food grade sodium benzoate was obtained from Protea Chemicals and food grade sorbic acid and potassium sorbate was obtained from Savannah Fine Chemicals. Antibiotic growth promoter (Avilamycin) obtained from local feed industry was added to have a final concentration of 30 ppm. The feed was extruded and then dried at 70ºC – 80ºC in a drying oven. Drying was monitored every hour until no further moisture loss occurred. In all cases, the drying process did not take longer than 4 hours. The proximate analysis of the basal diet had is shown in Table 1. Analysis was done by Resia Swart at the Department of Animal Sciences at the University of Stellenbosch

Table 1 Proximate analysis of basal diet used in all trials.

Proximate analysis of control	
Component	**%**
Dry Matter	100.00
Ash	14.67
Crude protein	48.89
Crude Fibre	3.58
Crude Fat	8.97

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4.4   Growth trials and stress experiment

Three different trials were conducted to monitor the growth performance of the test animals subjected to different treatments and under different conditions. The first trial was conducted under optimally controlled water temperature conditions and was run for a total of 124 days. Water temperatures within the range of 12ºC to 20ºC were found to be physiologically optimal for South African abalone (Britz *et al.*, 1997). Water temperature for this trial was set at 16.5 ºC and kept constant. 30 animals were placed randomly in each of the 40 containers in the laboratory facility. The number of animals was chosen such that the density of the animals in each container would not affect the production parameters (Lize Schoonbee, personal communication), and was based on previous on-farm experience at the particular facility.

At the initiation of the experiment, all animals were measured and weighed and the mean weight and length ± standard deviation (SD) was 2.1g ± 0.77 and 23.4mm ± 2.72 respectively. Shell length was measured along the longest axis to 2 decimal places using callipers (Figure 3) and animals were weighed accurately to 2 decimal places using a laboratory scale. All measurements in the laboratory were conducted while animals were anaesthetised with $MgSO_4$ in order to minimise injuries and stress due to handling.

Figure 3 Measurement of total shell length using callipers, accurate to two decimal places. Shell length is measured along the longest axis of the shell.

Containers were numbered and designated a specific treatment according to colour coded tags. Treatment NC acted as a negative control with no additives while treatment PC was the positive control containing an antibiotic growth promoter used in pig and poultry production. All containers were given the same amount of feed twice weekly. On every feeding day containers were emptied, cleaned with a brush and the remaining feed was collected using a sieve and weighed as is. Once a month animals were measured and weighed and one animal was removed from each container for characterisation of gut microflora. The different treatments are given in Table 2.

Table 2 Summary of additives used in the different treatments and positive control. All percentages are given in weight %

Treatment	Tag colour	Additives
NC	White	none
AF	Orange	1% acetic acid + 1% formic acid
PC	Green	30 ppm Avilamycin
SBPS	Blue	1% sodium benzoate + 1% potassium sorbate
BS	Red	1% Benzoic acid + 1% Sorbic acid

The controlled stress experiment was initiated immediately after completion of the controlled optimal conditions growth trial, using the same setup and animals that were used during the previous experiment. It was run for a total of 28 days. No animals were moved between containers and no animals were added to or removed from containers between experiments. Water temperature was raised to 20.5ºC (the maximum temperature that the laboratory water heaters could maintain) and kept constant. Breakdown of physiological processes at temperatures higher than 20ºC was suggested (Britz *et al.*, 1997) and on-farm experience showed that animal performance starts to deteriorate at 20ºC and above, which is an indicator of stress (Lize Schoonbee, personal communication). Animals were weighed and measured after 2 weeks at elevated water temperature, but no animals were removed for microbiology. After a further 2 weeks, the animals were weighed and measured for the last time and one animal was taken from each container for characterisation of intestinal microflora. The experiment was terminated after this and animals were moved to the commercial section of the farm.

The production conditions experiment was done in a section where animals were subjected to identical conditions to those in the commercial section of the farm. Animals were stocked at approximately 1000 animals per container and mean animal weight and length ± SD was 0.54 g ± 0.22 and 14.99 mm ± 2.05 respectively. Length and weight measurements were done in the same manner as in the other two experiments. Containers were numbered and designated a specific treatment according to colour coded tags. The feed used in this experiment was identical to that used in the other experiments. The experiment took place over a period of 90 days in total.

Animals were weighed monthly but due to the smaller animals size they were not anaesthetised prior to handling. 20 animals were sampled from each container for each measurement and replaced in the same container after all measurements were completed. No animals were taken from this experiment for microbiology work. Feeding was done twice weekly but remaining feed could not be recovered and the assumption was made that all feed given was consumed by the animals. The person overseeing the system is an experienced feeder and adjusted the amount of feed given so that the feed remaining from the previous feeding was minimal.

Calculation of the different parameters used for evaluation was done in the following way:

Specific growth rate (SGR) over a growth period starting at t_0 and ending at t_1

$$SGR = \frac{\ln\left(\frac{W_1}{W_0}\right)}{t_1 - t_0} \times 100 \qquad (d^{-1}) \qquad (1.1)$$

where W is animal weight and t is in days.

SGR is a non-linear calculation of growth rate. It has been shown that growth of *Haliotis midae* is non linear and can be modelled by the Von Bertalanffy growth function (Tarr, 1995), while growth of the abalone *Haliotis roei* has been modelled using a non-linear Gompertz growth function (Hancock, 2004). Reporting linear growth rates as a simple increase in weight or length per day are therefore not technically correct, especially if growth over very long periods is reported. *H. midae* is a very long lived species and can reach an age of over 30 years in the wild (Sales and Britz, 2001). Britz stated that growth in terms of length of *H. midae,* however, does not deviate much from a linear model for total animal length < 70 mm (Britz *et al.*, 1997). Industry also reports growth rates for both length and weight in linear terms and refers to these parameters as 'apparent growth rates' (Dr. Lourens de Wet, personal communication). In trials that are done during a short period when compared to the total life span of the abalone, the use of linear growth rates may be applicable but the use of these linear models should first be validated.

As can be seen from Figure 4, drawn from growth parameters calculated for the Von Bertalanffy growth curve by Tarr (Tarr, 1995), the linear approximation of length increase seems valid.

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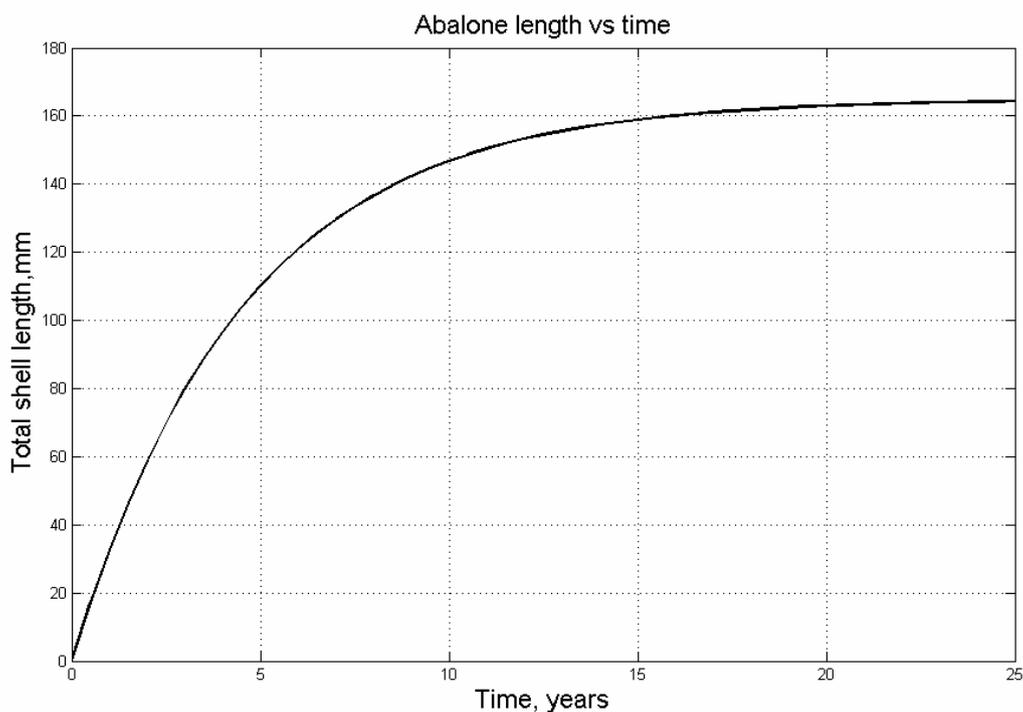
Figure 4 Von Bertalanffy growth curve of *Haliotis midae,* constructed with parameters determined by Tarr, 1995. Parameters values are: K = 0.22 y^{-1}, t_0 = 0, L_∞ = 165 mm.

The Von Bertalanffy growth curve is a non-linear, asymptotic growth function of the form

$$L_t = L_\infty \left(1 - e^{-K(t-t_0)} \right) \tag{1.2}$$

where L_t is animal length at time t in mm, L_∞ is the theoretical length in mm that an animal will reach if it grew for an infinite time, K is a growth parameter, y^{-1}, and t_0 is the theoretical time (assumed to be 0 in this instance) at which an animal would have a length of 0 mm if it had always grown according to the function (Gröger, 2001).

The linear growth rate based on animal length for a period starting at t_0 and ending at t_1 was calculated as:

$$AGRL = 1000 \times \left(\frac{L_{t_i} - L_{t_0}}{t_i - t_0} \right) \quad (\mu m / day) \tag{1.3}$$

Abalone weight is related non-linearly to the total length of the animal (Britz *et al.*, 1997), and when weight is plotted as a function of time using the Von Bertalanffy growth function, a growth curve of the form seen below in Figure 5 is obtained.
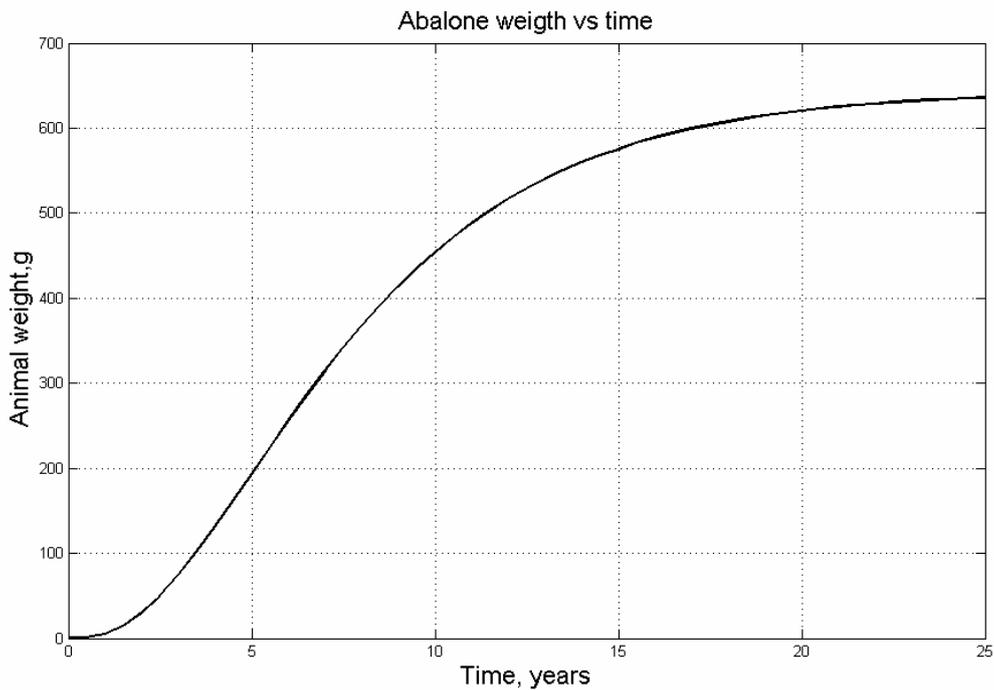
Figure 5 Growth curve for abalone vs. time, constructed using Von Bertalanffy growth curve, coupled with the allometric growth function with a = 167×10^{-6} and b = 2.97 (both values established in the current study) to relate animal length and weight (refer to Section 4.5).

From Figure 5 it is clear that the rate of mass increase initially increases, reaches an inflection point and then decreases. It is therefore not obvious from the graph whether it is appropriate to calculate weight growth rate using a linear function. It might be possible to approximate weight increase using linear equations under certain conditions. In order to establish whether this method could be used in this particular project, Figure 6 and Figure 7 were constructed from the data collected during the course of the experiments.

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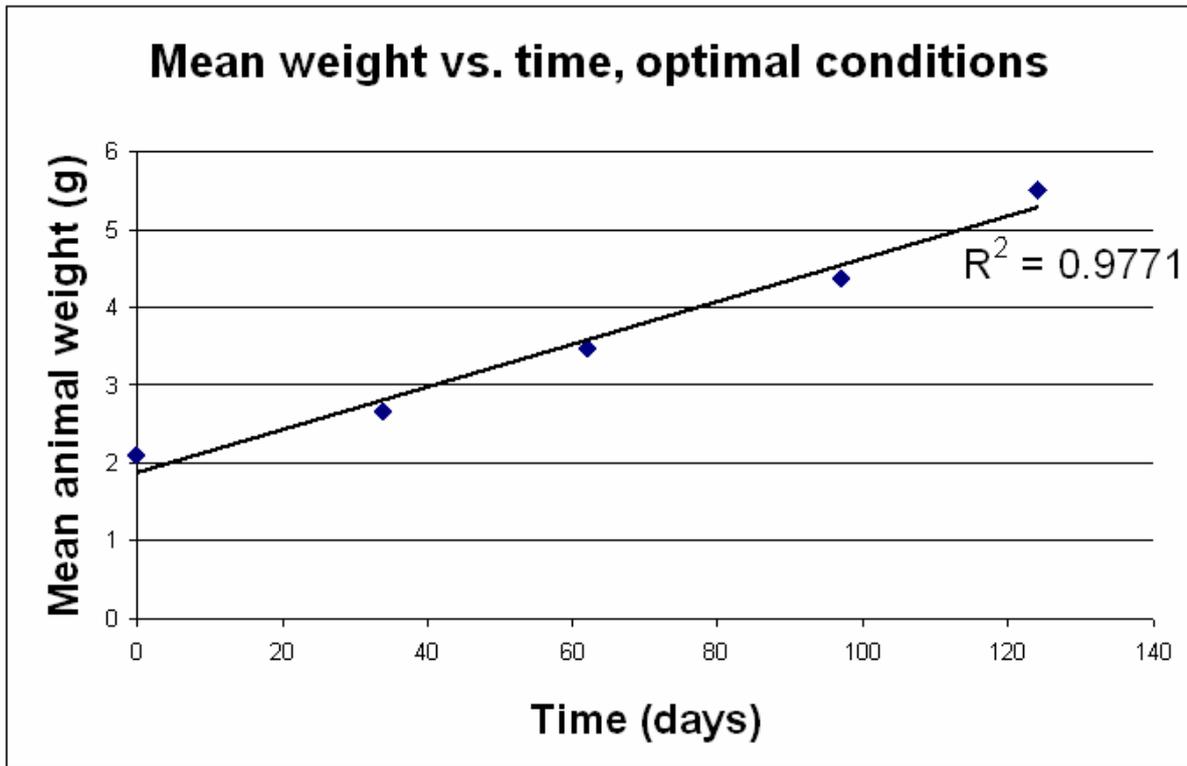
Figure 6 Plot of mean animal weight over the duration of the optimal conditions laboratory experiment, with a linear regression fit.
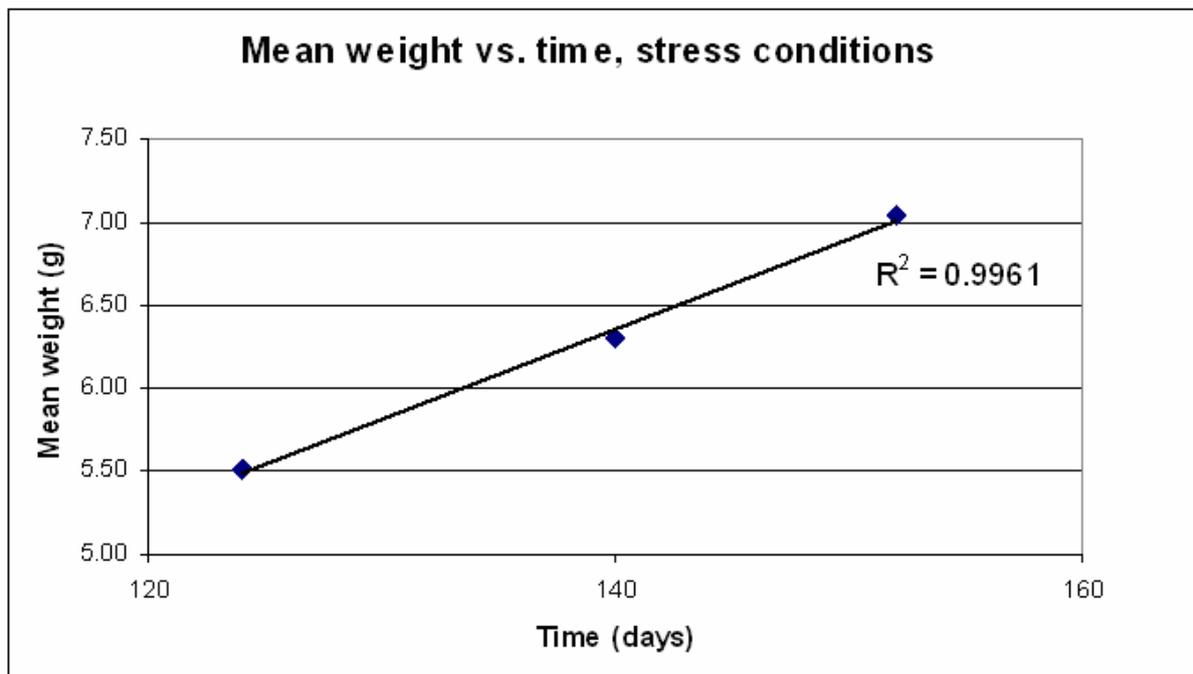
Figure 7 Plot of mean animal weight over the duration of the sub-optimal laboratory conditions experiment, with a linear regression fit.

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From Figure 6 and Figure 7 it can be seen that the growth in terms of weight can be approximated as a linear function for the duration of the respective trials, as the linear regression equations fitted to the data have R^2 values of 0.9771 for the optimal conditions laboratory experiment and 0.9961 for the sub-optimal conditions laboratory experiment, indicating very good fits. Consequently, linear growth rates in terms of animal weight were calculated as follows:

AGRW for a period starting at t_0 and ending at t_1 was calculated as

$$AGRW = 1000 \times \left(\frac{W_{t_i} - W_{t_0}}{t_i - t_0} \right) \quad \left(mg / day \right) \tag{1.4}$$

Other parameters that were used to evaluate the production performance of the animals are given below:

Feed conversion ratio (FCR) over a period starting at t_0 and ending at t_1 was calculated as

$$FCR = \frac{FI}{W_1 - W_0} \qquad \left(\frac{g \ dry \ feed \ utilised}{g \ wet \ weight \ gained} \right) \tag{1.5}$$

where *FI* is feed intake and *W* is animal weight, both in gram

Incidence cost (IC) over a period starting at t_0 and ending at t_1 was calculated as

$$IC = FCR \times CoF \qquad \left(\frac{R}{ton \ abalone} \right) \tag{1.6}$$

where *CoF* is the cost of the feed, including the cost of additives

The Fulton condition factor was calculated as:

$$Fulton \ CF = 1000 \times \frac{W}{L^3} \tag{1.7}$$

4.5   Establishing length vs. weight relationship

A large number of morphological and physiological variables in animals can be related by the general allometric equations of the form:

$$y = ax^b \tag{1.8}$$

where *y* and *x* are general dependant and independent variables respectively. This equation can be transformed into a linear equation by taking the natural logarithm of both sides, which yields the following:

$$\ln(y) = \ln(a) + b\ln(x) \tag{1.9}$$

The slope of this linear equation is given by the value of *b* and its value will differ according to which variables are plotted against one another. If the value of *b* is equal to 0, no relationship exists between *x* and *y,* when *b* = 1, the variables are related in a simple linear fashion etc.

Many biological variables in organisms can be related to body size by equation 1.8 including total body mass, and in aquaculture length is frequently related to mass using this equation. When mass is described in terms of a linear body dimension like length, a special case arises when the value of *b* is equal to 3. If *b* = 3, the organism is said to follow an isometric growth relationship.   This means that as the organism grows, all dimensions stay in proportion (in other words, animals of all ages and sizes are geometrically similar). Very few organisms follow an isometric growth pattern in nature. Growth that is not isometric is called allometric and implies a value of the exponent *b* other than 3, when weight and length are related (Schmidt-Nielsen, 1984).

7774 data points that were obtained in the laboratory experiments were subjected to the analysis described above to establish a relationship between length and weight for *Haliotis midae* of the form

$$W = aL^b \qquad (g) \tag{1.10}$$

where W is animal weight in gram and L is total animal length in mm.

 A linear regression equation was fitted to the linearised data and the value obtained for the exponent *b* was subjected to statistical analysis in order to establish whether it is not equal to 3.00

The 95% confidence interval for *b* was constructed using the equation

$$CI\left(b\right) = b \pm \left(t_{7774;0.025}\right)\sqrt{\frac{MSE}{SS_L}} \qquad (1.11)$$

where *CI*(*b*) is the confidence interval of *b*,

$t_{7774,\ 0.025}$ is the t-statistic for 7774 data points at a significance level of 95%

MSE is the mean square error

SS_L is the sum of squares for length

4.6   Characterisation of gut microflora

At the end of each growth period, one animal was removed from every container and treatments were grouped together.   Animals were transported from the trial site to Stellenbosch University on ice in an insulated cooler box. Animals were killed and the shell removed. One or two drops of 70% ethanol were applied to the stomach/intestine region to kill external bacteria. The intestine of each animal was removed aseptically and all the intestines from each treatment (8 intestines per treatment) were pooled, weighed and then homogenised using a sterilised mortar and pestle. The homogenised sample was added to 9 parts of sterile 0.7% NaCl solution and this was designated the 10^{-1} solution. A solution series was prepared from the 10^{-1} solution and selected dilutions were plated in triplicate onto four different media: Brain-heart infusion agar (BHI), enriched Anacker and Ordal agar (EAO), MRS agar and Thiosulfate-Citrate-Bile-salts-Sucrose agar (TCBS). This procedure was repeated for each treatment.

Plates were incubated aerobically, similar to Sawabe (Sawabe *et al.*, 2003) for 6-7 days at room temperature, after which colony counts were done on plates with 20 – 200 colonies. Distinctive colonies were selected and plated onto new plates of the same media that they were isolated from in order to get pure cultures and colony characteristics were noted. Pure cultures were grown up in liquid tryptone-soy broth (TSB) overnight at 30ºC.   If cultures would not grow in TSB, the media was supplemented with 2% NaCl. 750µl of culture was added to 750µl of 80% glycerol in a sterile plastic tube and put into long term storage at -80ºC.

At the conclusion of all abalone experiments, all colonies were plated onto TSB agar or TCBS media if they could not be grown on TSB. Gram stains were performed using standard protocols according to Isenberg (Isenberg, 1998). Genomic DNA was isolated by growing organisms up in liquid culture overnight and then using the standard CTAB/NaCl miniprep method according to Ausubel (Ausubel *et al.*, 1989). Genomic DNA was amplified using the polymerase chain reaction (PCR), cleaned up using a DNA cleanup kit and sent for 16S classification.

The methods used in this study differ from those used by either Sawabe *et al.* (Sawabe *et al.*, 2003) and Erasmus *et al.* (Erasmus *et al.*, 1997), due mainly to the fact that Sawabe and co-workers used a total of 3 specimens of *H. midae* in their study, and Erasmus *et al.* used a total of 12 animals. In this particular study, 40 animals had to be processed with each sampling and processing of all samples had to be done within the same day, as the composition of the microbial community might change significantly when samples are left overnight (Dr. H. Chenia, personal communication). There were no facilities where animals could be kept alive while samples were prepared, therefore all 40 animals had to be processed within the same day, which made it impractical to use time consuming microbiological techniques.

Methods also differed from Sawabe *et al.* (Sawabe *et al.*, 2003) and Erasmus *et al.* (Erasmus *et al.*, 1997) due to the fact that there was special interest in isolating potential pathogenic organisms found in the digestive tract of abalone. It is known that *Vibrio* bacteria are commonly associated with abalone and that these organisms are frequently pathogens of abalone (Cai *et al.*, 2006a; Liu *et al.*, 2000; Nicolas *et al.*, 2002). These bacteria have been isolated successfully from the gut of abalone and are present in large numbers in this part of the intestinal tract (Erasmus, 1996; Sawabe *et al.*, 2007; Sawabe *et al.*, 1998; Sawabe *et al.*, 2004b). It has been found that bacteria are not present in large numbers in the oesophagus of *Haliotis midae* (Erasmus, 1996), and this part of the digestive tract was therefore disregarded. *Vibrio* bacteria are able to grow aerobically (Dr. H. Chenia, personal communication), therefore anaerobic conditions were not used to incubate samples.

4.7   Statistical analysis

All data were subjected to statistical analysis using the ANOVA *F* test.   Differences were viewed to be significant for p < 0.05.   Only if the ANOVA analysis indicated the existence of significant differences, were the data subjected to Fischer's LSD post-hoc test to establish which treatments differed significantly, in accordance to Montgomery (Montgomery, 1997). All data were analysed using the Statistica software package.   In all data analyses, the following hypotheses were posed:

Null hypothesis: H_0: $\mu_{NC,x} = \mu_{AF,x} = \mu_{PC,x} = \mu_{PC,x} = \mu_{SBPS,x} = \mu_{BS,x}$

Alternative hypothesis: H_1: All treatments are not equal.

where μ is the mean value of parameter *x* for the different treatments.

Abalone have low growth rates, therefore differences relating to growth rates are hard to detect during trials, except when trials can be run for long periods.   In order to overcome this potential difficulty, the data were analysed with multiple ANOVA analyses.   Although multiple ANOVA analyses may inflate the chance of Type I error, this was an acceptable risk in this instance.   Due to the growth characteristics of abalone, Type II error must be avoided.   Type II error occurs when the null hypothesis is not rejected when it is in fact false.   Type II error would imply that the treatments did have an effect on the parameter tested, but that the effect was not detected.   Type I error implies that differences are detected when there are no true differences.   The slow growth rates of animals eliminated the risk of Type I error to a certain extent.

The assumption of normality that is inherent in ANOVA analysis was evaluated by plotting the residuals of the data on a normal probability plot.   Results of this can be seen in Appendix in Figure 15 to Figure 32.

Numerical data obtained during characterisation of gut microflora could not be treated statistically.   During preparation of samples for plate counts, all animals from each treatment were pooled in order to obtain an average value, and from this pooled sample, plates were prepared.   It would not be correct to treat the data obtained from these plates statistically as pseudo repetitions would be involved (D.G. Nel, personal communication).

5. RESULTS AND DISCUSSION

5.1 RESULTS

5.1.1 Choice of treatments

Due to limited resources in this project, it was necessary to limit the total number of treatments (including controls) to a maximum of 5. Two of these treatments had to be controls (one positive and one negative control), which left only 3 treatments in which acids and/or acid salts could be compared. A large number of certified organic acids and salts are currently available for use in human and animal nutrition. In order to decide which of these acids or salts should be included in the experiments, it is necessary to establish criteria that the substances must adhere to. It was decided that additive cost would not play a role in determining which acids/salts to use in this investigation and therefore the primary criterion set for this experiment was availability of high purity food grade products. The acids and salts that were readily available from local industry at the initiation of the experiments are given below in Table 3 along with some of their properties [the cation for all acid salts were either potassium (K^+) or sodium (Na^+)].

Table 3 Acids and salts available from suppliers. Also shown are chemical formulas, the phase of the substances at 25ºC and all the dissociation constants (pKa values) for the acids that have more than one acidic functional group.

Acid name	Chemical formula	pKa$_1$	pKa$_2$	pKa$_3$	Phase at 25ºC
Acetic acid	CH_3COOH	4.76	-	-	Liquid
Benzoic	C6H5COOH	4.19	-	-	Solid
Citric acid	$COOHCH_2C(OH)(COOH)CH_2COOH$	3.13	4.76	6.49	Solid
Formic acid	HCOOH	3.75	-	-	Liquid
Fumaric acid	COOHCHCHCOOH	3.02	4.76		Solid
Lactic acid	$CH_3CH(OH)COOH$	3.86	-	-	Liquid
Malic acid	$COOHCH_2CH(OH)COOH$	3.40	5.10	-	Solid
Phosphoric acid	H_3PO_4	2.14	7.2	12.4	Liquid
Propionic acid	CH_3CH_2COOH	4.88			Liquid
Sorbic acid	$CH_3CHCHCHCHCOOH$	4.76	-	-	Solid
Tartaric acid	COOHCH(OH)CH(OH)COOH	2.93	4.23	-	Solid
Benzoate	C6H5COO⁻	-	-	-	Solid
Citrate	$^-OOCHCH_2C(OH)(COO^-)CH_2COO^-$	-	-	-	Solid
Propionate	$CH_3CH_2COO^-$	-	-	-	Solid
Sorbate	$CH_3CHCHCHCHCOO^-$	-	-	-	Solid

From the acids available it was necessary to choose combinations in a way that would answer as many research questions as possible (see Section 3). To do this, it was decided that 2 substances would be included per treatment at equal levels (mass %) at a total inclusion level of 2%, i.e. each substance would be included at 1%. It was further decided that in one of the treatments, a relatively strong acid (low pKa value), which is not necessarily an organic acid, would be used in combination with a weaker organic acid in order to determine whether the lowering of pH has any effect on the efficacy of the treatment. It was also necessary to test whether the acids would leach from the feed when immersed in sea water and whether different acids had different leaching characteristics. Three different acids with very different properties were evaluated to determine their effects on the pH of the feed and their ability to resist leaching. Tartaric acid is a weak diprotic organic acid compared to strong mineral acids, sulphuric acid is a strong diprotic mineral acid and hydrochloric acid is a strong monoprotic mineral acid. The results of the test can be seen below in Figure 8.
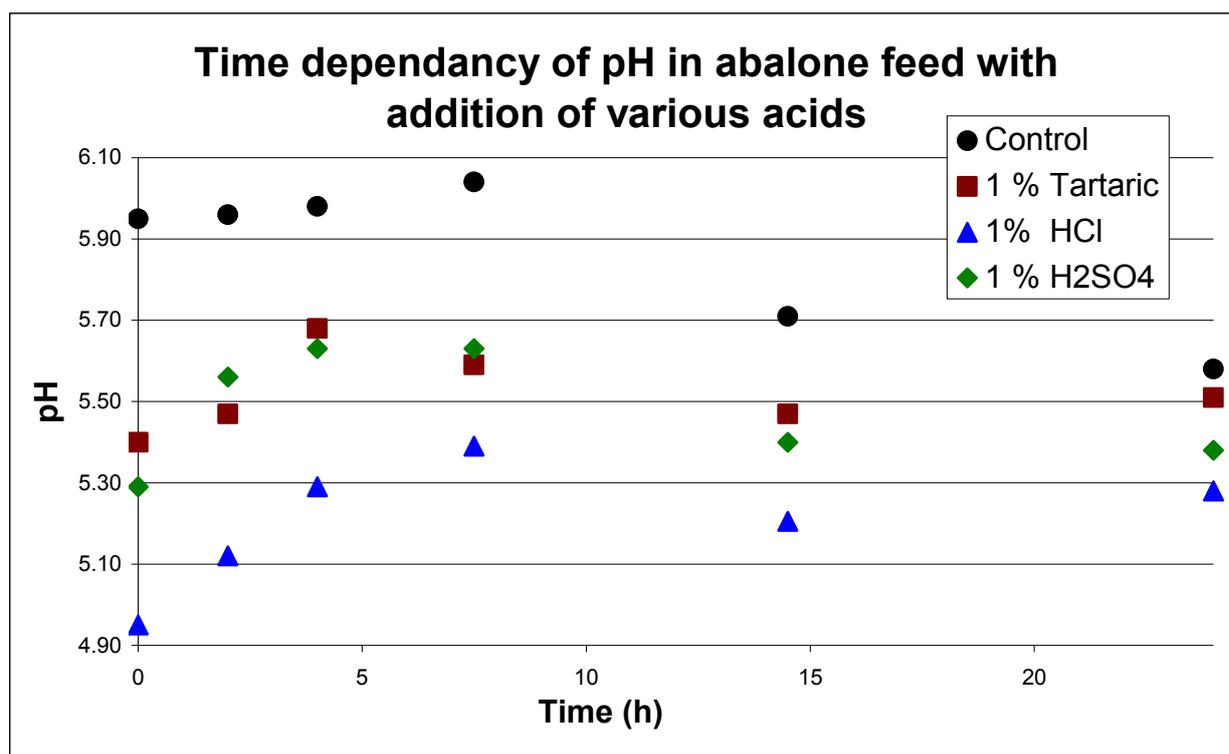

Figure 8 Effect of different acids on the pH of abalone feed and the change in pH over time when the feed is immersed in sea water.

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From Figure 8 it can be seen that all acids tested initially lower the pH of the abalone feed. The pH increases with time and reaches a maximum after approximately 7 hours after it was first immersed in seawater. When the pH reaches the maximum, it slowly decreases and the difference in pH between treatments decreases. Very little difference exists in the effect on pH of tartaric and sulphuric acid. Hydrochloric acid had the largest effect on the pH of the abalone feed.

From the results obtained in this acidification and leaching experiment it is clear that the acids do not have a large effect on the pH of the feed at 1% inclusion level. Based on these results it was therefore decided that only organic acids and their salts would be included in the experiments.

In an attempt to determine the mechanism responsible for the working of the acids, it was decided that one treatment should consist of acids known to have antimicrobial activity. The salts of these antimicrobial acids will be incorporated in another treatment to further determine whether the effects (if any) are due to the complete acid or only due to the anion of the acid. The only acid salts available are benzoate, citrate, propionate and sorbate. Of the acids of these salts, only benzoic and sorbic acid are known to have antimicrobial activity. Citric acid occurs in most living organisms and is used in the citric acid cycle and thus has no antimicrobial activity, while propionic acid has anti-mould activity but no antibacterial activity (Liebrand and Liewen, 1992). The only combinations of substances that adhere to both the above criteria are sorbic and benzoic acid coupled with sorbate and benzoate and the first two treatments were chosen as: 1% benzoic acid + 1% sorbic acid, and 1% benzoate + 1% sorbate.

The remaining treatment was chosen to investigate whether the acids can have their effects by serving as a metabolisable energy source for the abalone. A study done on enteric bacteria isolated from abalone found that the bacteria can ferment alginate to form acetic and formic acid. It further stated that these fermentation products could play a significant role in the energy metabolism of the abalone (Sawabe *et al.*, 2003). The remaining treatment was thus chosen as 1% acetic acid + 1% formic acid. A summary of the treatments used in the experiments can be seen in Table 2.

The antibiotic growth promoter was chosen on the basis of two criteria: it had to be readily available from the local feed industry and it had to be a recognised AGP in the industry. Avilamycin adhered to both these criteria and was used as the AGP in the positive control diet.

5.1.2 Laboratory Growth trials: Optimal conditions

The performance of the different treatments is evaluated according to a number of parameters, of which the primary parameter is the SGR, which is a non-linear growth rate. Linear growth rates were also calculated (AGRW and AGRL) to validate the results obtained from the SGR. FCR, IC, and Fulton CF are also evaluated. All data presented are given as the mean ± standard deviation (SD). All data were statistically evaluated using the standard ANOVA F-test and differences between treatments or time periods were evaluated using Fischer's LSD test. Differences were viewed as significant for p – values < 0.05.

Large amounts of data were collected throughout all experiments. During the duration of the controlled optimal conditions experiment, 5706 data points, comprising of separate measurements for length and weight, were taken and a total of 1240 measurements of uneaten feed were made. The number of animals measured and weighed at each measurement date can be seen in Table 15 to Table 19 in the Appendix.

It should be noted that the IC values given in this section are given relative to the value obtained for the negative control (treatment NC) over the whole controlled optimal conditions experiment. The SGR values were calculated over at least 2 growth periods in order to minimise the effect of any events within one growth period that could significantly influence results. Table 4 summarises the results obtained for SGR over the whole controlled optimal conditions experiment.

Table 4 SGR for controlled optimal conditions over different periods. Data are given as mean ± SD, all data treated with ANOVA, followed by Fischer's LSD test.

	SGR (d^{-1})			
	Period			
Treatment	**Overall (D0 – D124)**	**1 (D0 – D62)**	**2 (D34 – D97)**	**3 (D62 – D124)**
NC	$0.71^a \pm 0.054$	$0.74^a \pm 0.087$	$1.05^a \pm 0.082$	$1.39^a \pm 0.106$
AF	$0.83^b \pm 0.076$	$0.90^b \pm 0.119$	$1.29^b \pm 0.137$	$1.64^b \pm 0.150$
PC	$0.74^a \pm 0.036$	$0.75^a \pm 0.068$	$1.07^a \pm 0.086$	$1.46^a \pm 0.071$
SBPS	$0.82^b \pm 0.061$	$0.85^b \pm 0.093$	$1.23^b \pm 0.083$	$1.61^b \pm 0.121$
BS	$0.82^b \pm 0.051$	$0.89^b \pm 0.080$	$1.28^b \pm 0.133$	$1.61^b \pm 0.101$
p-value	0.0004	0.0025	0.0001	0.0004

From these results it can be seen that the negative control (Treatment NC) consistently had the lowest SGR of all treatments for all periods of calculation during the controlled optimal conditions experiment. Treatments AF, SBPS and BS consistently produced higher SGR values than both the negative (Treatment NC) and positive (Treatment PC) control, being significantly higher than both controls during this experiment.

The SGR increased from the start of the controlled optimal conditions experiment and the highest SGR observed during the trial was reached during the last growth period, for all treatments. SGR for all treatments for period D0 – D62 is significantly lower than that for period D34 - D97 which in turn is significantly lower than the SGR during period D62 – D124. In all cases the highest SGR observed is approximately double that of the overall SGR.

Differences in growth rates during the controlled optimal conditions experiment are given below in Table 5. All values are calculated relative to treatment NC. Measured over the whole experiment (D0 – D124), an improvement in SGR of 17.9%, 16.1% and 15.8% is seen for treatments AF, SBPS and BS respectively. The highest improvement in any single period is 22.9 % for treatment AF during period D34 – D97.

Table 5 % improvement in SGR over the negative control for different treatments for all growth periods during controlled optimal conditions experiment.

	% improvement in SGR over negative control			
Treatment	D0 – D124	D0 – D62	D34 – D97	D62 – D124
AF	17.9	21.8	22.9	17.9
PC	5.2	1.5	2.5	5.2
SBPS	16.1	15.1	17.6	16.1
BS	15.8	20.7	22.3	15.8

The values for the FCR were calculated over the same periods than for the SGR and are given in Table 6. There were no significant differences in FCR between treatments during any of the periods. A steady decrease in FCR is observed from the beginning of the controlled optimal conditions experiment to the end thereof.

Table 6 FCR values for controlled optimal conditions over different growth periods Data are given as means ± SD; all data treated using ANOVA followed by Fischer's LSD test.

Treatment	FCR over different periods			
	Period			
	Overall (D0 – D124)	1 (D0 – D62)	2 (D34 – D97)	3 (D62 – D124)
NC	0.82 ± 0.124	0.90 ± 0.180	0.88 ± 0.086	0.77 ± 0.169
AF	0.75 ± 0.105	0.88 ± 0.145	0.83 ± 0.125	0.67 ± 0.126
PC	0.80 ± 0.091	1.01 ± 0.133	0.97 ± 0.153	0.68 ± 0.120
SBPS	0.79 ± 0.171	1.00 ± 0.218	0.86 ± 0.180	0.66 ± 0.139
BS	0.75 ± 0.138	0.91 ± 0.192	0.76 ± 0.071	0.64 ± 0.148
p - value	> 0.05	> 0.05	> 0.05	> 0.05

IC is the feeding cost necessary to accomplish a certain amount of animal production e.g. R/ton abalone. The values are all given relative to the IC obtained for treatment NC over the whole controlled optimal conditions experiment. The IC values were calculated over the same periods as SGR and FCR and the values are given below in Table 7. There are no significant differences for IC between treatments for any of the periods, but a steady decrease in IC can be seen from the beginning of the controlled optimal conditions experiment to the end, for all treatments.

Table 7 IC for controlled optimal conditions over different growth periods. Data are given as means ± SD; all data treated using ANOVA followed by Fischer's LSD test.

Treatment	Index values for IC over different periods			
	Period			
	Overall (D0 – D124)	1 (D0 – D62)	2 (D34 – D97)	3 (D62 – D124)
NC	1.00 ± 0.163	1.10 ± 0.237	1.07 ± 0.113	0.95 ± 0.222
AF	0.94 ± 0.140	1.09 ± 0.194	1.04 ± 0.167	0.84 ± 0.168
PC	0.98 ± 0.119	1.24 ± 0.174	1.19 ± 0.200	0.83 ± 0.157
SBPS	1.04 ± 0.240	1.32 ± 0.306	1.14 ± 0.253	0.87 ± 0.196
BS	0.98 ± 0.194	1.20 ± 0.270	1.00 ± 0.100	0.84 ± 0.207
p - value	> 0.05	> 0.05	> 0.05	> 0.05

Various production parameters are summarised for the whole experiment below in Table 8. Instantaneous values for weight and length at the end of each growth period, and the linear growth rate obtained during the most recent growth period, can be seen for this experiment in the Appendix in Table 15 to Table 19. SGR, FCR and IC are represented for the controlled optimal conditions growth experiment in Figure 10 in the Appendix.

Table 8 Summary of total growth trial (D0 – D124) for controlled optimal conditions. All parameters are calculated over the whole trial period. All values are given as mean ± SD. Values without common superscripts in the same row differ significantly. All data evaluated using ANOVA, followed by Fischer's LSD test.

Abbreviations: W: weight, L: length, AGRW: apparent growth rate based on weight, AGRL: apparent growth rate based on length, FCR: feed conversion ratio

	Treatment					
Variable	**NC**	**AF**	**PC**	**SBPS**	**BS**	**p - value**
W_0 (g)	2.20 ± 0.233	2.05 ± 0.258	2.08 ± 0.258	1.97 ± 0.202	2.17 ± 0.279	> 0.05
W_{124} (g)	5.28^{ac} ± 0.413	5.73^{bc} ± 0.538	5.21^a ± 0.538	5.43^{ac} ± 0.411	5.94^b ± 0.479	**0.015**
L_0 (mm)	23.82 ± 0.842	23.40 ± 0.817	23.39 ± 0.817	22.92 ± 0.857	23.69 ± 0.936	> 0.05
L_{124} (mm)	32.16 ± 0.781	33.04 ± 1.127	32.24 ± 1.127	32.36 ± 1.166	33.29 ± 0.902	> 0.05
Fulton CF_0	0.163 ± 0.008	0.160 ± 0.008	0.162 ± 0.008	0.163 ± 0.007	0.162 ± 0.011	> 0.05
Fulton CF_{124}	0.158 ± 0.006	0.159 ± 0.007	0.155 ± 0.007	0.160 ± 0.008	0.161 ± 0.007	> 0.05
AGRW (mg/day)	24.78^a ± 2.45	29.67^{bc} ± 2.16	25.24^a ± 2.66	27.89^c ± 2.67	30.44^b ± 2.30	**0.00006**
AGRL (µm/day)	67.27^a ± 4.19	77.73^b ± 8.11	71.43^{ab} ± 6.69	76.16^b ± 8.88	77.42^b ± 5.09	**0.016**
Total feed intake (g)	70.2 ± 7.52	76.7 ±12.56	69.4 ± 10.94	73.5 ± 11.64	79.7 ± 15.08	> 0.05

At the end of the experiment there were significant differences in animal weights between treatments. Animals in treatments AF and BS were significantly heavier than those in treatments NC and PC, while animals from treatment BS was significantly heavier than those in treatment SBPS as well. No significant differences existed between treatments for animal length or Fulton CF and no difference was seen with regards to total feed intake. Linear growth rates in terms of weight (AGRW) for treatments AF, SBPS and BS were all significantly higher than those of the controls (NC and PC), as was the case for SGR. AGRW for treatment BS was significantly higher than AGRW for treatment SBPS, but did not differ from that of treatment AF. The use of AGRW to evaluate growth supports the results obtained with the use SGR as evaluation parameter. Growth rates in terms of length (AGRL) for treatments AF, SBPS and BS were all significantly higher than that of the negative control NC, but did not differ from the AGRL for the positive control PC.

5.1.3 Laboratory Growth trials: Stress conditions

The same parameters were used for evaluating the growth during controlled temperature stress conditions as those used for controlled optimal conditions. During the duration of the controlled temperature stress experiment, 2068 data points (not including those used in the controlled optimal conditions experiment), comprising of separate measurements for length and weight, were taken and a total of 320 measurements of uneaten feed were made. The number of animals measured and weighed at each measurement date can be seen in Tables 20 and 21 in the Appendix. The results obtained during the controlled stress experiment are given in Table 9.

Table 9 Results obtained during controlled temperature stress experiment. SGR, % improvement in SGR, FCR and IC. All data are given as mean ± SD. All data evaluated using ANOVA, followed by Fischer's LSD test.

| Treatment | Parameter | | | |
	SGR (d^{-1})	% improvement in SGR, relative to NC	FCR	IC (R/ton abalone)
NC	0.85 ± 0.246	0	1.11 ± 0.194	1.36 ± 0.257
AF	0.78 ± 0.145	-8.3	1.21 ± 0.345	1.52 ± 0.460
PC	0.93 ± 0.187	9.5	1.11 ± 0.190	1.36 ± 0.251
SBPS	0.99 ± 0.213	16.3	1.08 ± 0.310	1.43 ± 0.433
BS	0.79 ± 0.181	-6.3	1.14 ± 0.200	1.50 ± 0.287
p - value	> 0.05	-	> 0.05	> 0.05

There were no significant differences for SGR between treatments during the controlled stress experiment. Treatment SBPS yielded the highest SGR for this period, while treatment AF showed the lowest SGR. Treatment SBPS showed a 16.3% increase in SGR when compared to treatment NC, while treatment AF showed a decrease of 8.3%. The SGR values for the controlled stress experiment for all treatments was significantly lower than the highest SGR obtained during period D62 – D124 in the controlled optimal conditions experiment (compare Table 4).

No significant differences were seen between treatments when comparing FCR values. Treatment SBPS had the lowest FCR value, while treatment AF had the highest value. The FCR was significantly higher during the controlled stress conditions experiment than during any other period of the controlled optimal conditions experiment (compare Table 6). If IC values are compared, it can be seen that there are no significant differences between treatments, but IC values obtained during the controlled stress experiment are higher than any values obtained during the controlled optimum conditions experiment (compare Table 7).

Table 10 Summary of growth trial for laboratory conditions, stress experiment. All values are   given as mean ± SD. Values without common superscripts in the same row differ significantly. All data evaluated using ANOVA, followed by the Fischer's LSD test.

Abbreviations: AGRW: linear growth rate based on weight, AGRL: linear growth rate based on length, FCR: feed conversion ratio

Variable	Treatment					p - value
	NC	AF	PC	SBPS	BS	
Weight S_0 (g)	5.28^{ac} ± 0.413	5.73^{bc} ± 0.538	5.21^{a} ± 0.538	5.43^{ac} ± 0.411	5.94^{b} ± 0.479	**0.015**
Weight S_{28} (g)	6.69 ± 0.569	7.13 ± 0.627	6.75 ± 0.627	7.22 ± 0.749	7.42 ± 0.503	> 0.05
Length S_0 (mm)	32.16 ± 0.781	33.04 ± 1.127	32.24 ± 1.127	32.36 ± 1.166	33.29 ± 0.902	> 0.05
Length S_{28} (mm)	35.07 ± 0.994	35.72 ± 1.146	35.18 ± 1.146	35.45 ± 1.349	36.11 ± 0.940	> 0.05
Fulton CF S_0	0.158 ± 0.006	0.159 ± 0.007	0.155 ± 0.007	0.160 ± 0.008	0.161 ± 0.007	> 0.05
Fulton CF S_{28}	0.155^{a} ± 0.004	0.156^{a} ± 0.003	0.155^{a} ± 0.003	0.162^{b} ± 0.004	0.157^{a} ± 0.005	**0.019**
AGRW (mg/day)	50.59 ± 16.61	50.06 ± 11.77	55.05 ± 12.24	64.04 ± 20.38	52.63 ± 12.22	> 0.05
AGRL (µm/day)	103.99 ± 13.66	95.88 ± 14.91	104.72 ± 8.36	110.27 ± 14.77	100.69 ± 10.42	> 0.05
Total feed intake (g)	43.3 ± 3.71	41.0 ± 2.57	41.6 ± 2.96	43.9 ± 2.86	43.6 ± 2.99	> 0.05

Various other parameters evaluated for the whole of the controlled stress conditions experiment are given in Table 10. Instantaneous values for all sampling dates can be seen in the Appendix in Table 20 and Table 21 in the Appendix. SGR, FCR and IC are represented for the controlled stress conditions experiment in Figure 11 in the Appendix. It should be noted that the starting values for weight and length for the controlled stress experiment are the same as the final values for the controlled optimal conditions experiment.

From Table 10 it can be seen that there were no significant differences between treatments for length or weight at the end of the controlled stress experiment. Fulton condition factor differed significantly, with treatment SBPS having significantly higher values than all other treatments. No significant differences were seen in linear growth rates or feed intake between different treatments.

5.1.4 Growth trials: Production conditions

The same parameters were used for evaluating the growth during production conditions as those used for controlled optimal conditions. During the duration of the production experiment, 1842 data points comprising of separate measurements for length and weight were taken. No measurements of uneaten feed were made, due to the fact that no feed could be recovered from the containers used in this experiment.

No statistically significant differences were observed for SGR, FCR or IC between treatments in this experiment. This is mainly due to the large variance observed in data obtained in this experiment. The reason for this seems to be the sampling method, which is inadequate and should be adapted so as to either sample more animals per container, or to choose animals for sampling in another manner.

Table 11 SGR, FCR and IC for production conditions over whole experimental period. Data are given as means ± SD, all data treated using ANOVA, followed by Fischer's LSD test.

Treatment	SGR d^{-1}	FCR	IC index values
NC	0.80 ± 0.096	2.91 ± 0.61	3.56 ± 0.750
AF	0.88 ± 0.142	2.58 ± 0.75	3.22 ± 0.938
PC	0.79 ± 0.124	3.00 ± 0.80	3.68 ± 0.981
SBPS	0.84 ± 0.131	2.73 ± 0.68	3.60 ± 0.889
BS	0.78 ± 0.123	3.08 ± 0.82	4.04 ± 1.071

Treatment AF showed the highest SGR, and the lowest FCR and IC. Treatment BS has the lowest SGR and the highest FCR and IC. SGR values range from 0.78 – 0.88 d^{-1}, FCR values are between 2.58 – 3.08 and IC index values between 3.22 and 4.04.

No significant differences existed between animal weights, lengths or Fulton CF at the conclusion of the production conditions experiment. No difference was observed in apparent growth rates (AGRW or AGRL). Feed intake could not be estimated, as uneaten feed could not be recovered from the containers. At the end of the experiment, animals from treatment AF had the highest average weight and length. Observed AGRW and AGRL values were also highest in treatment AF.

Instantaneous values for weight, length and the Fulton CF at the end of each growth period are given for the production conditions experiment in the Appendix in Table 22 to Table 24. SGR, FCR and IC are represented for the production conditions growth experiment in Figure 13 in the Appendix.

Table 12 Summary of growth trial for production conditions. All parameters calculated over the whole trial period. All values are given as mean ± SD. Values without common superscripts in the same row differ significantly. All data evaluated using ANOVA, followed by the Fischer's LSD test.

Abbreviations: AGRW: linear growth rate based on weight, AGRL: linear growth rate based on length, FCR: feed conversion ratio.

	Treatment					
Variable	**NC**	**AF**	**PC**	**SBPS**	**BS**	**p - value**
W_0 (g)	0.54 ± 0.22	0.54 ± 0.22	0.54 ± 0.22	0.54 ± 0.22	0.54 ± 0.22	> 0.05
W_{90} (g)	1.10 ± 0.10	1.20 ± 0.16	1.10 ± 0.13	1.15 ± 0.15	1.08 ± 0.13	> 0.05
L_0 (mm)	15.00 ± 2.06	15.00 ± 2.06	15.00 ± 2.06	15.00 ± 2.06	15.00 ± 2.06	> 0.05
L_{90} (mm)	18.63 ± 0.50	18.89 ± 1.12	18.30 ± 0.62	18.83 ± 0.95	18.46 ± 0.65	> 0.05
Fulton CF$_0$	0.151 ± 0.017	0.151 ± 0.017	0.151 ± 0.017	0.151 ± 0.017	0.151 ± 0.017	> 0.05
Fulton CF$_{90}$	0.171 ± 0.008	0.177 ± 0.012	0.179 ± 0.011	0.172 ± 0.009	0.172 ± 0.009	> 0.05
AGRW (mg/day)	6.31 ± 1.14	7.33 ± 1.79	6.24 ± 1.44	6.84 ± 1.65	6.09 ± 1.41	> 0.05
AGRL (µm/day)	40.30 ± 5.60	43.18 ± 12.47	36.68 ± 6.85	42.51 ± 10.51	38.48 ± 7.26	> 0.05

5.1.5 Relationship between length and weight

The analysis was performed by the method discussed in Section 4.5. The values of *W* (g) were plotted against *L* (mm) for 7774 data points taken during the laboratory experiments (optimal and stress experiments) which yielded the following graph:
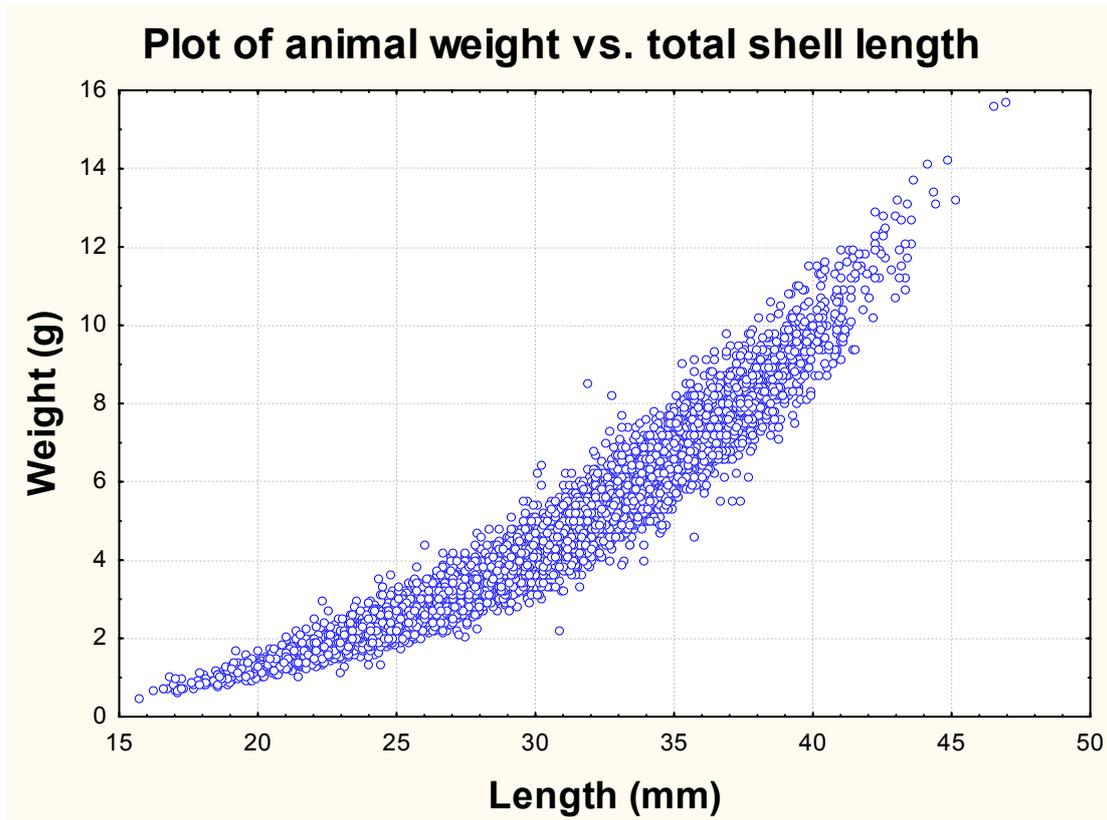
Figure 9 Plot of animal weight vs. total shell length.

After logarithmic transformation of the data followed by linear regression, it was found that *a* = - 8.6965 and *b* = 2.97 with R^2 = 0.9602. A confidence interval was constructed to evaluate the value of *b* obtained from the regression analysis. It was found that the 95% confidence interval of *b* ranges from 2.959 to 2.986, which does not include 3.00. Refer to Figure 12 for the linearised graph.

The equation that relates animal weight to total animal length in the system studied is given by:

$$W = 167 \times 10^{-6} L^{2.97} \qquad (1.12)$$

5.1.6 Characterisation of gut microflora

Results obtained from monthly microbiological studies are shown in Table 14. In total, 22 bacterial colonies and 4 yeast colonies were isolated. There were 7 Gram negative bacteria and 9 Gram positive bacteria, while 5 colonies were not stained or identified. Fifteen different bacterial colonies were identified up to genus level, of which 8 were of the genus *Bacillus*, three were *Vibrio*, two were *Pseudomonas* and there were one each of the genera *Staphylococci*, *Photobacterium* and *Shewanella*. The remaining bacteria could not be identified successfully. Some bacteria were present throughout the controlled optimal conditions experiment (e.g. V11 and V17), while others were only isolated at the end of certain periods (see Table 29 in Appendix).

Microbial diversity in the gut differed from growth period to growth period. In three periods, the diversity within the intestinal microbial community was relatively large, while in two periods there was less diversity. Results can be seen in Table 13.

Table 13 The total number of different bacteria and yeasts isolated at the end of each growth period.

Growth period	Bacteria	Yeasts
D0 - D34	7	3
D34 – D62	14	3
D62 – D97	12	3
D97 - D124	10	2
S0 – S28	4	1

At the end of the first growth period (D0 - D34) the total number of different culturable bacteria isolated were 7. This number increased to 13 at the end of the second period (D34 - D62) and then steadily decreased to 12 and then 10 for the two subsequent periods. At the end of the stress experiment, there were only 4 different bacterial colonies in total. During each of the growth periods D0 – D34, D34 – D62 and D62 – D97 3 different yeasts were present, during period D97 - D124 two different yeasts were isolated and during period S0 – S28 only 1 yeast was present.

Table 14 Results of 16S classification and growth periods during which organisms were present.

Symbol	Genus	Most likely species	Growth periods present
B2	Unknown		D34 – S28
B3	*Staphylococci*	*S. saprophyticus* *S. brasiliensis* *S. xylosus*	D97 - D124
B4	*Vibrio*	*V. harveyi* *V. rumosiensis* *V. alginolyticus* *V. parahaemolyticus*	D62 - D124
B8	Unknown		S0 - S28
B9	Unknown		D97 - D124, S0 - S28
E1	*Pseudomonas*	*P. putida* *P. fulva*	D62 – D124
E2	Unknown		D34 – D124
E3	*Bacillus*	*B. subtilis*	D34 - D97
E4	*Bacillus*	*B. sphaericus* *B. formis*	D34 - D97
E5	*Pseudomonas*	*P. putida*	D34 - D62
E6	*Bacillus*	*B. pumilus*	D62 - D97
E8	*Bacillus*	*B. sphaericus* *B. fusiformis*	?
E10	Unknown		D34 - D62
M2	*Bacillus*	*B. subtilis*	D0 - D34,D62 - D97
M4	*Bacillus*	*B. oteronius* *B. sporothermodurans*	D34 - D97
M6	*Bacillus*	*B. badius*	D34 - D62
M10	Unknown		D34 – D97
M15	*Bacillus*	*B. cereus* *B. thuringiensis* *B. antracis*	S0 - S28
V11	*Vibrio*	*V. cyclitrophicus* *V. tasmaniensis* *V. splendidus*	D34 – D124
V12	*Photobacterium*	*P. frigidiphilum*	D62 - D124
V17	*Vibrio*	*V. cyclitrophicus* *V. splendidus* *V. tasmaniensis*	D34 – D124
V18	*Shewanella*	*S. baltica* *S. pacifica*	D97 - D124
M1	Unidentified yeast		D34 – D97, S0 - S28
M3	Unidentified yeast		D62
V1	Unidentified yeast		D0 – D124
V5	Unidentified yeast		D0 - D34, D62 – D124

It can be seen from data (Table 30 to Table 33 in Appendix) that certain colonies are present in much higher numbers than others. During the first growth period (D0 - D34), B2 dominated on BHI media and E2 dominated on EAO media. Throughout the duration of the controlled optimal conditions experiment, V11 and/or V17 dominated on the TCBS media (it is not possible to distinguish between the two colonies accurately by visual inspection alone, therefore it is not clear whether one was dominant or whether both were present). On the MRS media, slight dominance was shown by colony M10.

During the second growth period (D34 - D62) there was less dominance by specific bacteria and a larger diversity was seen than in any other period. This changed during D62 - D97, where there was very clear dominance shown by colonies B2, E9 and M10. This continued in growth period D97 – D124. During the stress experiment, total dominance was observed by one colony (M15).

None of the *Vibrio* species isolated showed any growth on TSB enrichment agar neither was there any growth at 30ºC by the *Vibrio*'s on any solid media. V11 and V12 showed good growth at 30ºC in 2% NaCl enriched liquid TSB media. The *Vibrio* colonies were all successfully grown up at room temperature in TSB liquid media except V17, which could only be grown up in TSB broth supplemented with 2% NaCl.

Colony characteristics of the different bacteria are given in the Appendix in table 26 when cultured on the original isolation media and in table 27 when cultured on TSB media.

5.2   DISCUSSION

For the first time it is reported that growth of South African abalone *Haliotis midae* can be significantly enhanced due to dietary supplementation of organic acids to their feed. It has been clearly demonstrated that SGR, AGRW and AGRL can be enhanced in animals ranging in average length from 23 mm to 33 mm under controlled optimal rearing conditions by mixtures of 1% acetic and 1%formic acid (Treatment AF), 1% benzoic and 1% sorbic acid (Treatment BS), and 1% sodium benzoate and 1% potassium sorbate (Treatment SBPS) when compared to a negative control diet (see Table 4 and Table 9). Improvement in SGR due to the acid and acid salt treatment was between 15.8% and 17.9% when calculated over the whole optimal temperature conditions trial, which is much higher than the 8% improvement in SGR achieved with a probiotic treatment in *H. midae* (Macey and Coyne, 2005) in a trial using animals of the same size range (mean initial length of 20 mm, compared to mean initial length of 23 mm in the current trial). These acid and acid salt treatments also showed significantly higher SGR and AGRW values than the positive control containing Avilamycin, a commercial antibiotic growth promoter. Growth enhancement due to the addition of organic acids and their salts as a feed supplement has been reported before in pigs (Øverland *et al.*, 2007; Tsiloyiannis *et al.*, 2001a), poultry (Gauthier, 2005) and rainbow trout (De Wet, 2005) and it has been suggested that organic acids can act as substitutes to AGP's in cattle production (Castillo *et al.*, 2004). The current study adds another animal to the list of those that can benefit from dietary supplementation of organic acids in intensive rearing systems.

Avilamycin used as AGP at 30ppm inclusion did not have a favourable effect on the any of the growth rates (SGR, AGRW and AGRL) of the abalone in the current investigation (Table 4 and Table 9), even though inclusion levels correspond with industry standards for pigs and poultry. Even if the AGP did affect the intestinal microflora of the abalone, it did not have an effect on the growth of the animals. Avilamycin is an antibiotic that has an excellent activity against a broad range of Gram positive bacteria (Weitnauer *et al.*, 2004). Many Gram positive bacteria were isolated (*Bacillus* and *Staphylococci,* comprising 9 of the 16 known genera) from the gut of the abalone and it would be expected that these Gram positive organisms would be affected to some extent by the antibiotic, yet no evidence was found in the microbiological results to confirm this (refer to Table 28 and Table 29 in Appendix). It is possible that the inclusion levels of avilamycin were insufficient for aquaculture purposes or that the methods used to investigate the intestinal microflora of the abalone were not sensitive enough to detect any effects of the AGP. The use of AGP's in aquaculture does

not yield the same consistent results as in terrestrial conditions. Some investigations report enhanced growth in rainbow trout, tilapia and carp (De Wet, 2005; Viola and Arieli, 1987; Viola *et al.*, 1990), while others report a deterioration in animal performance in farmed catfish and arctic charr (Rawles *et al.*, 1997; Toften and Jobling, 1997a, b). Further investigation is necessary in order to establish whether AGP's can be used successfully in abalone culture and in aquaculture in general.

The question of the mechanism by which the acid and acid salt treatments enhance the growth rate of the abalone could not be answered with any certainty. Mechanisms suggested from the literature for the mode of action of organic acids include a pH effect (affecting digestion and the intestinal microbial community), a metabolic effect and a microbial effect (Gauthier, 2005; Partanen and Mroz, 1999; Tsiloyiannis *et al.*, 2001a). To investigate a mechanism by which the growth rate of the abalone studied in the current investigation was enhanced, it may be best to look at the mechanisms that are not consistent with the observed data and to eliminate these first.

It seems unlikely that the treatments enhanced the animal growth rates due to a pH effect. There are no differences between the results obtained with the acid treatments and that of the acid salts, even though the different substances would have different effects on the gut pH. Further, the abalone feed used in this trial seemingly had a high buffering capacity (refer to Figure 8). It has been noted that certain feeds may have significant buffering capacity (Partanen and Mroz, 1999) and that animals generally can maintain the pH of their gastro intestinal tract in order to maintain homeostasis (Gauthier, 2005). The gut pH of adult abalone has been reported as 5.2 (Knauer *et al.*, 1996), which is already acidic and the effect of the weak organic acids at the low concentrations used in the treatments will be minimal at this pH. If the above factors are combined it can be assumed that the treatments will have a minimal effect in lowering gut pH and this mechanism can be discarded as the one by which growth is enhanced in this investigation.

The organic acids did apparently not enhance growth by acting as an additional carbon source to the abalone in the present study. It was suggested that short chain volatile fatty acids (SCVFA) could make a significant contribution to the abalone's energy requirements with specific reference to acetic and formic acid (Sawabe *et al.*, 2003). It was further shown under laboratory conditions that *Vibrio halioticoli* can produce these acids via fermentation of alginate which is found in most natural food sources of the abalone and the conclusion was reached that conditions in the abalone gut are such that it is possible for this particular fermentation to take place (Sawabe *et al.*, 2003). *Vibrio halioticoli* was isolated from various

abalone species in that particular study, including *Haliotis midae* (Sawabe *et al.*, 1995; Sawabe *et al.*, 2003). Other authors have shown that volatile fatty acids contribute significantly to the energy supply of some animals. In cattle it was found that volatile fatty acids from rumenal fermentation are the primary energy source and that acetate and butyrate can be utilised efficiently by cattle, (Russell *et al.*, 1992) while another study found that lambs can utilise acetic, propionic and butyric acid salts as energy source (Essig *et al.*, 1959). It is also known that certain SCFA's (especially acetic, propionic and butyric acid) play an important role as energy source of the human colonic mucosa (Roediger, 1980; Royall *et al.*, 1990; Wong *et al.*, 2006). The data obtained in the present study do not support the above mode of action. The acetic and formic acid combination has clear potential to enhance the growth because of an energy contribution, but it is unclear whether the benzoic/sorbic acid and benzoate/sorbate combinations can have the same effect. No information could be found on the absorption or metabolism of benzoic acid, sorbic acid, benzoate or sorbate by marine herbivores or abalone. Benzoic acid and benzoate are readily absorbed in the intestinal tract by some mammals (humans, rats, rabbits) and metabolised to hippuric acid which is excreted in the urine. There appears to be no difference between benzoic acid and the sodium and potassium salts in toxicology (Cong *et al.*, 2001; Griffith, 1925; WHO, 1974). The metabolism of benzoic acid and benzoate to hippuric acid involves an addition reaction to the aliphatic part of the benzoic acid and leaves the aromatic ring unchanged. None of the carbon of the benzoic acid is therefore available to the animal as an additional carbon and/or energy source. No indication from literature whether this is also true for any other animals could be found. Sorbic acid is readily metabolised via the same route as other fatty acids (Deuel *et al.*, 1954), quoted from (Lück *et al.*, 2000; Partanen and Mroz, 1999) and can therefore serve as an energy source to the abalone, with all the carbon in the structure being metabolisable. It is not known if sorbic acid salts (sorbate) can also be metabolised by animals, but it is assumed that the sorbate will readily accept a proton from the gut (pH 5.2) and then be metabolisable. If all the above is taken into account and the assumption is made that abalone cannot utilise any of the carbon in benzoic acid or benzoate, it can be calculated that the acids/salts in Treatment AF consists of 33% utilisable carbon, Treatment SBPS has 24% and Treatment BS has 32% carbon. The calculated amount of carbon present only in the crude protein and crude fat of the basal diet (Anonymous, 2005) is 33% of the total weight of the feed, with the assumption that none of the other carbon is available to the animal. This implies that the acid treatments cannot enhance the carbon content of the feeds, but at best can only match the current content. Further, it is not known whether data obtained in human and mammal studies regarding SCFA's effects in the colon are applicable to abalone, as the abalone does not

have a differentiated small intestine and colon, but only an intestine (Erasmus *et al.*, 1997). The effects of SCFA's as possible energy source to the intestine was therefore disregarded.

It could not be shown that the growth rate enhancement of the abalone in response to organic acid and acid salt supplements was due to a microbial effect of the treatments, although the possibility could also not be excluded based on the available data. The mainstream idea is that the organic acids and/or their salts have a microbial effect acting either as a bactericidal substance which lowers overall bacterial numbers or that the treatments alter the intestinal environment in such a way that certain beneficial bacteria colonise preferentially (Doyle, 2001; Gibson and Roberfroid, 1995; Partanen and Mroz, 1999). Both effects could translate into health benefits to the host. The data in this investigation support neither theory (see Table 28 and Table 29 and compare Table 30 to Table 33). No obvious differences were found between treatments in the composition or the number of bacteria in the microbial community of the gut, therefore no conclusions can be made about the effects of the different treatments on the intestinal microflora of the abalone.

The acid treatments do not have the same favourable effect in *Haliotis midae* during times of temperature stress than during optimal conditions. During the stress experiment no significant differences were observed in SGR between treatments, differing from the results obtained in the controlled optimal conditions experiment in this regard. The variance of the data increased during the stress experiment, causing the lack of significant differences. The acid treatments (treatment AF and BS) yielded the lowest SGR values during the stress period, while the acid salts (treatment SBPS) yielded the highest SGR. The benzoic/sorbic acid treatment performed markedly worse in the production conditions experiment than under controlled optimal conditions. Under controlled optimal conditions its performance was nearly identical to that of the other acid treatment, but under production conditions it showed the lowest SGR, highest FCR and highest Incidence costIC, making it the least effective treatment. Treatments AF and SBPS again show improved SGR over both control diets during the production conditions trial, although statistical significance could not be shown due to much higher variance than for the controlled optimal conditions experiment. This variance is attributed to relatively large water temperature variations observed during the course of the experiment, coupled with the occurrence of an algal bloom (see Figure 140 and Table 25 in Appendix) which has been shown to be potentially toxic to abalone (Botes *et al.*, 2003).

During both laboratory experiments, the different acids and salts used did not significantly affect the feed intake of abalone at the total inclusion levels of 2% used in the trial. Acetic and formic acid both have aggressive odours, but this did not affect the feed intake. No significant differences were found in total feed intake between treatments for either the controlled optimal conditions experiment or the controlled stress experiment. Feed intake could not be determined in the production conditions experiment. Certain acids have been known to affect feed intake in some animals. Due to the aggressive odours of some acids, feed intake can be reduced, while other acids stimulate feed intake (De Wet, 2005; Partanen and Mroz, 1999). Propionic acid decreased feed intake in chicks (Cave, 1984) quoted from (Gunal *et al.*, 2006). Other investigators reported that feed intake increased upon the addition of lactic and citric acid in diets for piglets (Tsiloyiannis *et al.*, 2001a, b), although it is not clear whether the acids stimulated feed intake or whether feed intake was higher due to improved growth rates observed in the animals.

The choice of which acids or salts to include in feeds is very important, as these substances differ in physical and chemical properties that may lead to different results. It is known that certain acids can affect palatability of feed (Partanen and Mroz, 1999), they differ in antibacterial properties (Hsiao and Siebert, 1999) and in their metabolic activities (Partanen and Mroz, 1999). Acids should be chosen according to the function they have to fulfil in the feed and/or animal ingesting the feed. In this study, the acids in treatment BS were chosen for their antibacterial activities, while the acids in treatment AF were chosen due to their apparent contribution in the energy metabolism of the abalone. Although none of the above modes of action could be verified in the abalone during this study, both these treatments showed potential as growth promoters in the South African abalone and therefore justified the choice of treatments.

The animals in the laboratory experimental system underwent an adaptation period that led to the increase in SGR and simultaneous decrease in FCR values observed over the duration of the controlled optimal conditions experiment. The steady increase in SGR observed throughout the experiment is contrary to what is expected, as SGR normally decreases with increasing animal age (Dr. Lourens de Wet, personal communication). FCR values decreased over the duration of the experiment (from 0.88 – 1.01 during period 1 to 0.64 – 0.77 during period 3). When the trial was initiated, the abalone had to adapt to another type of feed with a different appearance, texture, composition and possibly taste which caused an initial lag in growth and high FCR values. Animals were subjected to an acclimatisation period for 4 weeks in a trial done on *Haliotis midae* (Macey and Coyne, 2005) but the same was not possible in the present trial. The FCR values observed during the

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controlled optimal conditions experiment range from 0.64 – 1.01, which falls in the range previously published by Britz *et. al* of 0.6 – 1.3 for *Haliotis midae* ranging from 15 mm – 30 mm shell length (Britz *et al.*, 1994). It is possible to obtain FCR values that are < 1 because FCR is calculated as the amount of wet weight gained for an amount of dry feed consumed. Abalone can also absorb nutrients like calcium from their environment (Fleming *et al.*, 1996), which could further lead to FCR values < 1.

When the animals were subjected to temperature stress, performance parameters deteriorated significantly. This is reflected in the significant increase in IC and FCR from the values observed during the period D62 – D124 in the controlled optimal conditions experiment, with a simultaneous decrease in SGR during the same period. The decrease in SGR and increase in FCR is in agreement with the findings of Britz *et. al*, who found that weight gain declined sharply at temperatures above 20ºC and FCR increased significantly for temperatures above 20ºC in *Haliotis midae* (Britz *et al.*, 1997). The increased FCR and simultaneous decrease in SGR are related. The increased FCR points to a decreased ability of the animals to efficiently utilise feed at the elevated water temperatures and as a result of this, the growth rates of these animals decrease, which is reflected by the decreased SGR values. In a study conducted in the open sea in different areas around the South African coastline it was found that temperature did not have a direct effect on growth rate of the South African abalone *Haliotis midae* (Tarr, 1995). This is contradictory to both the current study and the one conducted by Britz and co-workers (Britz et. al, 1997). The probable reason for the different results is that the conditions in the open sea cannot be controlled and it is likely that other factors that were not taken into account were responsible for results obtained in that study. In the current study FCR ranged from 1.08 -1.21 at 20.5ºC. At 22ºC, the FCR value was found to be 1.82 and a gradual breakdown of physiological processes in abalone at temperatures higher than 20ºC was hypothesised (Britz *et al.*, 1997), which would explain the decrease in SGR and increase in FCR observed at a water temperature of 20.5 ºC. This research serves as further evidence that water temperatures higher than 20ºC fall outside the range of optimal conditions (based on SGR and FCR) for *Haliotis midae*.

The feeding cost of production of abalone under practical production conditions is significantly higher than production under controlled conditions, even though growth rates are very similar. The values obtained for the SGR under production conditions are between 0.78 – 0.88 d^{-1} and those of the controlled optimal conditions fall between 0.71 – 0.83 d^{-1}. The high IC for production conditions is due to the very high FCR values obtained in this experiment. Feeding was done in such a way that the animals had permanent access to feed, as it is done under true rearing conditions. The reason for the high FCR values is that

the unutilised feed could not be recovered from this system and it was assumed that all feed given was ingested by the abalone. Although this is not necessarily true, the FCR values obtained in this way give an indication of achievable values under practical rearing conditions, even though the person overseeing the system is an experienced feeder. FCR values reported in literature for *Haliotis midae* are between 0.6 - 1.3 (Britz *et al.*, 1994) using a formulated diet, 0.97 - 1.37 using an artificial pellet diet over a temperature range of 12ºC – 20ºC (Britz *et al.*, 1997) and 0.7 – 1.0 in 5 formulated feeds containing different protein sources (Britz, 1996a). All these studies were conducted under controlled conditions, resulting in the relatively low FCR values compared to those found under production conditions.

It appears as if abalone shell growth and weight increase responded differently to a temperature increase from 16.5ºC to 20ºC in the system investigated. AGRL increased when compared to the growth period D97 – D124 after water temperatures were raised from 16.5 ºC to 20.5 ºC, while the SGR (which is based on weight) decreased (compare Table 19 and Table 21, and Table 4 and Table 9), for all treatments. This means that the increase in shell length of the animals sped up, while the increase in mass slowed down. This is reflected in the decrease in Fulton CF from the start of the controlled stress experiment to the end, even though the differences are not statistically significant. Fulton CF values decreased during the controlled stress experiment for all treatments except treatment SBPS, even though the same decrease in SGR and increase in AGRL was observed in treatment SBPS. The reason why the increased shell growth is not reflected in the Fulton CF in treatment SBPS at the end of the controlled stress experiment is probably due to the fact that the decrease in SGR during the stress experiment for this treatment is smaller when compared to the decrease observed in the other treatments, and because the period for which these respective growth rates were valid was relatively short (only 28 days), not allowing the animal weights and lengths to change enough in order to reflect the effects of the different growth rates. A similar result was obtained in a study done on *Haliotis midae* when investigating the effect of temperature on the growth of the animals. It was found that the condition factor of the animals decreased as water temperature increased for a temperature range of 12ºC to 24ºC (Britz *et al.*, 1997). The decrease in condition factor in the temperature range 12ºC to 20ºC could not be explained by the authors, although the decrease in condition between 20ºC and 24ºC was attributed to stressful conditions. The decrease in condition factor observed between 12ºC and 20ºC is unexpected, as growth rates (both shell growth rate and weight growth rate) increased within this temperature interval (Britz *et al.*, 1997). If these increases in growth rates are proportional, the condition factor should stay the same. The data of Britz *et. al* 1997 and the current investigation therefore point to differing responses in

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shell growth rate and weight increase when water temperature varies, which results in different condition factors at different water temperatures. An investigation done on the abalone *Haliotis fulgens* also showed that there was a difference in the ratio of dry flesh weigh to shell weight at different water temperatures, with the higher ratio occurring at lower temperatures (García-Esquivel *et al.*, 2007).

A relationship between the shell length and total weight of the animal was established. After plotting the logarithm of the weight as a function of the logarithm of the length (using 7774 data points) and fitting a straight line to the resulting graph it was found that the relationship between length and weight for abalone within the size range of 15 mm – 47 mm is given by the equation:

$$W = 167 \times 10^{-6} L^{2.97}$$

where *W* is in gram and *L* is in mm. An almost identical result was obtained using 3000 specimens of *Haliotis midae* ranging from 6mm – 150mm, where an exponent of 2.99 was found (Britz *et al.*, 1997). The authors concluded that the growth relationship was allometric. If the value of the exponent of *L* is equal to 3.00, the abalones follow an isometric growth relation. The value of 2.97 was evaluated with statistics and it was found that the 95% confidence interval excludes 3.00, which confirms that the South African abalone follows an allometric growth relation. This result has possible practical implications where the length of animals can be used to estimate their weight (e.g. where image analysis of a large number of animals could be used to estimate weight as opposed to weighing each animal).

A variety of microorganisms were isolated at various stages from the selected abalones, but the role of these organisms in relation to growth of the host was not clear. 16S classification results showed that the organisms belonged to the genera *Staphylococci, Vibrio, Pseudomonas, Bacillus, Photobacterium* and *Shewanella*. A previous study done on the enteric bacteria of *Haliotis midae* found bacteria belonging to the following genera: *Alcaligenes, Pseudomonas, Micrococci, Flavobacteria, Enterobacteria, Vibrio, Aerococci, Aeromonas, Bacillus, Moraxella* and *Chromobacteria* (Erasmus, 1996). *Photobacterium*, *Shewanella*, and *Staphylococci* were isolated in the current study but not in the previous one, while *Alcaligenes, Micrococci, Flavobacteria, Enterobacteria, Aerococci, Aeromonas, Moraxella* and *Chromobacteria* were isolated during the previous study but not in the current one. The reason for this difference is probably because the current study eliminated the stomach and crop from the investigation while the previous one did not, and because different isolation media were used in the two studies as a result of the different aims of the

two studies. Further, Erasmus *et al.* (Erasmus *et al.*, 1997) focused on bacteria that degrade complex polysaccharides found in the natural diet of abalone, while the current study was mainly concerned with the effects of the treatments on potential pathogens (mainly *Vibrio* species), and the intestinal microbial community as a whole. The role that the different organisms isolated during this study play in polysaccharide degradation were not investigated, as it was not part of the scope of the project. It should be noted that iIn the study done by Erasmus, the organisms were only identified to genus level using morphological and biochemical tests which does not give an indication to which particular species the organisms belong. From literature it is evident that a variety of *Vibrio* species are commonly isolated from abalone and other marine animals and it has been reported as a pathogen as well as a potential digestive partner in abalone (Cai *et al.*, 2006a; Erasmus *et al.*, 1997; Lee *et al.*, 2001; Liu *et al.*, 2000; Nicolas *et al.*, 2002; Sawabe *et al.*, 2003). From 16S sequencing results, the most probable matches for the *Vibrio* isolates are *V. harveyi, V. rumosiensis, V. alginolyticus, V. parahaemolyticus, V. cyclitrophicus, V. tasmaniensis* and *V. splendidus*. Of these possible species, *V. rumosiensis, V. cyclitrophicus, V. tasmaniensis* and *V. splendidus* are not commonly associated with abalone, and there role in the intestine is unknown. *Bacillus* spp. has been shown to have health and growth benefits and to enhance digestive enzyme activity when used as a probiotic treatment in shrimp (Wang, 2007; Ziaei-Nejad *et al.*, 2006) and common carp (Wang and Zirong, 2006), but its role in abalone is unknown. *Alcaligenes, Pseudomonas, Flavobacteria, Vibrio, Bacillus* and *Moraxella* isolated from the digestive tract of *Haliotis midae* all showed polysaccharolytic enzyme activity and could possibly play a role in the digestion of seaweed in abalone (Erasmus, 1996). No enzymatic studies were performed in the current investigation, therefore no conclusions could be made with regards to the effect of the isolated bacteria on the digestion of *H. midae*. *Shewanella alga* has been isolated as a pathogen of the abalone *Haliotis diversicolor supertexta* (Cai *et al.*, 2006b). It is not known what the possible role of the remaining genera of bacteria play in the abalone, whether beneficial or detrimental or neither.

It was found that during certain periods specific organisms dominated the culturable portion of the bacteria, but no differences were observed between treatments (see Table 30 to Table 33 in Appendix). In the controlled optimal conditions experiment, colony B2 was dominant among the organisms isolated on BHI media during all periods from D34 – D124, E1 (a *Pseudomonas* species) was dominant on EAO media during the periods D62 – D97 and D97 – D124 and colony M10 was dominant on MRS media during period D62 – D97. During the controlled stress experiment (period S0 – S28), one particular *Bacillus* species was dominant on MRS, BHI and EAO media. This dominance of certain bacteria in abalone was also observed by other investigators. It was found that up to 61% of culturable bacteria in the intestine of the South African abalone *Haliotis midae* belonged to only 3 different genera (Erasmus, 1996). Another investigation found that one species of bacteria, *Vibrio halioticoli* comprised between 40.2% and 60.2% of the total intestinal bacteria in various abalones, including *Haliotis midae* (Sawabe *et al.*, 2003).

Significant changes occurred to the composition of the microbial community of the gut during the temperature stress experiment. The total number of different culturable colonies decreased from a total of 10 at the end of period D97 - D124 to only 4 at the end of period S0 – S28. M15, an organism of the genus *Bacillus* which was not previously isolated during the controlled optimal conditions trial, was dominant at the end of the stress experiment. At the end of the stress experiment no growth was observed on the TCBS plates, suggesting that the particular *Bacillus* species replaced all the *Vibrio, Photobacterium* and *Shewanella* spp.

The possibility exists that there was a change in the composition of the intestinal microbial community of the abalone as a result of the different treatments, but that this change could not be detected by the methods used. It appears that the culturing methods used in the current study are not sensitive enough to show any minor changes in the composition of the microbial community of the gut that might have occurred during this study. Plate counts yielded erratic results and in some instances quantitative data could not be obtained. Erasmus found large differences [of up to 4 orders of magnitude (10^4)] in bacterial numbers isolated from the gut of *Haliotis midae* using culturing methods and culture independent methods (Erasmus, 1996). Culturing methods introduce a large bias in the results, as not all bacteria that are found in the abalone are culturable (Dr. Anna Mouton and Dr. Kim ten Doeschate, personal communications). It has been shown that some bacteria that are very abundant in marine environments are not culturable (Eilers *et al.*, 2000), while another study found that non culturable bacteria were abundant in the seawater tested in that study and comprised more than 99.9% of the marine bacterial community of that specific sample (Ferguson *et al.*, 1984). It is further known that certain bacteria enter a viable but non-

culturable (VBNC) state in response to certain stress factors, including changes in salinity, nutrient concentration, temperature and light (McDougald *et al.*, 1998). These bacteria that enter this condition will not be culturable, and would therefore not be detectable with culturing techniques. It is possible that no change in bacterial composition could be detected as a result of certain species of bacteria entering the VBNC state as a result of conditions that changed from pre-sampling (when the organisms were in the abalone gut), to post sampling (when the organisms were being grown on selective isolation media). No attempt was made to isolate organisms on media that contain complex polysaccharides as did Erasmus (Erasmus, 1996), as this study was focused on potential pathogens. This may have accounted for the lack of observed differences between treatments, as the treatments may have affected organisms that are part of the polysaccharide degrading population of the abalone gut. Finally, changes in bacterial populations may occur as a result of sub-lethal injury of organisms as a result of the sample preparation technique (McDougald *et al.*, 1998), which also may have affected the results obtained.

It is possible that the temperature at which bacteria were incubated in the laboratory had an effect on the results obtained. Some evidence of this was found during the classification of the intestinal microflora of the abalone. The *Vibrio* bacteria were not culturable on solid media at 30ºC, although they could be grown up in liquid media at 30ºC and on solid media at room temperature. This indicates that the organisms are sensitive to a combination of incubation temperature and the phase of the media. Bacteria are known to have certain temperature ranges in which they are able to grow, specific to each species. If the temperature at which organisms are incubated falls outside of this range, growth will not occur (Bailey and Ollis, 1986). It is also accepted that temperature has a very significant effect on the growth rate of bacterial colonies within their specific temperature ranges (Ratkowsky *et al.*, 1982). Water temperatures were kept constant at 16.5ºC throughout the controlled optimal conditions experiment and at 20.5ºC during the controlled stress experiment, but agar plates prepared from animals kept at these temperatures were incubated at laboratory room temperature. It is therefore possible that the difference in experimental and incubation temperatures had a significant effect on the species and numbers of bacteria that were culturable. It is then also further possible that the portion of the intestinal microbial community that was affected by the different treatments was not culturable due to a temperature effect. This would explain the apparent lack in response observed within the microbial community of the gut after the different treatments were administered.

5.3   IMPLICATIONS OF RESULTS IN INDUSTRY

Some of the results obtained during this study have particular applications to the abalone aquaculture industry. These are summarised shortly and the implications that these results may have for the industry is highlighted.

-        Improved growth rates as a result of dietary addition of certain organic acids to abalone feed.

-        AGP did not improve growth rates.

-        The acids used in the current trial at the chosen inclusion levels did not affect feed intake.

-        The acids and salts did not produce different results.

-        The animals had to adapt to new conditions and production parameters suffered as a result.

-        Feed costs are much higher for practical production conditions than for controlled laboratory conditions.

-        A relationship between animal length and weight was established.

The industry can benefit from the current research if it is applied correctly. Improved SGR implies that abalone production time can be shortened, or heavier animals can be produced during current production times, improving profitability of producers. Producers can claim improved sustainability and environmentally friendly production of abalone as the feed is free of any antibiotics, yet it still promotes growth. The feed industry will also benefit from having a new product to market. Except for the on-farm benefits to producers, the marketing potential of the product is significant and should not be underestimated.

Acid salts have certain benefits to the pure acid form, which may warrant the inclusion of the salt rather than the acid in animal feeds. Certain acids are in the liquid phase (e.g. both acetic and formic acid) which implies that they may be volatile, pure acids can have very strong odours and are more corrosive to feed manufacturing equipment than salts.

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Generally, working with acid salts will avoid most of these problems, as they exist as solids, are mostly odourless and less reactive toward equipment and they may be more soluble in water (Partanen and Mroz, 1999). The acid salts therefore have the potential benefit of simplifying and reducing the cost of the feed manufacturing process by eliminating the need to handle, administer and/or mix volatile and corrosive liquids. The reduced volatility and reactivity of the organic acid salts compared to pure organic acids will also enhance the storage capability of feeds, as the active ingredient will not vaporise or react and become inactive. If the acid salts show the same efficacy as growth promoters than the pure acids (as was seen for the case for the acids and salts tested in South African abalone in the current project), feed manufacturers may prefer the salts to the pure acids.

The inclusion of the various acids and salts in the abalone feed did not affect the feeding cost of abalone in the current trial. The different treatments had no significant effect on the IC in any of the experiments (laboratory or production conditions). This result has the potential to benefit the industry, as abalone can be produced at higher growth rates and at no significant extra cost.

New, improved husbandry practices could be developed from some of the current results. It is clear that abalones need a considerable amount of time to adapt to a new diet before growth rates and FCR reach optimal levels. This should be kept in mind by producers when working out feeding regimes for abalone farms, as each time the diet of the animals change, they may have to go through the adaptation period and thereby lower production. Further, there was a large amount of feed wastage in the current experiment in the practical production conditions trial that had a direct influence on FCR and IC. Feed wastage is also reported as being a general problem in aquaculture (Ang and Petrell, 1997) and the possible detrimental effects it can have on feeding cost was seen in this trial. It is clear that there is a large scope for further investigation to improve feed utilisation under production conditions, either by establishing optimal feeding levels or by increasing feed availability to animals by improving tank designs, feed water stability etc. Optimisation of production and minimisation of production costs will both improve profitability of the enterprise.

The establishment of a length-weight relationship for abalone could prove to be an invaluable management tool for on-farm managers. It is now easy to establish whether animals are in bad condition (possibly due to stress) and the cause can be established and remedied. Alternatively, animal weight can be established quickly and accurately from image analysis for many different containers and sections of the farm and feed rations adjusted accordingly, thereby improving management efficiency. In both instances, the result has the potential to improve farm management and profitability.

6. CONCLUSIONS

It has been shown that organic acids and organic acid salts can act as growth promoters in the South African *Haliotis midae*, causing a significant increase in SGR compared to a negative control diet and to a diet containing a commercial antibiotic growth promoter. The mode of action of the organic acids in *Haliotis midae* could not be established in this trial, but some mechanisms were eliminated as being inconsistent with data. It was found that the organic acids did not enhance the growth of abalone in the current trial by having a pH effect in the gut, or by acting as an additional carbon and/or energy source for the animals. It could not be shown that the SGR of the animals in the system was enhanced due to a microbial effect of the different treatments on the microflora found in the intestinal tract, but this mechanism could not be eliminated either (mainly as a consequence of the microbiological methods used).

Avilamycin as dietary antibiotic growth promoter had no effect on growth rate or feed utilisation of *Haliotis midae* in the current trial, even though inclusion levels were comparable to industry standards for terrestrial animals and the substance has activity against many of the organisms isolated from the abalone.

It could not be shown with the methods used that the different treatments had a significant effect on the intestinal microflora of the abalone during the experiment. It is possible that the chosen methods are not sensitive enough to detect any changes that occurred as a result of the different treatments in the composition of the intestinal microbial community of the abalone. It is further also possible that a temperature effect significantly influenced the microbiological results obtained.

A variety of microorganisms were isolated at various stages during the study, but the role that these organisms play in the growth, health and/or digestion of the abalone is not known. During certain growth periods, specific bacteria were dominant among the culturable organisms of *Haliotis midae*.

It was seen that the animals in the laboratory experiment underwent and adaptation period that initially caused low growth rates and high FCR values, but both parameters improved throughout the controlled optimal conditions experiment. Under sub-optimal temperatures the performance of the animals in the laboratory experiment deteriorated significantly and the acids did not have the same favourable effects as observed during controlled optimal conditions. Though growth rates under production and controlled optimal conditions seem to be very similar, the production of abalone under practical conditions is less efficient as a result of decreased feed utilisation (mainly caused by feed wastage).

A relationship between shell length and animal weight has been established for *Haliotis midae*. This result has the potential to develop into an additional farm management tool that could improve production. It has further been shown that the South African abalone *Haliotis midae* does not follow an isometric growth relationship. It appears as if shell growth and weight increase in *Haliotis midae* respond differently to changes in water temperature.

The choice of which acid(s) and in which form (pure acid or acid salt) to include the substances, in experiments or commercial products, is very important as it can significantly influence results and feed manufacturing practices. In this investigation the different acids and salts did not affect feed intake in either of the laboratory experiments.

7.   RECOMMENDATIONS

There are many difficulties associated with working or experimenting with abalone, even under controlled conditions. To simplify the task of subsequent experimenters, the following recommendations are made:

In trials investigating growth characteristics, ensure that the trial is run over a long enough period of time so that any effects due to treatments have opportunity to manifest. Abalones are slow growing and effects take time before they become significant. It is suggested that the minimum period for growth trials be approximately 120 days. If the animals undergo a period of stress or if large fluctuations in conditions are experienced during the trial period, it might be necessary to extend the trial, as the variance of data may increase, masking any significant differences. It is further recommended that animals be acclimatised prior to beginning the trial in order to avoid inconsistent results. Acclimatisation should extend for at least 4 weeks.

In trials where entire experimental units (e.g. the entire population of animals within a container) are not sampled, attention should be given to the sampling method. The number of animals taken for sampling should be standardised and the selection of animals should be such that it is truly random. In this trial, 20 animals were sampled from containers containing approximately 1000 animals, but results were not consistent and it is recommended that at least 25 – 30 animals be sampled in similar situations. If at all practically possible, the animals should be anaesthetised in order to immobilise them (to simplify sampling) and to avoid injury.

Further trials investigating the effect of organic acids and their salts should focus on various aspects. It is very important to establish at which concentrations these substances have their effects and what the optimal concentrations are. The mechanism by which the acids have their effect and the site in the gastro-intestinal tract where this effect occurs is also very important. Different organic acids should be investigated to determine their efficacy and whether any differences exist between acids in abalone culture. This could not be established in this investigation. More accurate microbiological studies should be done to ascertain whether the effect is microbial. Focus should be on culture independent methods in order to eliminate the shortcomings of the current study. Bacteria could be investigated as possible probiotics, starting with the dominant species found among the natural intestinal microflora of the abalone, as there is a great need for research into specific host-microbe

interactions in the marine environment. Animals could also be challenged with a known pathogen in order to determine the effects of dietary organic acids in abalone on disease resistance.

An accurate method must be found to record mortalities during experiments. Animals moved around inexplicably between containers in the laboratory experiment, even though containers were covered with nets to prevent this. This, coupled with the fact that very few animals died throughout the duration of the experiments, made the recording of mortalities impractical. In the production conditions experiment the exact number of animals in each container was not known and a high number of animals were present which also rendered the recording of mortalities impractical.

The following recommendations are made to the industry:

In order to formulate an optimal product, it is recommended that some additional investigations be conducted in future to answer certain crucial questions. There are still many questions regarding the use of organic acids as growth promoters, especially in aquaculture. It is necessary to establish which acids are effective, whether different acids have different effects and what the optimum inclusion levels of these substances are. Further, it is not known whether there are any significant differences in effects between the pure acids or the acid salts, or whether performance can be further enhanced by using a mixture of acids and salts (as suggested by literature). The acids and/or salts could also be tested in combination with other products e.g. plant extracts and essential oils. The scope for new investigations is very broad.

It was clearly shown that organic acids can enhance the growth rate of South African abalone in the size classes used in this study, but it is not known whether these acids will have the same beneficial effect in larger animals. Future investigations should attempt to answer this question.

Organic acids have been shown to be effective in the South African abalone, but there are many more abalone species for which the effects are not known. There are also many other aquaculture species in which the effects have not been tested. It should be investigated whether organic acids have the same beneficial effects in other species of abalone and in other aquaculture organisms, as the potential exists to develop an environmentally friendly product that enhances aquaculture production. The marketability of products containing non-antibiotic growth promoters could further increase returns, as the possibility exists that

consumers might pay higher prices for products that are known to be environmentally friendly.

The abalone industry should investigate ways to improve on farm feed management. It has been shown that FCR is much higher for practical production conditions than for controlled laboratory conditions and feed wastage is the main reason for this. Seeing that feeding cost is one of the main operating costs for abalone culture, any improvement in feed management will directly impact economic return. Wastage can be minimised by either improving feeding regimes allowing optimal feeding, or improving feed characteristics and properties that would allow improved utilisation. The relationship between length and weight established in the current study may be useful to keep track of animal weight, which in turn could be used to adjust the amount of feed given to suit the need of animals at any particular time. The possibility of utilising this tool should be investigated further.

If probiotic organisms for abalone are investigated, species from the indigenous microflora should be investigated first. Many bacteria from the genus *Bacillus* were isolated from *Haliotis midae* in the current investigation and it has been shown that organisms belonging to this genus have beneficial effects on various aquaculture organisms under practical production conditions. It is proposed that preliminary screening be done on these organisms when evaluating potential probiotic organisms for *H. midae*.

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9. APPENDIX

9.1 Growth trials

9.1.1 Laboratory experiment: Optimal conditions

All data presented are given as means ± SD.

Table 15 Animal weight, length, condition and number per treatment at initiation of controlled optimal conditions experiment.

	D0			
Treatment	**Weight (g)**	**Length (mm)**	**Fulton CF**	**n**
NC	2.20 ± 0.233	23.82 ± 0.842	0.163 ± 0.008	240
AF	2.05 ± 0.258	23.40 ± 0.817	0.160 ± 0.008	240
PC	2.08 ± 0.258	23.39 ± 0.817	0.162 ± 0.008	240
SBPS	1.97 ± 0.202	22.92 ± 0.857	0.163 ± 0.007	240
BS	2.17 ± 0.279	23.69 ± 0.936	0.162 ± 0.011	240

Table 16 Animal weight, length, condition and number per treatment at the end of growth period D0 – D34, as well as linear growth rates experience during that particular growth period.

	End of period D0 - D34					
Treatment	**Weight (g)**	**Length (mm)**	**Fulton CF**	**n**	**AGRL (µm/day)**	**AGRW (mg/day)**
NC	2.66 ± 0.199	26.10 ± 0.750	0.150 ± 0.006	243	67.0 ± 7.25	13.44 ± 3.16
AF	2.69 ± 0.311	26.07 ± 0.939	0.152 ± 0.005	243	78.4 ± 9.81	18.89 ± 3.41
PC	2.59 ± 0.311	25.79 ± 0.939	0.150 ± 0.005	236	70.8 ± 9.81	14.89 ± 3.41
SBPS	2.57 ± 0.313	25.69 ± 1.064	0.151 ± 0.004	233	81.5 ± 13.87	17.59 ± 4.96
BS	2.80 ± 0.365	26.53 ± 0.865	0.149 ± 0.008	236	83.5 ± 10.74	18.74 ±5.04

Table 17 Animal weight, length, condition and number per treatment at the end of growth period D34 - D62, as well as linear growth rates experience during that particular growth period.

Treatment	End of period D34 - D62					
	Weight (g)	Length (mm)	Fulton CF	n	AGRL (µm/day)	AGRW (mg/day)
NC	3.45 ± 0.224	27.71 ± 0.689	0.162 ± 0.007	233	58.3 ± 10.18	28.51 ± 2.19
AF	3.55 ± 0.371	27.92 ± 1.046	0.163 ± 0.002	228	66.5 ± 10.47	30.51 ± 5.01
PC	3.29 ± 0.371	27.32 ± 1.046	0.161 ± 0.002	229	54.7 ± 10.47	25.15 ± 5.01
SBPS	3.31 ± 0.310	27.31 ± 1.020	0.163 ± 0.010	226	58.2 ± 16.93	26.65 ± 3.50
BS	3.73 ± 0.376	28.32 ± 0.713	0.164 ± 0.006	231	64.1 ± 10.67	33.18 ± 3.32

Table 18 Animal weight, length, condition and number per treatment at the end of growth period D62 - D97, as well as linear growth rates experience during that particular growth period.

Treatment	End of period D62 - D97					
	Weight (g)	Length (mm)	Fulton CF	n	AGRL (µm/day)	AGRW (mg/day)
NC	4.21 ± 0.303	30.16 ± 0.825	0.153 ± 0.004	225	70.1 ± 11.04	21.63 ± 4.15
AF	4.54 ± 0.488	30.86 ± 1.146	0.154 ± 0.005	219	84.4 ± 5.37	28.51 ± 5.46
PC	4.05 ± 0.488	30.11 ± 1.146	0.148 ± 0.005	218	80.0 ± 5.37	21.83 ± 5.46
SBPS	4.23 ± 0.412	30.30 ± 1.083	0.152 ± 0.008	218	85.6 ± 11.47	26.27 ± 6.82
BS	4.78 ± 0.468	31.30 ± 0.839	0.156 ± 0.007	224	85.4 ± 7.62	30.25 ± 5.71

Table 19 Animal weight, length, condition and number per treatment at the end of growth period D97 - D124, as well as linear growth rates experienced during that particular growth period.

Treatment	End of period D97 - D124					
	Weight (g)	Length (mm)	Fulton CF	n	AGRL (µm/day)	AGRW (mg/day)
NC	5.28 ± 0.413	32.16 ± 0.781	0.158 ± 0.006	215	73.3 ± 9.73	39.26 ± 8.77
AF	5.73 ± 0.538	33.04 ± 1.127	0.159 ± 0.007	211	80.0 ± 13.82	43.89 ± 8.27
PC	5.21 ± 0.538	32.24 ± 1.127	0.155 ± 0.007	211	78.5 ± 13.82	42.67 ± 8.27
SBPS	5.43 ± 0.411	32.36 ± 1.166	0.160 ± 0.008	209	75.9 ± 14.29	44.16 ± 7.94
BS	5.94 ± 0.479	33.29 ± 0.902	0.161 ± 0.007	218	73.4 ± 12.56	42.64 ± 11.51
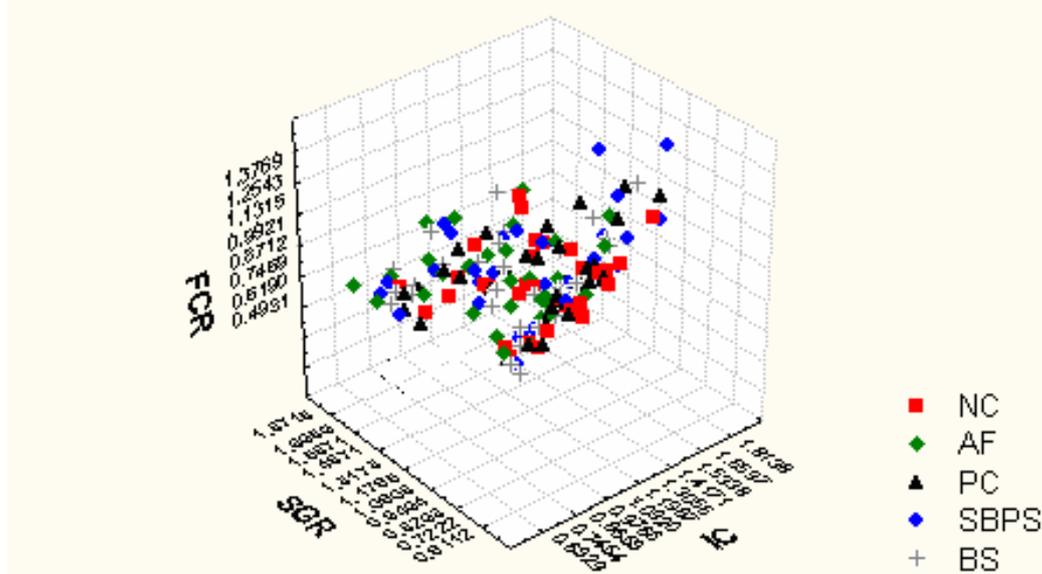
Figure 10 Representation of SGR, FCR and IC for all treatments for all growth periods during the controlled optimal growth conditions experiment.

Table 20 Animal weight, length, condition and number per treatment at the end of growth period S0 - S16, as well as linear growth rates experience during that particular growth period.

	End of period S0 - S16					
Treatment	**Weight (g)**	**Length (mm)**	**Fulton CF**	**n**	**AGRL (µm/day)**	**AGRW (mg/day)**
NC	6.04 ± 0.582	33.64 ± 1.041	0.158 ± 0.004	209	92.4 ± 23.30	47.57 ± 28.06
AF	6.48 ± 0.537	34.46 ± 1.102	0.158 ± 0.004	205	88.6 ± 11.56	46.99 ± 12.22
PC	6.03 ± 0.537	33.84 ± 1.102	0.155 ± 0.004	205	100.0 ± 11.56	51.46 ± 12.22
SBPS	6.32 ± 0.578	34.11 ± 1.313	0.159 ± 0.007	201	108.9 ± 15.45	53.14 ± 14.06
BS	6.66 ± 0.385	34.82 ± 0.931	0.158 ± 0.005	214	95.6 ± 12.60	44.57 ± 19.19

Table 21 Animal weight, length, condition and number per treatment at the end of growth period S16 - S28, as well as linear growth rates experience during that particular growth period.

Treatment	End of period S16 - S28					
	Weight (g)	Length (mm)	Fulton CF	n	AGRL (µm/day)	AGRW (mg/day)
NC	6.69 ± 0.569	35.07 ± 0.994	0.155 ± 0.004	208	119.5 ± 20.15	54.62 ± 16.02
AF	7.13 ± 0.627	35.72 ± 1.146	0.156 ± 0.003	204	105.6 ± 14.29	54.14 ± 11.65
PC	6.75 ± 0.627	35.18 ± 1.146	0.155 ± 0.003	203	111.0 ± 14.29	59.82 ± 11.65
SBPS	7.22 ± 0.749	35.45 ± 1.349	0.162 ± 0.004	203	112.0 ± 21.04	75.10 ± 29.76
BS	7.42 ± 0.503	36.11 ± 0.940	0.157 ± 0.005	216	107.5 ± 12.20	63.38 ± 14.00
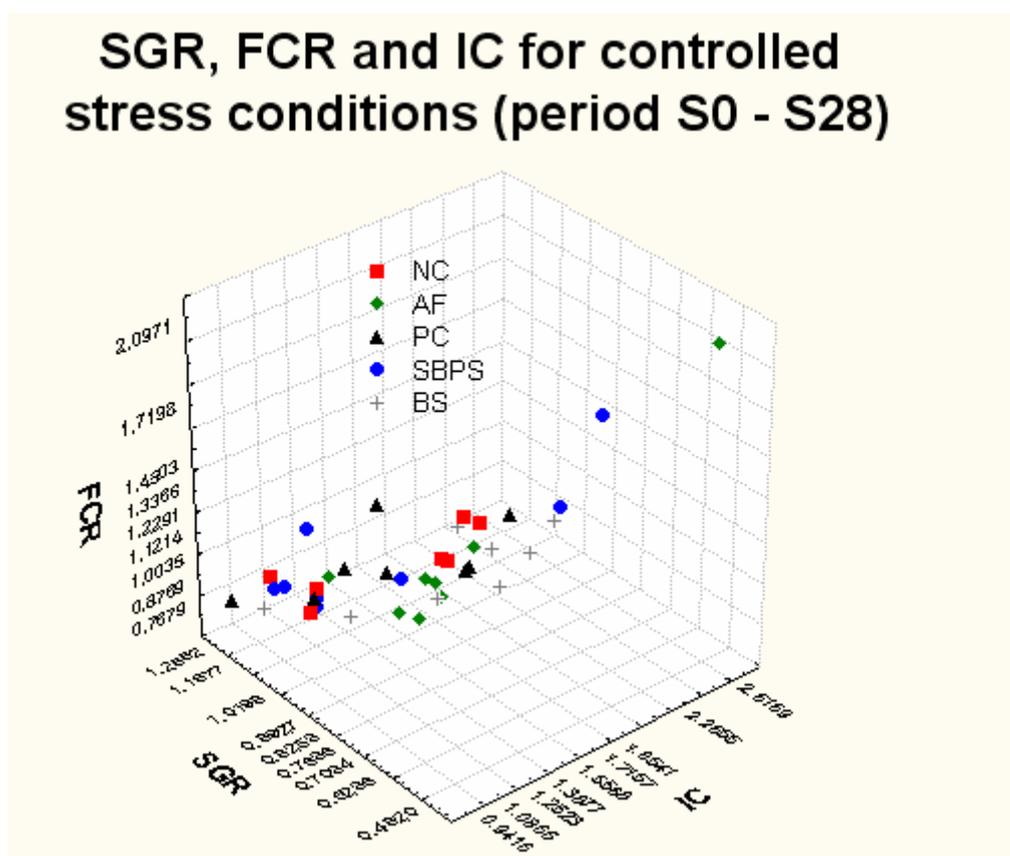
Figure 11 Representation of all data for SGR, FCR and IC for the controlled stress conditions experiment.

Linear regression of logarithmically transformed length and weight data

ln(W) = -8.6965 + 2.9729 ln(L)
95% Prediction intervals shown

r² = 0.9602
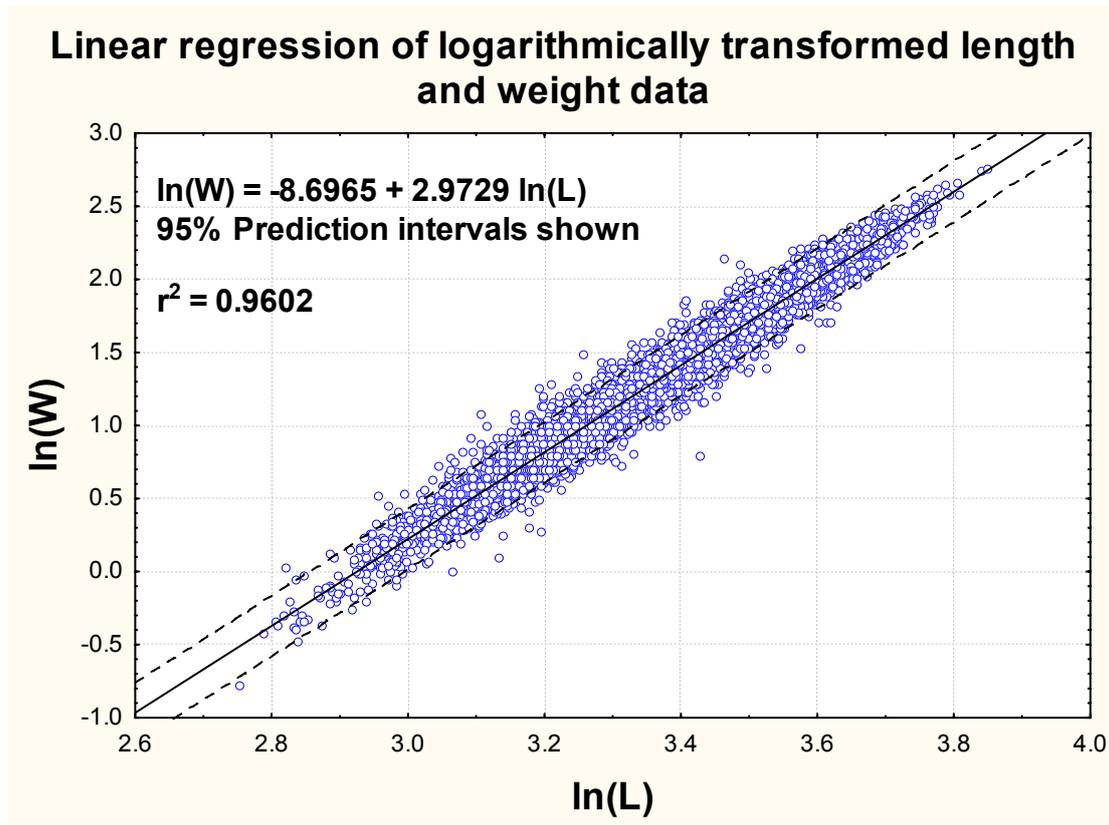
Figure 12 Logarithmic transformation and linear regression for length and weight data for laboratory experiments.

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9.1.2 Production conditions

Table 22 Animal weight, length and condition at the end of growth period D0 – D27 for production conditions.

Treatment	D0 – D27		
	Weight (g)	Length (mm)	Fulton CF
NC	0.92 ± 0.12	17.65 ± 0.46	0.167 ± 0.021
AF	1.09 ± 0.22	18.28 ± 1.03	0.177 ± 0.011
PC	0.97 ± 0.14	17.62 ± 0.79	0.177± 0.014
SBPS	1.01 ± 0.11	18.11 ± 0.78	0.171 ± 0.014
BS	0.98 ± 0.13	18.24 ± 0.66	0.161 ± 0.011

Table 23 Animal weight, length and condition at the end of growth period D27 – D62 for production conditions

Treatment	D27 – D62		
	Weight (g)	Length (mm)	Fulton CF
NC	0.99 ± 0.09	17.86 ± 0.52	0.174 ± 0.012
AF	1.20 ± 0.12	18.96 ± 0.96	0.177 ± 0.011
PC	0.96 ± 0.15	17.71 ± 0.92	0.171 ± 0.003
SBPS	1.09 ± 0.10	18.64 ± 0.58	0.169 ± 0.007
BS	1.13 ± 0.20	18.92 ± 1.18	0.166 ± 0.003

Table 24 Animal weight, length and condition at the end of growth period D62 – D90 for production conditions

Treatment	D62 – D90		
	Weight (g)	Length (mm)	Fulton CF
NC	1.10 ± 0.10	18.63 ± 0.50	0.171 ± 0.008
AF	1.20 ± 0.16	18.89 ± 1.12	0.177 ± 0.012
PC	1.10 ± 0.13	18.30 ± 0.62	0.179 ± 0.011
SBPS	1.15 ± 0.15	18.83 ± 0.95	0.172 ± 0.009
BS	1.08 ± 0.13	18.46 ± 0.65	0.172 ± 0.009
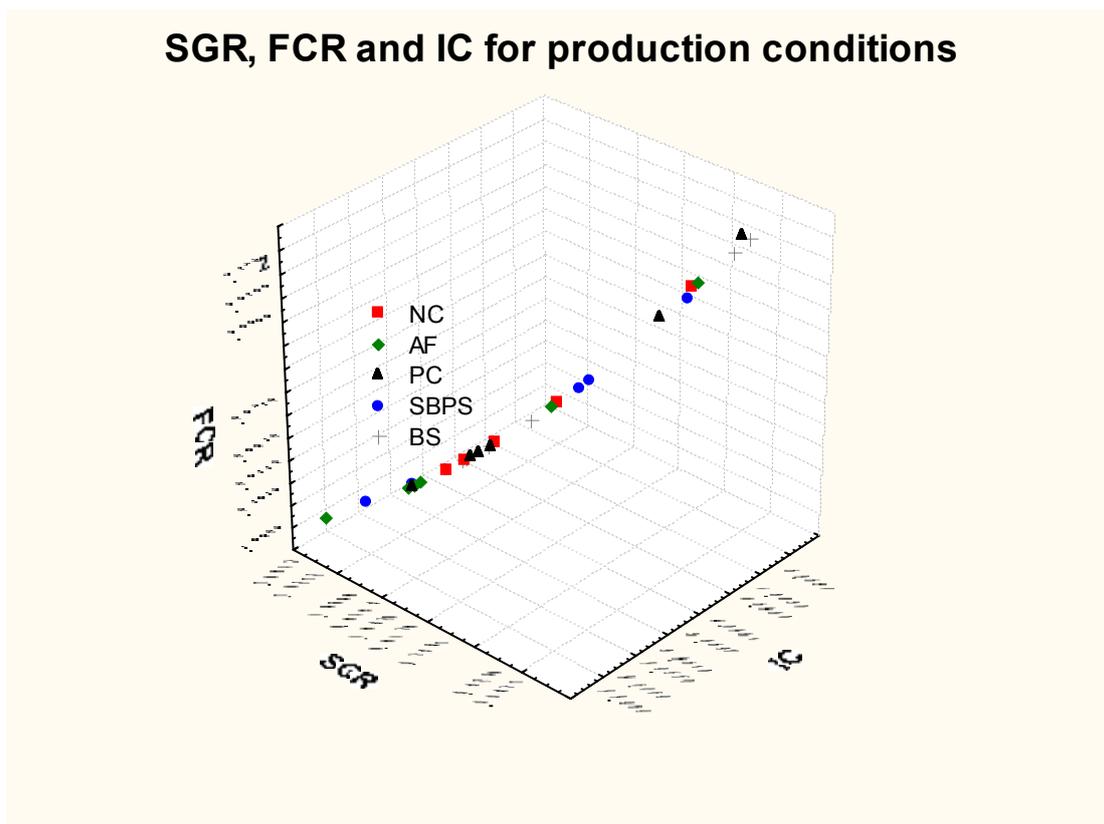
Figure 13 Representation of all data for SGR, FCR and IC for production conditions experiment.

Table 25 Temperature profile from December 2006 to June 2007 for Danger Point, Gansbaai, Western Cape, South Africa

	Temperature ºC						
	December	**January**	**February**	**March**	**April**	**May**	**June**
Mean	18.90	19.47	19.04	17.35	17.28	17.26	16.78
SD	1.523	2.110	1.915	1.607	0.613	0.600	0.390
Min	15.00	15.50	14.80	13.70	16.20	15.80	16.20
Max	21.70	22.50	22.20	20.60	18.30	18.20	17.40

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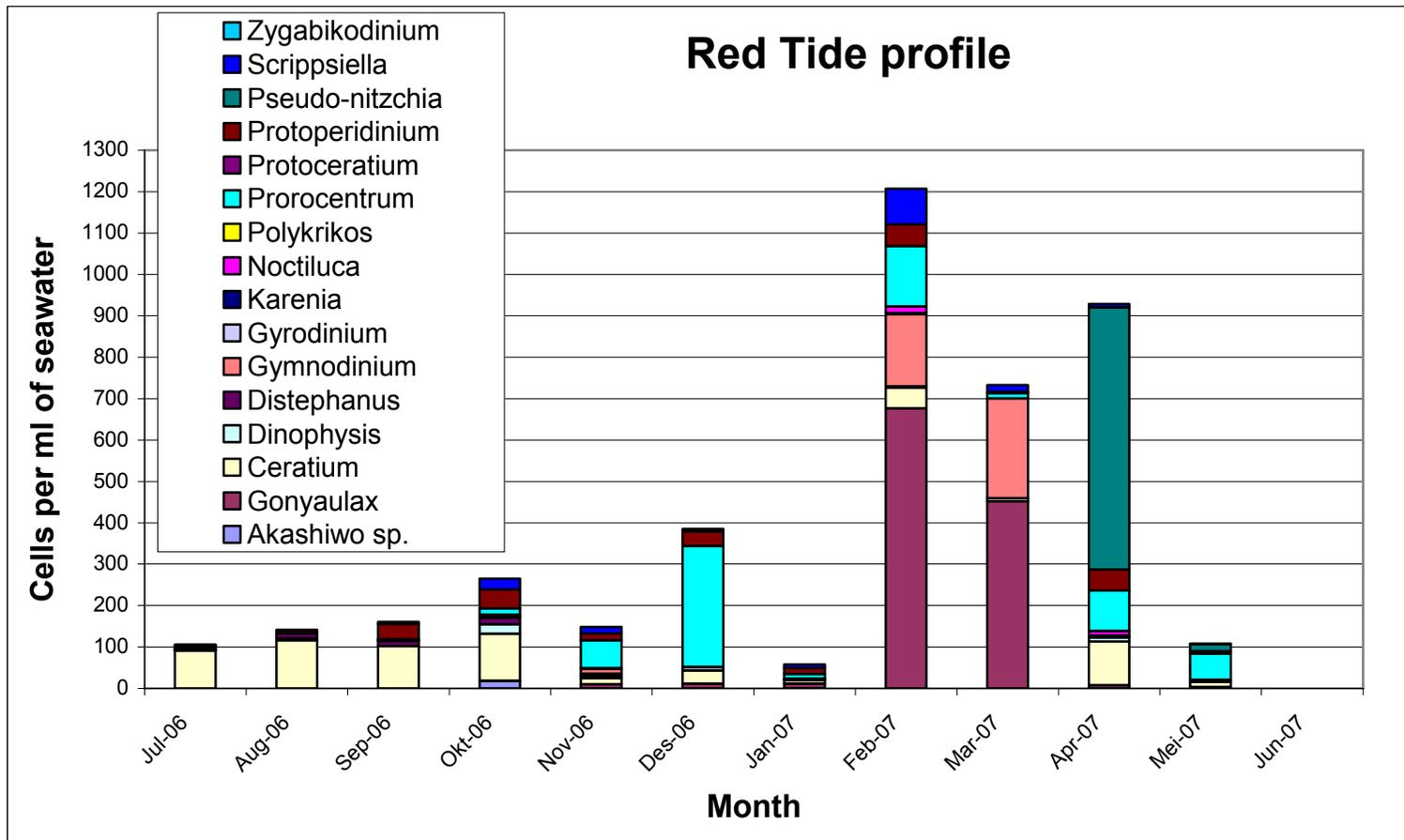
Figure 14 Red tide profile for July 2006 to June 2007 for Danger Point, Gansbaai, South Africa

9.2   Characterisation of gut microflora

Table 26 Bacterial colony characteristics when grown on original isolation media.

Name	Genus	Form	Elevation	Margin	Appearance	Optical properties	Colour	Texture	Shape
B2	Unknown	Round	Raised	Entire	Shiny	Opaque	Yellow	smooth	
B3	*Staphylococci*	Round	Raised	Entire	Dull	Opaque	Cream, slightly orange at edges	smooth	Round
B4	*Vibrio*	Round	Flat	Erose	Shiny	Transparent	-	smooth	
B5	Unknown	Round	Flat	Erose	Dull	Transparent	-	Rough	
B7	Unknown	Round	Raised	Entire	Shiny	Opaque	Yellow	smooth	Round
B8	Unknown	Round	Convex/raised	Entire	Shiny	Opaque	Bright white	smooth	
B9	Unknown	Round	Raised	Entire	Shiny	Translucent	Orange	smooth	
E1	*Pseudomonas*	Round	Raised/convex	Entire	Shiny	Opaque	Yellow	smooth	Rods
E2	Unknown	Round	Flat	Entire	Shiny	Translucent	Bright yellow	smooth	Round
E3	*Bacillus*	Round	Flat/raised	Entire	Shiny	Opaque	Bright white	smooth	
E4	*Bacillus*	Round	Raised	Entire	Shiny	Translucent	Cream	smooth	Rods
E5	*Pseudomonas*	Round	Raised	Entire	Shiny	Opaque	Pale white	smooth	Rods
E6	*Bacillus*	Round	Flat	Entire	Dull	Opaque	Pale white	smooth	
E8	*Bacillus*	Round	Flat	Entire	Dull	Opaque	Pale white	smooth	
E9	Unknown	Round	Flat	Entire	Shiny	Transparent	Yellow	smooth	Rods
E10	*Pseudomonas*	Round	Flat	Entire	Shiny	Translucent	Pink	smooth	Rods
M2	*Bacillus*	Round	Raised	Curled	Dull	Opaque	Pale white	Rough	Rods
M4	*Bacillus*	Round	Raised	Entire	Shiny	Opaque	Fudge/human colour	smooth	Rods
M6	Unknown	Round	Flat	Entire	Shiny	Opaque	Dark pale white	smooth	Rods
M8	Unknown	Round	Raised	Entire	Shiny	Opaque	Fudge/human colour	smooth	Rods
M10	Unknown	Round	Raised	Entire	Shiny	Opaque	Light yellow	smooth	Rods
M15	*Bacillus*	Round	Flat	Erose	Dull	Translucent	Pale white	Rough	Rods?
V11	*Vibrio*	Round	Raised	Entire	Shiny	Translucent	Orange/yellow	smooth	Oval
V12	*Photobacterium*	Round	Raised	Entire	Shiny	Translucent	Greenish	smooth	
V17	*Vibrio*	Round	Flat	Entire	Shiny	Translucent	Yellow/orange	smooth	Rods
V18	*Shewarella*	Round	Flat/raised	Entire	Shiny	Translucent	Greenish	smooth	Rods
M1	Unknown	Round	Undulate	Entire	Shiny	Opaque	Bright white	smooth	
M3	Unknown	Round	Raised/convex	Entire	Shiny	Opaque	Bright pink	smooth	
V1	Unknown	Round	Undulate	Entire	Shiny	Opaque	Dark green	smooth	
V5	Unknown	Round	Undulate	Entire	Shiny	Opaque	White	smooth	

Table 27 Table of characteristics and names for selected bacteria when grown on TSB media.

Symbol	Genus	Most likely species	Colony form	Elevation	Margin	Appearance	Optical properties	Colour	Texture	Shape
B2	Unknown		Round	Flat	Entire	Shiny	Translucent	Creamy/pale mustard	smooth	
B3	*Staphylococci*	*S. saprophyticus S. brasiliensis S. xylosus*	Round	Flat	Entire	Shiny	Opaque	Mustard/fudge	smooth	Round
B4	*Vibrio*	*V. harveyi V. rumosiensis V. alginolyticus V. parahaemolyticus*	No growth on TSB							
E1	*Pseudomonas*	*P. putida P. fulva*	Round	Raised/convex	Entire	Shiny	Translucent	White	smooth	Rods
E2	Unknown		Round	Flat	Entire	Shiny	Translucent	Pale fudge	smooth	Round
E3	*Bacillus*	*B. subtilis*								
E4	*Bacillus*	*B. sphaericus B. formis*	Round	Flat	Entire	Shiny	Opaque	Fudge	smooth	Rods
E5	*Pseudomonas*	*P. putida*	Round	Flat	Entire	Shiny	Opaque	Light yellow	smooth	Rods
E6	*Bacillus*	*B. pumilus*								
E8	*Bacillus*	*B. sphaericus*								
E9	Unknown		Round	Raised	Irregular	Shiny	Opaque	Light brown	smooth	Rods
E10	*Pseudomonas*	*P. putida, P. fulva*	Round	Flat			Translucent	Pink	smooth	Rods
M2	*Bacillus*	*B. subtilis*	Round	Raised	Irregular	Dull	Opaque	Pale white	Rough	Rods
M4	*Bacillus*	*B. oteronius B. sporothermodurans*	Round	Flat	Entire	Shiny	Opaque	Light brown	smooth	Rods
M5	Unknown		Round	Flat	Erose	Shiny	Opaque	Light brown	smooth	Rods
M6	*Bacillus*	*B. badius*	Round	Convex	Erose	Shiny	Opaque	Light brown	smooth	Rods
M10	Unknown		Round	Flat	Entire	Shiny	Translucent	Pale yellow	smooth	Rods
M15	*Bacillus*	*B. cereus, B. thuringrenss, B. antracis*	Round	Raised	Filamentous	Dull	Opaque	Pale white	Rough	Rods
V11	*Vibrio*	*V. cyclitrophicus V. tasmaniensis, V. splendidus*	No growth on TSB							Oval
V12	*Photobacterium*	*P. frigidiphilum*	No growth on TSB							
V17	*Vibrio*	*V. cyclitrophicus V. splendidus V. tasmaniensis*	No growth on TSB							
V18	*Shewarella*	*S. baltica, S.pacifica*	No growth on TSB							Rods

Table 28 Occurrence of organisms for different treatments.

Treatment	Period	Bacteria																					Yeasts			
		B2	B3	B4	B8	B9	E1	E2	E3	E4	E5	E6	E10	M2	M4	M6	M10	M15	V11	V12	V17	V18	M1	M3	V1	V5
NC	D0 - D34	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	+	+
NC	D34 – D62	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	+
NC	D62 – D97	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+
NC	D97 – D124	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
NC	S0 - S28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
AF	D0 - D34	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-	+	-
AF	D34 – D62	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-
AF	D62 – D97	+	-	-	-	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+
AF	D97 – D124	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+
AF	S0 - S28	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
PC	D0 - D34	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-
PC	D34 – D62	+	-	-	-	-	+	-	-	+	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-
PC	D62 – D97	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+
PC	D97 – D124	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
PC	S0 - S28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
SBPS	D0 - D34	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-
SBPS	D34 – D62	+	-	-	-	-	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	-
SBPS	D62 – D97	+	-	-	-	-	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+
SBPS	D97 – D124	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
SBPS	S0 - S28	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
BS	D0 - D34	+	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	-	+	-	+	-	-	-	+	-
BS	D34 – D62	+	-	-	-	-	+	+	+	+	-	-	-	+	-		+	-	+	-	+	-	+	-	+	-
BS	D62 – D97	+	-	-	-	-	+	-	+		-	-	-	+	-		+	-	+	+	+	-	-	-	+	+
BS	D97 – D124	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
BS	S0 - S28	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-

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Table 29 Occurrence of different organisms during different growth periods.

Period	Treatment	B2	B3	B4	B8	B9	E1	E2	E3	E4	E5	E6	E10	M2	M4	M6	M10	M15	V11	V12	V17	V18	M1	M3	V1	V5
									Bacteria														Yeasts			
D0 – D34	NC	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	+	+
D0 – D34	AF	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-	+	-
D0 – D34	PC	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-
D0 – D34	SBPS	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-
D0 – D34	BS	+	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	-	+	-	+	-	-	-	+	-
D34 – D62	NC	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
D34 – D62	AF	+	-	-	-	-	+	-	-	+	-	-	-	-		-	+	-	+	-	+	-	+	-	+	-
D34 – D62	PC	+	-	-	-	-	+	-	-	+	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-
D34 – D62	SBPS	+	-	-	-	-	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	+	+	-
D34 – D62	BS	+	-	-	-	-	+	+	+	+	-	-	-	+	-	-	+	-	+	-	+	-	+	-	+	-
D62 – D97	NC	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+
D62 – D97	AF	+	-	-	-	-	+	-	+	+	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+
D62 – D97	PC	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+
D62 – D97	SBPS	+	-	-	-	-	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	+	-	+	+
D62 – D97	BS	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	+
D97 – D124	NC	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
D97 – D124	AF	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+
D97 – D124	PC	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
D97 – D124	SBPS	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
D97 – D124	BS	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
S0 – S28	NC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
S0 – S28	AF	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
S0 – S28	PC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
S0 – S28	SBPS	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
S0 – S28	BS	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-

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Table 30 Available plate count data for the first growth period, D0 – D34. Organisms for which no data are available are not shown. TNTC: Too numerous to count.

Organism	Treatment	Growth period	(CFU/ml)
V1	AF	D0 – D34	TNTC
V1	NC	D0 – D34	19.5×10^3
V1	BS	D0 – D34	467
V1	PC	D0 – D34	4×10^3
V1	SBPS	D0 – D34	TNTC
M2	AF	D0 – D34	2.5×10^3
M2	NC	D0 – D34	3×10^3
M2	BS	D0 – D34	800
M2	PC	D0 – D34	2.7×10^3
M2	SBPS	D0 – D34	2.2×10^3
M4	AF	D0 – D34	400
M4	BS	D0 – D34	100
E1	AF	D0 – D34	TNTC
E1	NC	D0 – D34	5.4×10^3
E1	BS	D0 – D34	467
E1	PC	D0 – D34	4.2×10^3
E1	SBPS	D0 – D34	3×10^3

Table 31 Available plate count data for the first growth period, D34 – D62. Organisms for which no data are available are not shown.   TNTC: Too numerous to count.

Organism	Treatment	Growth period	(CFU/ml)
V1	AF	D34 – D62	767
V1	NC	D34 – D62	500
V1	BS	D34 – D62	1×10^3
V1	PC	D34 – D62	1.1×10^3
V1	SBPS	D34 – D62	1×10^3
M2	AF	D34 – D62	2.7×10^3
M3	SBPS	D34 – D62	1.3×10^3
M4	NC	D34 – D62	100
M4	SBPS	D34 – D62	100
M6	NC	D34 – D62	67
E2	NC	D34 – D62	5.1×10^3
E3	NC	D34 – D62	900
E4	NC	D34 – D62	50
B2	AF	D34 – D62	1.515×10^6
B2	NC	D34 – D62	1.875×10^6
B2	BS	D34 – D62	915×10^3
B2	PC	D34 – D62	1.16×10^6
B2	SBPS	D34 – D62	8.75×10^6

Table 32 Available plate count data for the first growth period, D62 – D97. Organisms for which no data are available are not shown.   TNTC: Too numerous to count.

Organism	Treatment	Growth period	(CFU/ml)
V1	AF	D62 - D97	34.3×10^3
V1	NC	D62 - D97	110×10^3
V1	BS	D62 - D97	32×10^3
V1	PC	D62 - D97	303×10^3
V1	SBPS	D62 - D97	217×10^3
V5	AF	D62 - D97	16×10^3
V5	NC	D62 - D97	40×10^3
V5	BS	D62 - D97	105×10^3
V5	PC	D62 - D97	26.7×10^3
V5	SBPS	D62 - D97	6.7×10^3
B2	All	D62 - D97	Dominant on BHI media
E1	All	D62 - D97	Dominant on EAO media
M10	All	D62 - D97	Dominant on MRS media

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Table 33 Available plate count data for the first growth period, D97 – D124. Organisms for which no data are available are not shown.   TNTC: Too numerous to count.

Organism	Treatment	Growth period	(CFU/ml)
V1	AF	D97 – D124	40×10^3
V1	NC	D97 – D124	223×10^3
V1	BS	D97 – D124	253×10^3
V1	PC	D97 – D124	593×10^3
V1	SBPS	D97 – D124	430×10^3
V5	AF	D97 – D124	667
V5	NC	D97 – D124	73×10^3
V5	BS	D97 – D124	10×10^3
V5	PC	D97 – D124	76×10^3
V5	SBPS	D97 – D124	26×10^3
B3	BS	D97 – D124	200×10^3
B4	AF	D97 – D124	733×10^3
B4	NC	D97 – D124	6.33×10^6
B4	BS	D97 – D124	2.03×10^6
B4	PC	D97 – D124	5.93×10^6
B4	SBPS	D97 – D124	2.53×10^6
B2	All	D97 – D124	Dominant on BHI media
E1	All	D97 – D124	Dominant on EAO media

No plate counts could be done at the end of growth period S0 – S28. The dominant bacterial species (colony M15) grew very aggressively, which made it impossible to distinguish colonies with any degree of certainty.

9.3   Statistical methods: Model checking

9.3.1   Controlled optimal conditions
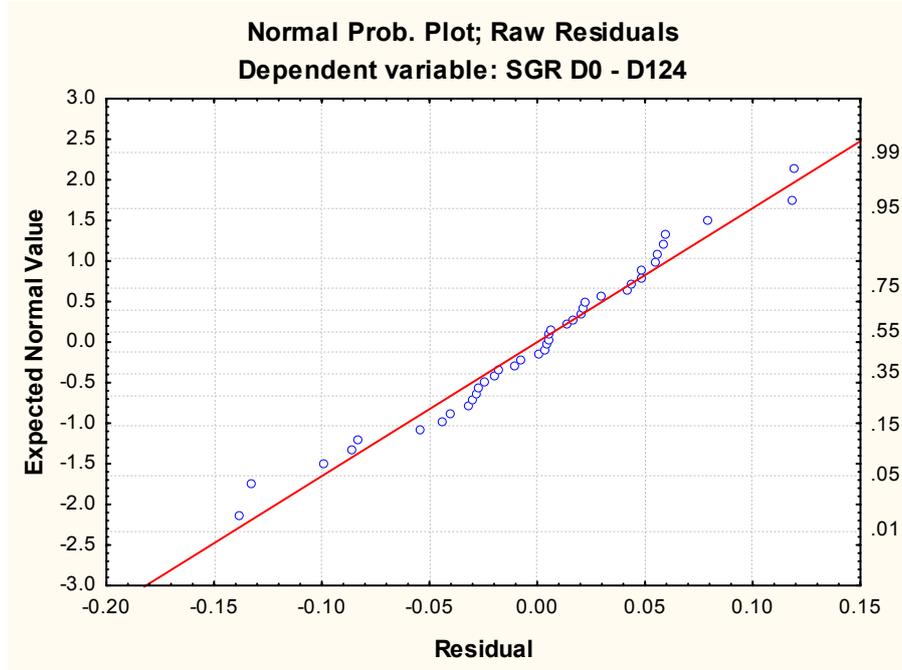
Figure 15 Normal probability plot of residuals of SGR, period D0 – D124
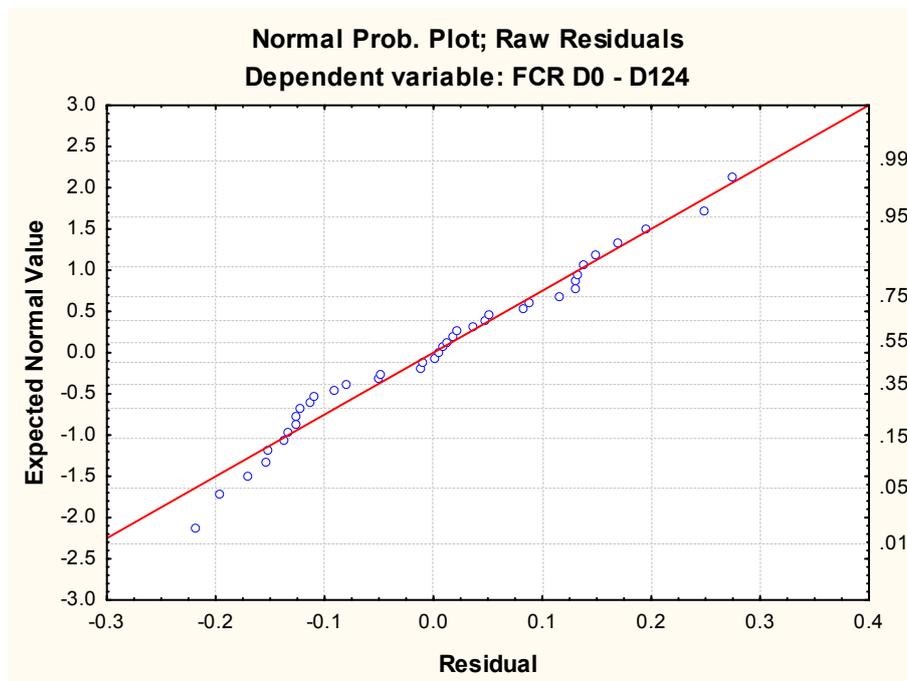
**Figure 16** Normal probability plot of residuals of FCR, period D0 – D124

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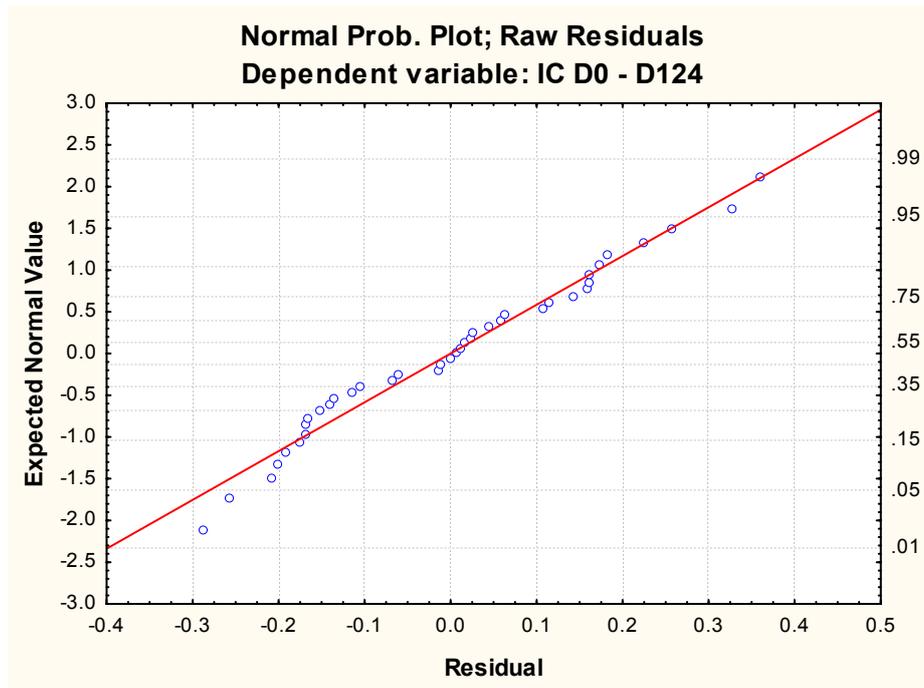
**Figure 17** Normal probability plot of residuals of IC, period D0 – D124
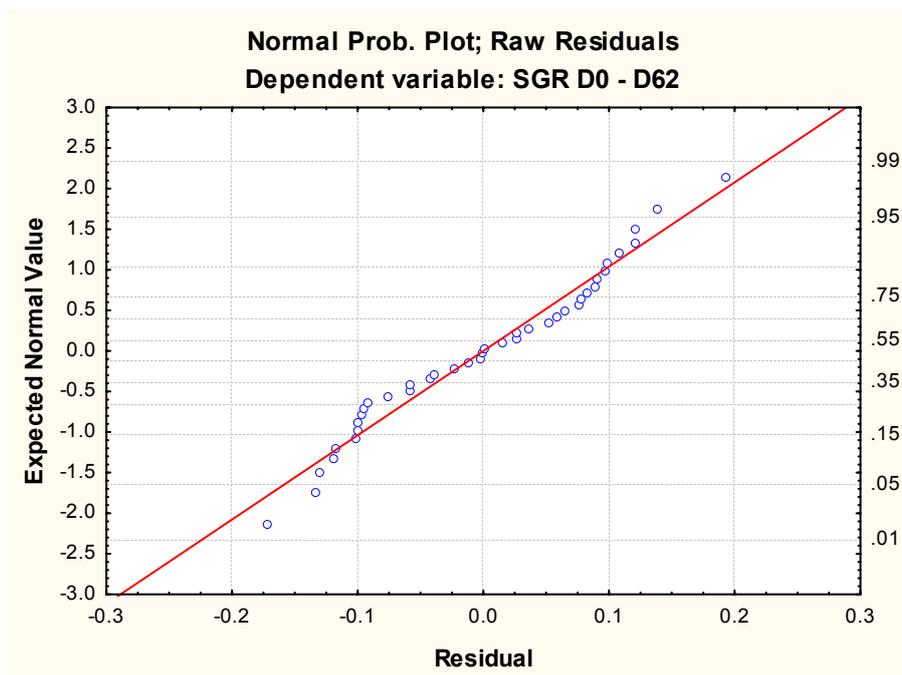
**Figure 18** Normal probability plot of residuals of SGR, period D0 – D62

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Figure 19 Normal probability plot of residuals of FCR, period D0 – D62
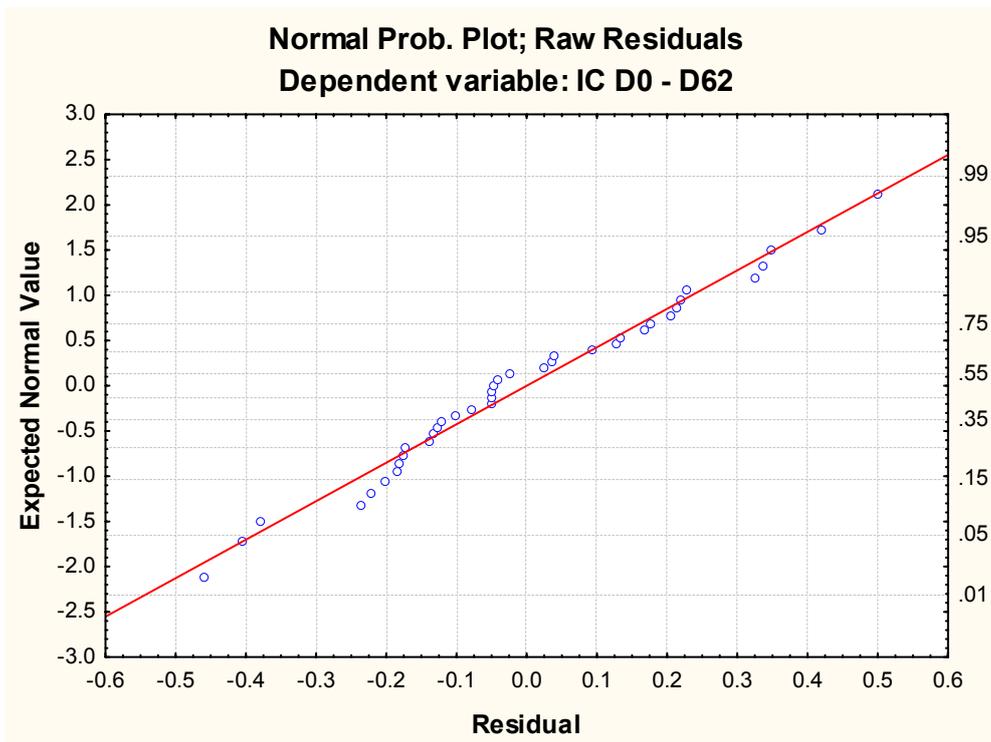
Figure 20 Normal probability plot of residuals of IC, period D0 – D62

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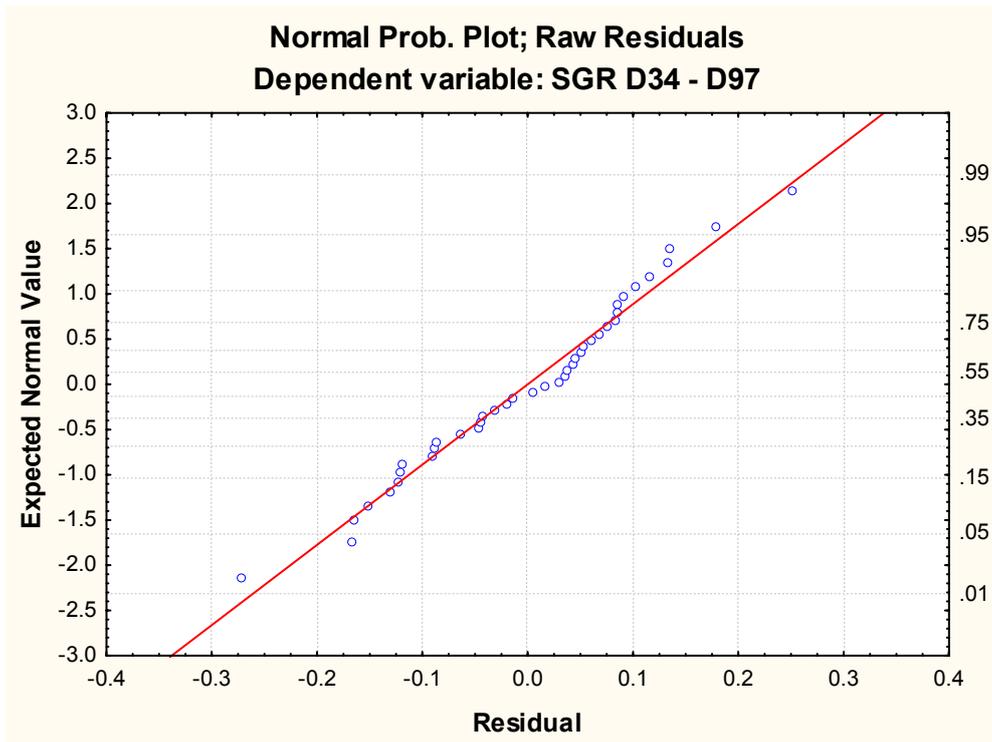
Figure 21 Normal probability plot of residuals of SGR, period D34 – D97
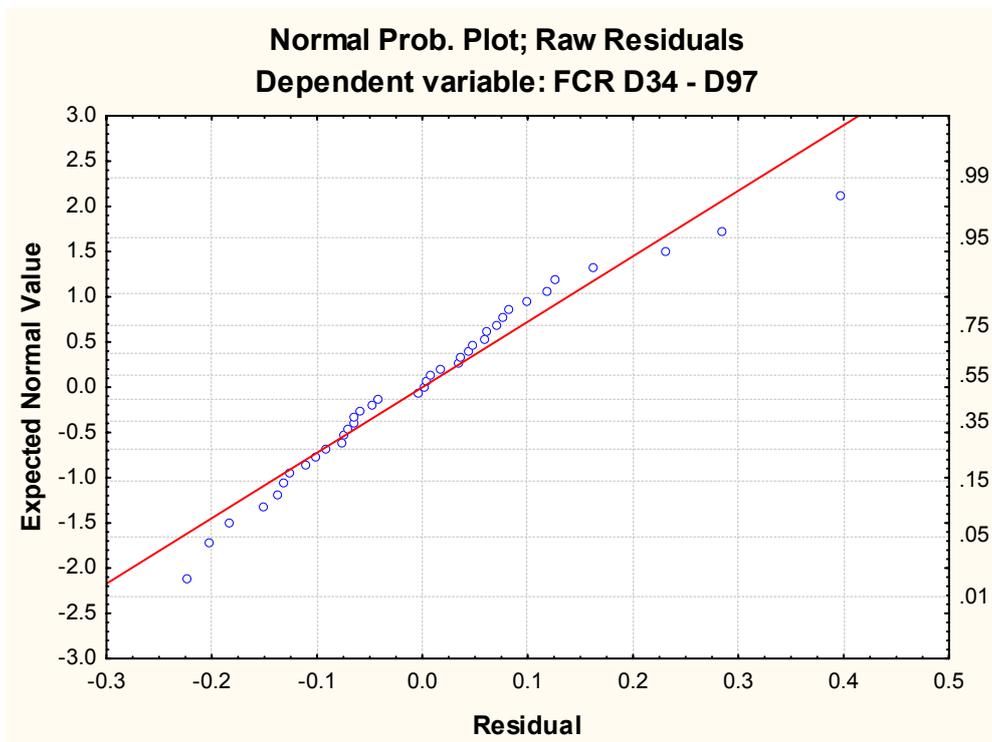
Figure 22 Normal probability plot of residuals of FCR, period D34 – D97

113
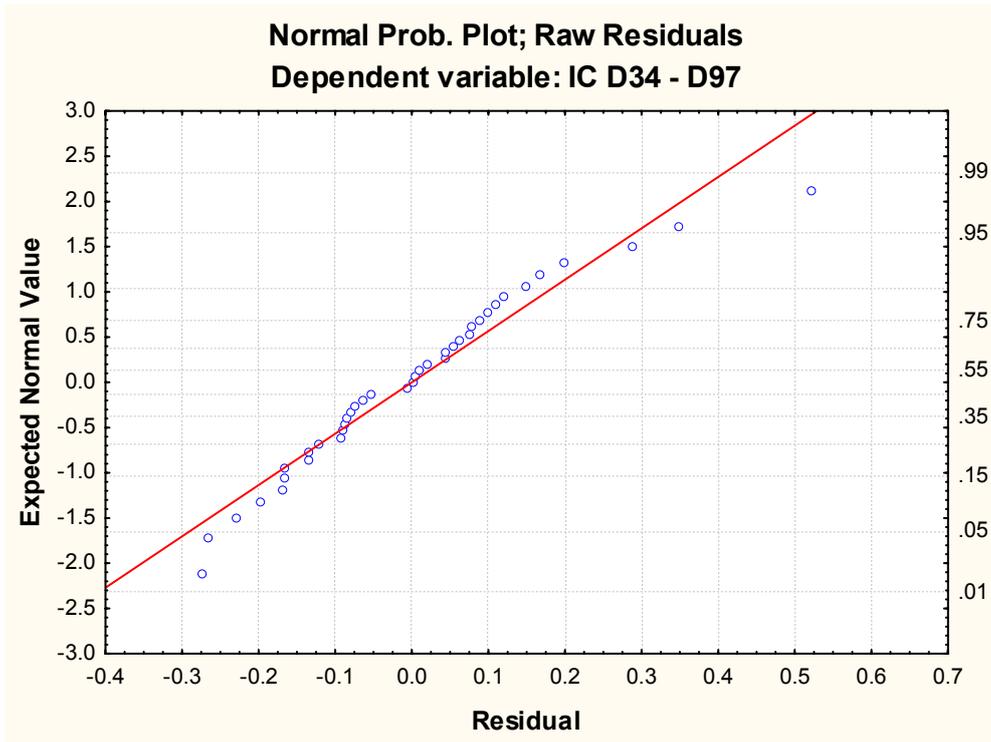
Figure 23 Normal probability plot of residuals of IC, period D34 – D97
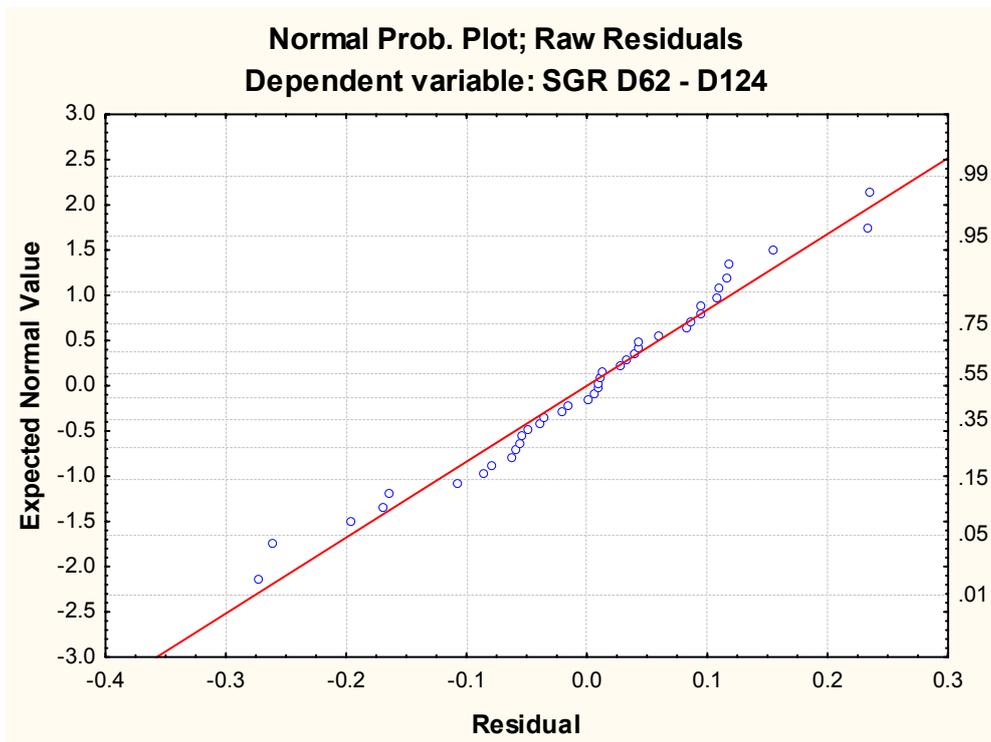
Figure 24 Normal probability plot of residuals of SGR, period D62 – D124

114
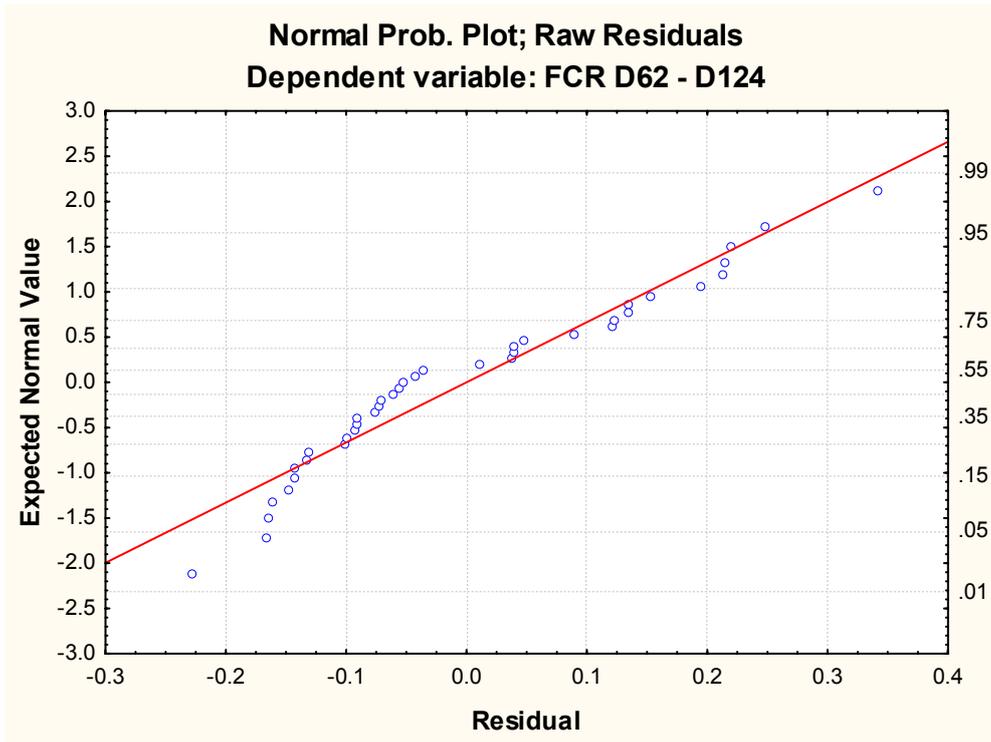
**Figure 25** Normal probability plot of residuals of FCR, period D62 – D124
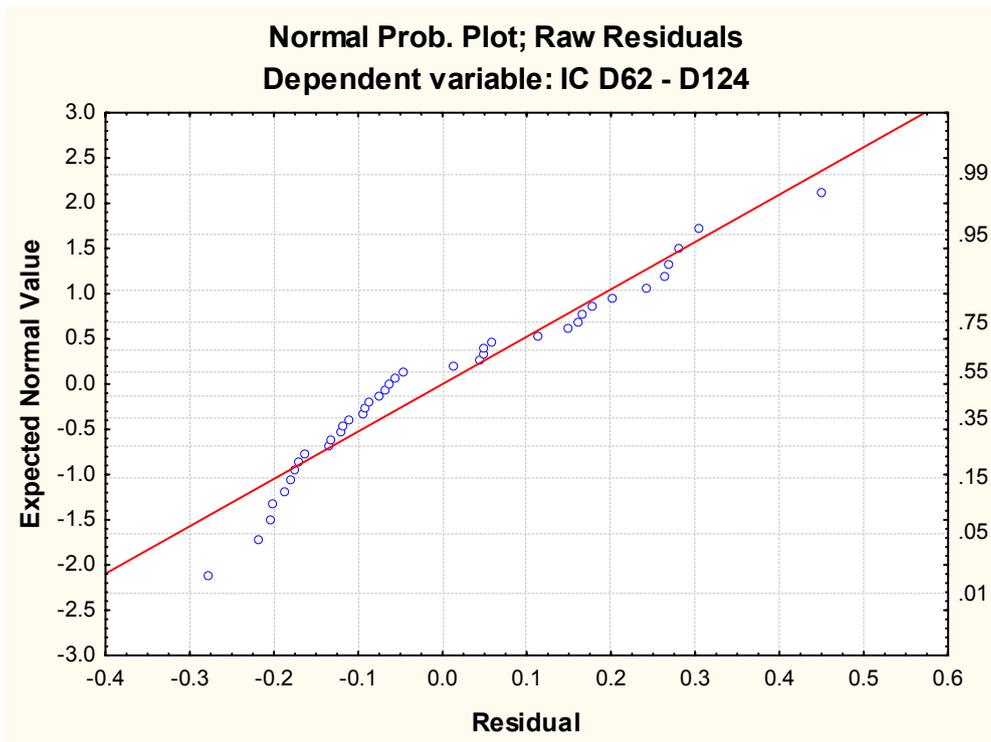
Figure 26 Normal probability plot of residuals of IC, period D62 – D124

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9.3.2 Controlled stress conditions

Figure 27 Normal probability plot of residuals of SGR, period S0 – S28
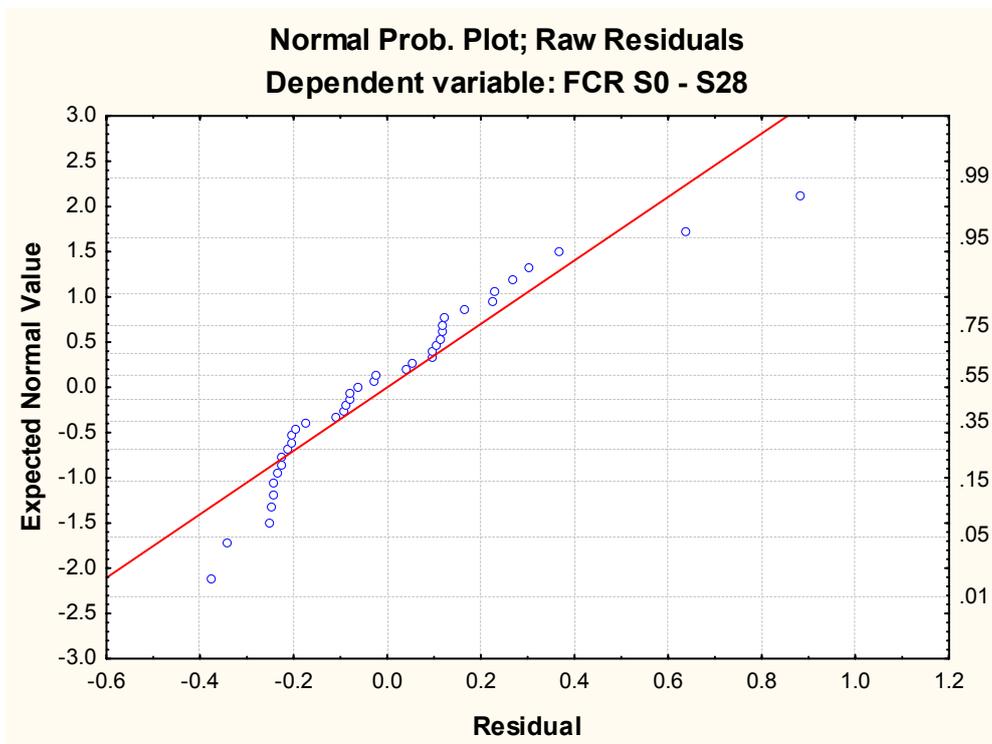
Figure 28 Normal probability plot of residuals of FCR, period S0 – S28

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**Normal Prob. Plot; Raw Residuals**
Dependent variable: IC S0 - S28
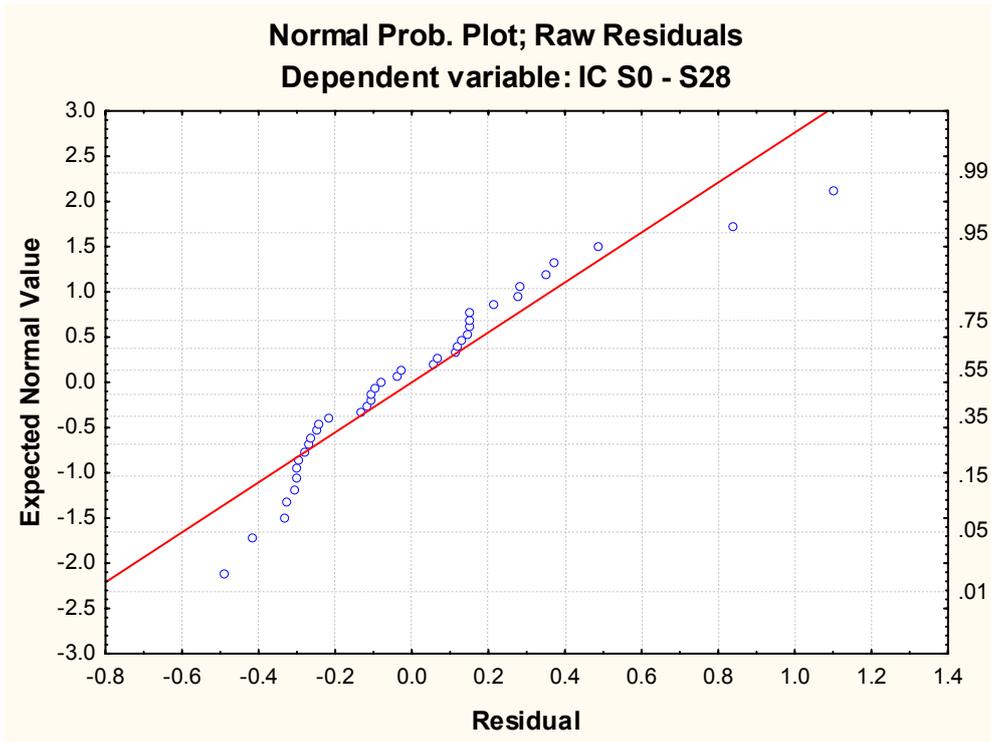
Figure 29 Normal probability plot of residuals of IC, period S0 – S28

9.3.3   Production conditions

Normal Prob. Plot; Raw Residuals
Dependent variable: SGR Production Conditions
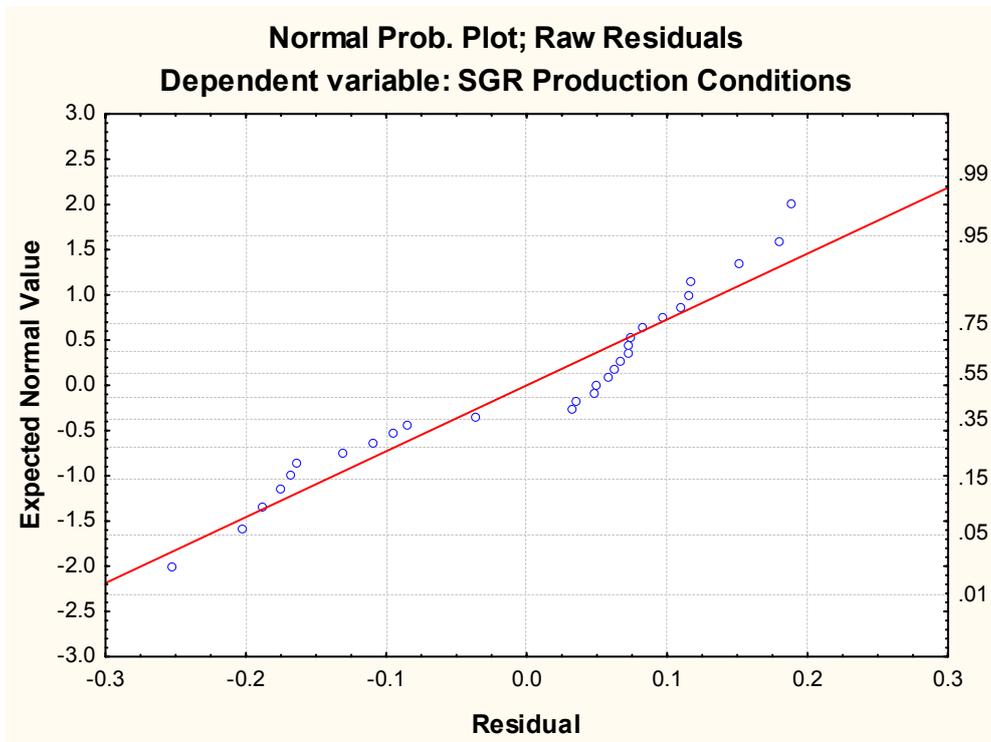
Figure 30 Normal probability plot of residuals of SGR, production conditions

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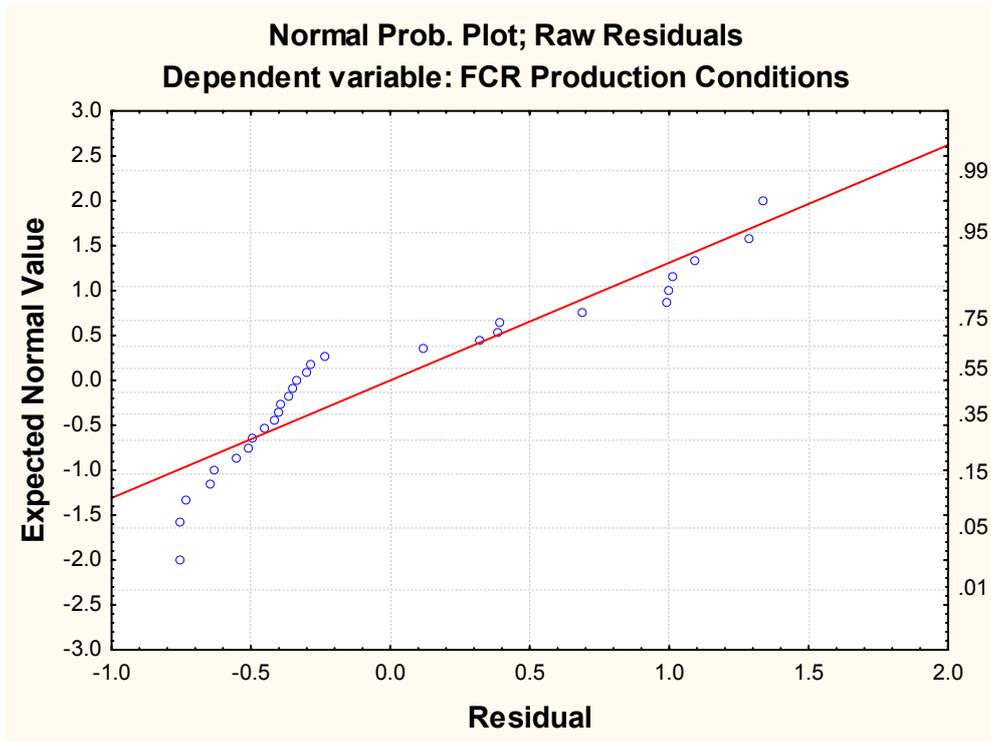
Figure 31 Normal probability plot of residuals of FCR, production conditions
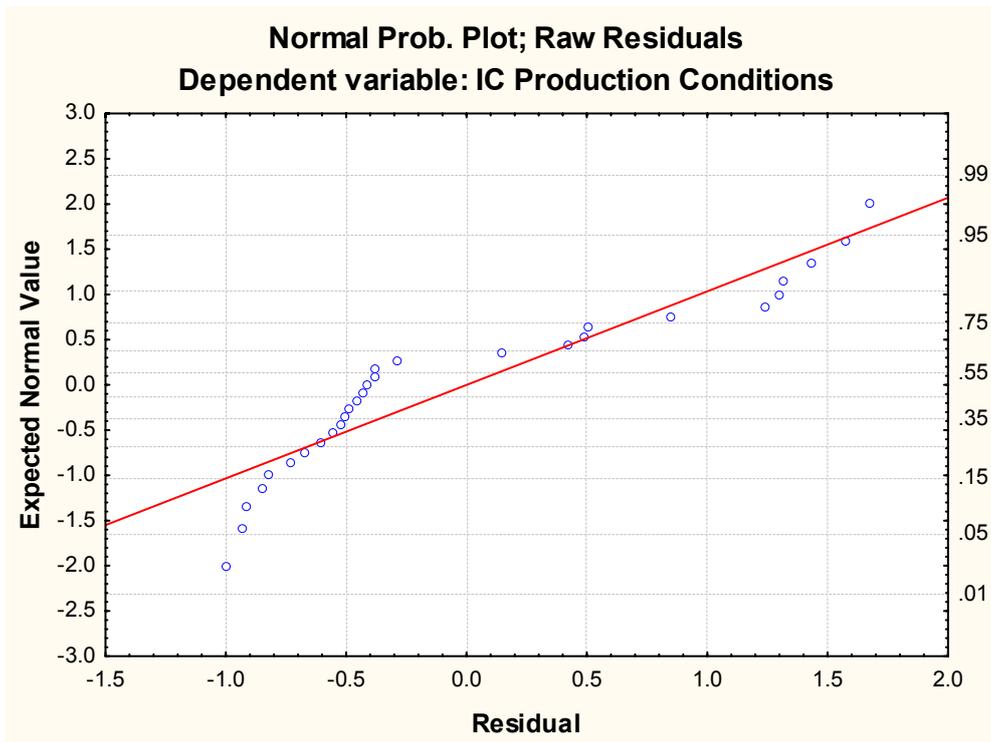

Figure 32 Normal probability plot of residuals of ICR, production conditions

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