CULTURE-BASED STUDY OF THE DIGESTIVE ABILITIES POSSESSED BY ANAEROBIC BACTERIA ISOLATED FROM THE HINDGUT OF THE MARINE HERBIVOROUS FISH *Kyphosus SYDNEYANUS*

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fulfilment of the requirements of the degree of Doctor of Philosophy

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a University or other institution of higher learning, except where due acknowledgment is made in the acknowledgements.

Signed ………………………………………..

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List of Abbreviations

ANOVA	analysis of variance
BHT	butylated hydroxytoluene
BLAST	Basic Local Alignment Search Tool
CFU	colony forming units
GF	growth factor
MAF	Ministry of Agriculture and Forestry
MBIE	New Zealand Ministry of Business, Innovation and Employment
NCBI	National Centre for Biotechnology Information
NZ	New Zealand
OD	optical density
PCR	polymerase chain reaction
sccm	standard cubic centimeter per meter
SCFA	short chain fatty acids
VFA	volatile fatty acids
YCFA	**Y**east extract-**c**asein hydrolysate-volatile **f**atty **a**cids medium
YCFA-GF	YCFA medium with the addition of *Kyphosus sydneyanus* gut fluid
YCFA-M	YCFA medium with the addition of mannitol
YCFASWU	YCFA media with the addition of an aqueous extract from *Undaria pinnatifida*
YCFA-X	Control YCFA media, with water in place of nutrient additions

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Co-Authored Works

Included in ‘Chapter 4, Section A: Final Multi-Well Growth Rate Measurement Method from Paper’ Is the contents of a research paper, published during this PhD (Wornell, et al., 2022). I am the first author on this paper and contributed 75% of the work. I contributed all of experimental work from which results were reported in this publication. I also contributed the majority of the analysis, conceptualising planning and novel methodology of this work. I also contributed all of the writing of this publication. Bikiran Pardesi and Linsey White each contributed 7% of the work, in conceptualisation, planning and reviewing of the manuscript. Anthony M. Roberton contributed 7% of this work in conceptualisation by sharing the results of his previous studies, which provided a basis to this research. Anthony M. Roberton, along with Bikiran Pardesi, set up the laboratory at which I carried out these experiments, and decided on the early methodology used. Kevin Lee contributed 2% of this work in analysis of some results. Sevetlana Boycheva contributed 2% of the work in conceptualisation of some methodology used.

Signed ………………………………………………….

…………………………Bikiran Pardesi……………

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Abstract

This project investigated novel growth factors for fish gut bacteria present in brown algae. This is an *in vitro* study of strictly anaerobic bacteria isolated from the gut of *Kyphosus sydneyanus* (Silver Drummer), a fish common to the Northeastern New Zealand coast. The strictly anaerobic gut bacteria require specialised culture methods and media. Fish gut bacteria have the potential to be used in industry for large scale fermentation, producing high value natural products.

There are two major findings of this study, which improved further analysis of the functional digestive abilities possessed by these bacteria. The first is a novel methodology for high throughput *in vitro* culture-based investigation of fastidious and strictly anaerobic bacteria. Allowing direct comparison of bacterial growth across conditions. The second is novel media for optimal *in vitro* growth of *K. sydneyanus* gut bacteria. Three different nutrients from brown algae *Undaria pinnatifida* were found to produce significantly faster bacterial growth than the standard of fish gut fluid.

Using these method developments, it was found that the growth rate of the *K. sydneyanus* gut microbiome was significantly increased in a mixed isolate culture, in a demonstration of community digestive action. Also, a significant variation in digestive ability at the family level was found, showing functional diversity in substrate preference. These findings help industry to improve fermentation conditions for *K. sydneyanus* gut bacteria.

Impact of COVID Statement

This PhD was significantly impacted by COVID-19.

Successive lockdowns in Auckland reduced time available in the laboratory. Some samples prepared for analysis were lost between lockdowns. Major delays to deliveries of scientific equipment and consumables slowed project progress.

Due to these factors, the experimental work was necessarily reduced in scope.

Chapter 1: Introduction

Background

Raising fish in captivity can cause stress and a loss of gut flora, this limits the nutrients these fish can access from certain plant material (Fisherson, et al., 1985). Currently these farmed fish need to be fed captured fish to have access to the essential short-chain fatty acids (SCFAs) and other algal fermentation products usually provided by gut flora (Mountfort, et al., 2002). For example, farmed carnivorous fish currently get their required protein from captured herbivorous fish, which like all other ruminating organisms get most of their protein from their gut microbiota (Cotta & Russell, 1981).

The lab of Clements et al. at the University of Auckland has successfully cultured some of these fish hindgut microbes *in vitro* (Clements, et al., 2007). Optimising this culture process is essential to allow industrial microbial conversion of brown seaweed to protein biomass, which can be used as a feed, providing missing nutrients to the diet of fish and reducing farming costs. Other applications are the use of fermentation products as a sustainable agricultural fertiliser and as a food source for black soldier fly larvae, which are important feed components for aquaculture and poultry.

Previous research, conducted through a Marsden project and developed through a Smart Ideas grant, revealed that fish hindgut microbes provide an important source of dietary protein to their host, and that these novel organisms can be grown in culture. A key discovery was the potential presence of a novel bacterial growth factor in brown algae, thought to be essential for vigorous growth of fish hindgut bacteria in laboratory conditions. Previous work by Robertson et al. at the University of Auckland [*unpublished*] demonstrates that addition of the water extract from brown algae to media is key to vigorous growth of fish hindgut bacteria *in vitro*. The aim of this PhD is to further identify specific compounds causing this enhanced growth. Growth factor discovery and culture media improvements will enable the isolation of novel bacteria and improvement of large-scale fermentation projects.

This study uses an extract taken from the blade of the brown seaweed *Undaria pinnatifida*. The *Kyphosus sydneyanus* diet is usually dominated by brown seaweed (Barsanti & Gualtieri, 2006). The seaweed *U. pinnatifida* was chosen due to growth on the New Zealand coast making collection of fresh samples possible, there is also a large industrial interest in this seaweed (Hay & Luckens, 1987; Nayar & Bott, 2014). The global seaweed industry is estimated to have an annual value of around US$6 billion, of which US$5 billion is food products and US$1 billion is based on seaweed extracts for use in our market of interest: animal feeds, fertilisers, and bioactives (Nayar & Bott, 2014). *U. pinnatifida* was the fourth most-produced cultivated seaweed in 2010, at 1.5 million tonnes it accounted for 10% of global seaweed production (Nayar & Bott, 2014). Therefore, the new insights into the biochemical make-up of this organism generated by this study will attract interest from a large range of invested parties, fuelling advances in related industries.

These improvements work towards achieving the larger project goals. The group works to generate novel bacterial products for the global food supply chain. These natural products will address four global problems: the cost of feeding capture fish to farmed fish; the lack of critical aquaculture nutrients present in terrestrial protein sources; the issues with using seaweed biomass to produce animal feeds (particularly arsenic accumulation); and the demand for sustainable agricultural fertiliser. This is a completely novel idea on an international level, as this is one of the first studies on the microbial and physiological processes present in the natural fish-microbe symbiosis.

Aims

The overarching topic of this study is the use of *in vitro* culture to identify the substrate preferences of certain strictly anaerobic bacteria. This work includes the development of novel culture-based methodologies, used to investigate the growth effects of various nutrients on fish gut bacteria. The overall aim of this study is to achieve optimal growth of chosen *K. sydneyanus* gut bacteria *in vitro*. Previous unpublished work by the Clements laboratory found that the aqueous extract of the brown algae *Undaria pinnatifida* was essential for optimal growth of *K. sydneyanus* gut bacteria *in vitro*. These previous results suggested the presence of growth factors in brown algae aqueous extract.

In this study, culture-based *in vitro* experiments utilised an adjustable **Y**east extract-**c**asein hydrolysate-volatile **f**atty **a**cids (YCFA) media recipe. Novel methodology was developed to investigate the growth effects of various nutrients on three bacterial isolates, and a mix of all three, cultured on YCFA medium. Specialist media was developed by adapting YCFA media to provide the nutrients that these microorganisms require for optimal *in vitro* growth. These required nutrients would usually be available in the natural environment of these gut bacteria. Each bacterium used in this experimental work was isolated from the gut of *K. sydneyanus* and genetically identified at the Family level by 16S rRNA gene sequencing.

A range of factors make up the background to this study, this includes first the diet and gut morphology of the host fish *K. sydneyanus*, secondly the nutritional profile of brown algae and thirdly the published methodologies used for *in vitro* culture of anaerobic bacteria. An extensive literature review of these topics is presented in **Chapter 2**. The work in this literature review provides context to the discussion in **Chapter 6**.

Terms and scope of the study

The scope of this study is limited to one fish species, *K. sydneyanus*, caught off the North-Eastern coast of New Zealand. Then further limited to bacteria isolated from one section of the *K. sydneyanus* gut, Section IV (Figure 1). Throughout the method development stage, detailed in **Chapter 4**, up to sixteen different isolates were used in culture-based experiments. Once the novel growth rate measuring methodology was published (**Chapter 4**), the scope of this study was narrowed down to three isolates, each from a different bacterial family. The isolates used were BP38 of the Rikenellaceae Family, BP47G of the Oscillospiraceae Family, and BP5G of the Lachnospiraceae Family.

The use of bacteria from three different but commonly-isolated Families from the *K. sydneyanus* gut provides growth results that reflect the diversity of the gut environment. These three isolates were also chosen for these experiments due to their full genome sequences being available in public databases, along with the good performance of these isolates in preliminary studies. Good performance in preliminary studies is required to generate control data for these isolates in the most minimal medium (YCFA), that can then be compared to the growth generated by the addition of various potential growth factors.

Isolates were used in growth rate experiments to discover the growth effects of various nutrients. Candidate nutrients were added to the standard YCFA media. YCFA was the most minimal media that can be used to culture these isolates, based on the extensive growth experiments conducted by Prof. Tony Robertson and Dr Bikiran Pardesi. Growth rate experiments reveal divergence in nutrient preference between the three isolates.

A mixture of all three isolates was also used, with the aim to discover the effect of mutually beneficial digestion on the *in vitro* growth of *K. sydneyanus* gut bacteria. These co-culture experiments were carried out under the same varying nutrient conditions as the single isolates. Growth rate experiments using a mixture of all three bacteria reveal any growth advantages provided by the presence of multiple isolates, and any community digestion behaviour occurring within these bacteria.

Contribution to knowledge

A major contribution of this study is the development of a novel methodology for high-throughput measurement of gut bacteria *in vitro* growth responses to various nutrients. This important development greatly reduces the workload for researchers investigating fastidious anaerobic bacteria. Work that would usually take months can now be completed in weeks, with all growth rate results directly comparable, allowing for analysis of the gut microbiome. This publication also includes methodology for general *in vitro* culture and media preparation methods. This is an update to older methodologies, such as Hungate (1975), a greatly needed contribution for modern *in vitro* anaerobic work.

In this study these novel methodologies were used to measure effects of various brown algae derived nutrients on the growth rates of three *K. sydneyanus* gut bacterial isolates. Understanding the *in vitro* nutrient requirements of gut bacteria helps us to understand their function, and their relationship with the host fish. The nutritional complexity of the gut environment is difficult to replicate *in vitro*, hindering the study of these organisms. In any symbiotic relationship between bacteria and the host, there are many factors affecting the nutrient environment, making identification of precise growth factors for *K. sydneyanus* gut bacteria a difficult task. The development of simple and replicable growth media for *in vitro* culture of *K. sydneyanus* gut bacteria will greatly increase culture success and ease the metabolomic and functional studies of these organisms.

Nutrients trialled in these novel media experiments were chosen due to their presence in the aqueous extract of *U. pinnatifida*. This aqueous extract was found to produce optimal growth rates of *K. sydneyanus* gut bacteria in intial experimental work. The results of growth rate experiments of this study contributed four alternative YCFA-based media recipes, which utilised these nutrients of interest. These novel media provide defined and replicable alternatives to the more complex and less defined YCFA media with gut fluid or YCFA with brown algae aqueous extract. Some of the novel media developed in this study were even found to produce faster growth than the known media alternatives listed. The development of these defined and replicable media increases reliability and replicability of the results of future work.

Research questions

The research questions addressed by this study are varied and include: What substrates are preferred by *K. sydneyanus* gut bacteria cultured *in vitro*? Are there novel growth factors for these bacteria in the aqueous extract of the brown algae *Undaria pinnatifida*? Is the aqueous extract of brown algae required for the optimal growth of *K. sydneyanus* gut bacteria cultured *in vitro*?

Two major research problems were addressed by this study. First the poor growth of *K. sydneyanus* gut bacteria when cultured in liquid media, whereas growth on a solid agar plate was good. This was a roadblock to measuring the growth rates of these bacteria in a high-throughput manner. Current solid media-based methodologies for growth rate measurement are based on colony counting, which is not appropriate for these isolates as they often grow in a solid mat. A second problem addressed by this study is that due to the fastidious nature of these strict anaerobes, a single experiment can take longer than a month to prepare and carry out. There was a need to develop a high-throughput alternative methodology to speed up this process and generate the data needed to answer the study questions. To overcome these problems a novel high-throughput method of measuring the growth rates for bacteria on a solid media was developed in this study. This methodology was published, and this paper makes up **Chapter 4**.

Hypotheses

The major hypothesis of this study is that there are growth factor(s) present in the aqueous extract of brown algae that are required for optimal growth of *K. sydneyanus* gut bacteria *in vitro*. The end goal of this work is to find a defined and simplified medium for the culture of these bacteria, incorporating these novel growth factors. To investigate this issue a total of twelve nutrient additions to YCFA media were trialled. The effects of these nutrients on the growth rates of three individual *K. sydneyanus* gut bacterial isolates and a mixture of all three together were measured using novel methodology. Along with answering the major hypothesis of this study, the results of these growth experiments also help to answer questions in the literature regarding coevolution and the consortial nature of bacteria in the herbivorous fish gut.

Concepts and variables

A key concept of this study is the methodology used for *in vitro* culture of fastidiously anaerobic bacteria. The fastidious nature of strictly anaerobic bacteria is well known to frustrate culture-based work with these isolates. Experiments that take weeks with aerobic bacteria can take months with anaerobes due to their slow growth rate *in vitro*, the bespoke anaerobic media that must be prepared, and the specialist equipment that must be used when working with these isolates to prevent any oxygen from entering the culture environment at any stage.

Another key concept of this study is the potential co-evolution between gut bacteria and their host and the consortial nature of herbivorous fish gut bacteria. Co-evolution between the host and their gut microbiota is well known among terrestrial vertebrates (Macke, et al., 2017). It occurs when selective forces are exchanged between both parties, resulting in reciprocal evolutionary change (Macke, et al., 2017). Environment precipitating a co-evolutionary relationship between herbivorous fish and some gut bacteria has been proven by Flint et al. (2005) in a study that found parallel lineages of *Epulopiscium* symbionts present in the guts of surgeon fish from distant sampling sites of the Red Sea and the Great Barrier Reef. The host fish *K. sydneyanus* have evolved a dependency on gut bacteria for digestion and have evolved a specialised gut Section IV with a separating second sphincter, in which many bacteria can perform fermentation of brown algae for the nutritional benefit of the host (Clements & Choat, 1993; Rimmer & Wiebe, 1987). This level of co-evolution on the part of the host fish suggests there is also some level of co-evolution on the part of the core microbiota, as this host-associated phase is likely to dominate the lifecycle of these symbionts (Macke et al., 2017).

The extent to which the isolates of different families from the *K. sydneyanus* gut benefit from co-culture is explored in this experimental work. It is important to investigate to what extent bacteria of the herbivorous fish gut rely on community digestive action. This concept ties into co-evolution, with experimental evidence of consortial digestion supporting the idea that these bacteria have co-evolved with the host fish and with other bacteria. This co-evolution and consortial digestion optimise nutrient usage in this niche. The concept of a consortium of fish gut bacteria is evidenced by the results of this study, as a mixture of three isolates grew faster than any individual isolate on average across all twelve media trialled. These findings support the concept of co-evolution between the herbivorous fish and their gut microbiota. These bacteria were all isolated from the same gut section and were found to best utilise the usual nutrients present in this gut section. This is evidence of likely selection for these digestive abilities.

There are many variables in this study, these are divided into the controlled, dependent and independent variables. The controlled variables of this study are bacterial isolates used, and nutrient additions added to the media. The dependent variables of this study are growth rates data for each isolate, plus the mix, on each media. These results are set out in **Chapter 5** and discussed in **Chapter 6**. The independent variables of this study are substrate preference of each isolate, which is based on the digestive pathways they possess, and therefore the nutrients these bacteria can make use of. Substrate preferences directly affect the rate of growth on different media types trialled. This is how the dependent variables shown in the growth results can reflect the interaction between the controlled variables.

Methodology

This study is concerned with three individual isolates from the *K. sydneyanus* gut, and a mixture of all three. The isolates used were BP38 of the Rikenellaceae Family, BP47G of the Oscillospiraceae Family, and BP5G of the Lachnospiraceae Family. A novel multi-well method was used to test the effect of various nutrient additions to YCFA media on the growth rates of these bacteria. For fastidious anaerobic microbes the process necessary to develop novel culture media is laborious and time consuming due to the long incubation times and strict culture conditions required by these obligate anaerobes (Bryant, 1972; Hungate, 1969).

The novel protocols developed within this PhD research project, which are detailed in **Chapter 4,** are designed for maximum recovery and performance of anaerobic bacteria *in vitro*. The methodology presented in **Chapter 4 Protocols 1 to 3** pertains to single bacterial isolates of known genetic identity, cultured on a rich media containing host gut fluid. Solid media is used in all protocols in this study, as many anaerobic bacteria are unable to grow in a liquid medium (Speers, et al., 2009). **Chapter 4 Protocol 4** presents a method of high-throughput growth rate analysis on solid media. The usual way of measuring growth on a solid media, colony counting, cannot be used as bacteria isolated from the gut may grow in mats. These bacteria may also grow in colonies invisible to the naked eye (Tramonatano, et al., 2018). This protocol is used to compare bacterial growth rates on novel replicable media including the twelve nutrient additions to those on rich media. The results of this study include the development of multiple replicable media that can be used to improve the *in vitro* performance of *K. sydneyanus* gut bacteria.

The twelve nutrient additions were trialled in batches, the results of this study are presented in **Chapter 5**. **Chapter 5 Section A** presents the growth rates of each isolate on YCFA media with the complex nutrient addition of *K. sydneyanus* gut fluid. This addition of host gut fluid represents a standard rate of *in vitro* growth against which the alternative nutrient additions can be compared. **Chapter 5 Section B** presents four different polysaccharides present in brown algae: mannitol, laminarin, alginic acid and glucose. Four common sugars in brown algae. **Chapter 5 Section C** presents the growth rate effects of the aqueous extract from four seaweed species: *Undaria pinnatifida, Ecklonia radiata, Cystophora torulosa* and *Caulacanthus ustulatus*. **Chapter 5 Section D** presents the growth rate effects of a normal amount of vitamin solution in YCFA, versus ten times the normal vitamin solution, versus the vitamin extract from the aqueous extract of *U. pinnatifida*. In each section of **Chapter 5** the growth rates of chosen isolates with the novel nutrient additions are compared with the growth rates with either *K. sydneyanus* gut fluid or *U. pinnatifida* aqueous extract. These are complex and undefined nutrient additions that most closely resemble the nutrient make-up of the environment these bacteria were isolated from the *K. sydneyanus* hindgut. These two media are the media recipes most used to culture *K. sydneyanus* gut bacterial isolates *in vitro* in the Clements laboratory. These rich media recipes that attempt to replicate the nutrient composition of the gut environment are highly nutritionally complex, but problematically, chemically undefined. Use of media containing gut fluid reduces experimental reproducibility as the nutrient profile varies between individual host organisms, including fish (Egerton, et al., 2018), so is subject to nutrient variation from batch to batch. The use of rich media with gut fluid or blood limits the accuracy of metabolic and physiological investigation of microbes in culture (Tramonatano, et al., 2018). A control YCFA media, with water in place of further nutrient additions, named YCFA-X, was also included in all cases and used as a control across the board.

Thesis layout

This PhD thesis is split into six chapters. **Chapter 1** introduces the main aims of the project and sets out the original contribution to knowledge that this work makes to the field of Microbiology. **Chapter 2** is a literature review, providing context to the work of this PhD. The final methods seen in **Chapter 3** were used to measure the growth rates of three individual isolates and a mix of all three on twelve different varieties of media. **Chapter 4** details the development process for the *in vitro* culture and investigation of fish gut bacteria, which led to the publication of the novel methodologies seen in **Chapter 3**. The results of these growth rate experiments are set out in **Chapter 5** and then discussed in **Chapter 6**.

Chapter 2: Literature Review

Introduction

The *in vitro* study of anaerobic bacterial isolates from the hind gut of seaweed-eating fish requires specialised methodology and a tailored culture medium. Previous unpublished work by the Clements laboratory found that the aqueous extract of the brown algae *Undaria pinnatifida* was an essential ingredient for optimal growth of *Kyphosus sydneyanus* (Silver drummer) gut bacteria *in vitro [unpublished work]*. These results suggested that there are possible growth factors for *K. sydneyanus* gut bacteria present in the aqueous extract of *U. pinnatifida*.

This study investigates the effect of various nutrients present in brown algae on the *in vitro* growth rate of *K. sydneyanus* gut bacteria. Also investigated are the effects of other brown and red algae present in New Zealand on *K. sydneyanus* gut bacteria growth rates. These experiments test the likelihood of the presence of a novel essential growth factor for *K. sydneyanus* gut bacteria in *U. pinnatifida*.

This literature review details what is known about the diet and gut morphology of *K. sydneyanus*. The functions of the gut microbiota of herbivorous fish are covered, with particular attention paid to the known mechanisms of algae carbohydrate digestion by *K. sydneyanus* gut bacteria. This review informs the **Chapter 6** discussion of growth rate experiments carried out within this study using these algae carbohydrates (and sugar alcohols) as bacterial growth factors.

This literature review also explores the background to the hypothesis that there are unique growth factors for *in vitro* growth of *K. sydneyanus* gut bacteria present among the nutrients of the brown algae *U. pinnatifida*. The nutritional profile of *U. pinnatifida* is reviewed in this chapter. Also reviewed is the history and recent developments in the methodology used for *in vitro* culture of strictly anaerobic gut bacteria. These reviews inform **Chapter 4** method development in choosing nutrients to test for growth factor effects on *K. sydneyanus* gut bacteria.

Current state of the literature

The search for novel bacterial growth factors (GFs) to improve culture media has become a rare approach to solving the unculturable bacteria issue, leaving a gap in the literature on this subject. This study provides the opportunity to resume this work after a forty-year lull, which could be the key to a renaissance in the discovery of novel bacterial products. Within this study, novel methods were developed that enable high-throughput analysis of fish gut bacteria growth rates (Wornell, et al., 2022). These methods were used to investigate possible growth factors for *K. sydneyanus* gut bacteria, and to develop novel improved media recipes for the culture of these bacteria. This novel media can be used to investigate fish gut bacteria in future work, particularly the large-scale fermentation carried out by this research group. The novel methodology developed within this study can be used in future work to investigate bacteria from a range of anaerobic environments, with many research and industrial applications.

This study explores the concepts of bacterial co-evolution, niche partitioning and mutually beneficial digestion among the *K. sydneyanus* gut microbiota. The extent to which each of these bacterial interactions occur in the herbivorous fish gut is currently unknown, based on a review of the literature. Previous findings in this area are in support of a co-evolutionary relationship between herbivorous fish and their gut bacteria (Flint, et al., 2005) With herbivorous fish being found to harbour a complex and specialised indigenous community of gut bacteria (Liston, 1957; Fishelson, et al., 1985; Cahill, 1990), phylogenetically distinct from bacteria in the surrounding environment (Lindsay and Gooday, 1985).

The short bacterial regeneration time means that the host-associated phase is likely predominant in the lifecycle of these symbionts, therefore a level of co-evolution in likely (Macke, et al., 2017). The use of a mixture of three bacterial isolates in a co-culture in this study goes some way to answering these questions for *K. sydneyanus* gut bacteria. Analysis of the results of this study suggest that these are all active features of the *K. sydneyanus* gut microbiota, which has a consortial nature. Therefore, this study fills a gap in the literature, where functional studies demonstrating the consortial nature of *K. sydneyanus* gut bacteria are needed.

Seaweeds

The seaweeds can be separated into the exclusively marine multicellular algae, and the unicellular or colonial microalgae that inhabit both marine and freshwater environments (Uzov & Zelinsky, 2013). The marine multicellular algae can be further divided into Rhodophyta (red), Phaeophyceae (brown) and Chlorophyta (green) algae (Uzov & Zelinsky, 2013). The general seaweed metabolome has been broken down into amino acids (36%), lipids (23%), nucleotides (15%), carbohydrates (12%), cofactors, prosthetic groups and electron carriers (8%), peptides (4%) and secondary metabolites (2%) (Belghit, et al., 2017). However, as marine macroalgae vary in protein, fibre, fatty acid, vitamin and mineral content at the species level (Belghit, et al., 2017), it is difficult to make generalisations about these organisms, except that algae are a diverse set of photosynthetic organisms within which polysaccharides make up the majority of total biomass (Uzov & Zelinsky, 2013). Algal chemical composition is closely related to taxonomy, with the main consistency being the importance of polysaccharides as the main chemical component across all algae (Uzov & Zelinsky, 2013), where they can have storage or structural functions (Kloareg & Quatrano, 1988; Painter, 1983).

There are approximately 2000 species of brown algae, all of which share features with both terrestrial plants and green and red algae in their multicellular nature, with cells surrounded by a cell wall, and their photosynthetic abilities (Charrier, et al., 2012). However, despite these similarities, these organisms evolved separately from their closest relatives 200 million years ago, evolving 400 million years after terrestrial plants and other multicellular algae had already diversified (Charrier, et al., 2012). Due to their unique evolutionary history, brown algae have unique developmental mechanisms and morphologies (Charrier, et al., 2012).

Brown algae contain various polysaccharides, including alginates, laminarin, fucoidans (Arijón, et al., 2021). In contrast, cellulose is the only glycan present in both terrestrial plants and brown algae, reflecting the high degree of adaption these organisms have to their respective terrestrial and marine environments (Painter, 1983). Laminarin, discovered in the brown algae *Laminaria*, is the principal food reserve of brown algae (Painter, 1983). A range of other glycans are also present in brown algae including cellulose, lichenan, alginate, fucoidan, sulfated hexurono-xylofucans and complex sulfated heteroglycans (Painter, 1983). Laminarin content of brown algae varies throughout the year, where it may comprise up to 35% of the dry weight in the winter and autumn and is absent in the spring (Black & Dewar 1949).

Undaria pinnatifida

The brown algae *U. pinnatifida* is ranked in the top invasive seaweed species worldwide, with a great capacity for dispersal and survival in a range of environments (Nyberg and Wallentinus, 2005). It is the only winter annual of the Laminariales order, with sporophytes that germinate and grow through winter and early spring (Yoshikawa, et al., 2001; Dean, et al., 2007). The first discovery of *U. pinnatifida* growing in New Zealand was at Wellington harbour in 1986 (Hay & Luckens, 1987). This organism has since spread across much of the New Zealand coastline (Russel, et al. 2008). Being a non-native and invasive species in New Zealand, *U. pinnatifida* was classified as an unwanted organism under the Biosecurity Act by the Ministry of Agriculture and Forestry (MAF, 2010). Due to the failure of various *U. pinnatifida* eradication attempts across New Zealand (Hunt, et al. 2009), and the massive export value of farming this alga, the New Zealand government permitted the farming and harvesting of *U. pinnatifida* in established areas from May 2010 (MAF, 2010). The long New Zealand coastline (15000 – 18000 km), which spreads across latitudes from a subtropical

34.5°S to a cool temperate 47.5°S (Gordon, et al., 2010), provides good growing conditions for this alga (Dean, et al., 2007).

The global seaweed industry is estimated to have an annual value of around US$6 billion, of which US$5 billion is from food products and US$1 billion is from seaweed extracts for use in our market of interest: animal feeds, fertilisers, and bioactives (Nayar & Bott, 2014). *U. pinnatifida* was the fourth most-produced cultivated seaweed in 2010, at 1.5 million tonnes it accounted for 10% of global seaweed production (Nyar & Bott, 2014). In Asia, *U. pinnatifida* farming generates around US$600 million per annum, primarily through conversion into human food products (White, et al., 2014). This organism can also be used in many other industries, including soap, toothpaste, animal feed, fertiliser and fuel (White, et al., 2014).

Brown algae have long been used as a food source, and as a gelling or thickening agent in food and pharmaceuticals (Mohammed, et al., 2012). However, recently there has been growing interest in the use of brown algae as complementary medicine (Mohammed, et al., 2012). Metabolites present in macroalgae are of great commercial interest for their health-promoting benefits (Belghit, et al., 2017). In a study by Belghit, et al., (2017), on 21 seaweeds across three phyla and eight orders, a total of 391 biochemical compounds were identified. Sulfolipids and fucoidans are used for their anti-inflammatory effects and fatty acids and phenolic compounds for their anti-microbial effects (Mohammed, et al., 2012). Sulphated polysaccharides, sulfolipids and polyphenols have anti-mutagenic effects (Mohammed, et al., 2012). Polyphenols have anti-diabetic effects and water-soluble polysaccharides and sulfolipids have anti-cancer properties (Mohammed, et al., 2012). Brown algae are also commercially valuable as a food and feed resource, with application depending on the species, which vary in protein, fibre, fatty acid, vitamin and mineral content (Lovstad & Kraan, 2011; Mæhre, et al., 2014).

The nutritional content of Asian and European grown *U. pinnatifida* has been well defined (Kolb, et al. 2004; Jurkovic, et al., 1995; Yamada, et al., 2007). The overall nutrient content of Asian *U. pinnatifida* dried products has been reported as: crude protein (16.7 ± 0.9), carbohydrates (33.5 ± 3.4), nucleic acids (0.9 ± 0.12) (g/100 g dry weight) (Jurkovic, et al., 1995). The protein content of Asian *U. pinnatifida* has been found to be very similar, at 16.3 ± 1.4 g/100 g dry weight (Kolb, et al., 2004). This value is high for a brown alga, with the typical range being between 7 and 16 g protein/100 g dry weight (Zhou, et al., 2015). However, the chemical composition of macroalgae is known to vary with temperature, season and location (Galland-Irmouli, et al., 1999; Nelson, et al. 2002; Boulom, et al. 2014), and *U. pinnatifida* is known to have a “plastic” quality, where the sporophytes can match their physiology to a range of environments, enhancing the success of this invasive seaweed (Dean, et al., 2007).

In New Zealand waters *U. pinnatifida* is not limited by light or nitrogen availability (Dean, et al., 2007), and so this organism thrives in many places along the New Zealand coast (Russel, et al. 2008). There have been several studies by New Zealand universities into the physiology and ecology of New Zealand sourced *U. pinnatifida* (White, et al., 2014). These include the seasonal variation studies carried out on the protein content by Zhou, et al. (2012), and on the lipids, fatty acids, phytosterol, and α-tocopherol by Boulom et al. (2012). Also, the study by Hau, et al. (2012) into the heavy metal content of *U. pinnatifida.* The unique nutritional composition of New Zealand grown *U. pinnatifida* has already been proven by Boulom et al., 2014, who found a significantly different fatty acid profile in the NZ grown variety compared to that grown in Korea or Japan (Bouloum, et al., 2014). Other studies have also shown key differences in nutrient content in comparison to samples grown in Europe and Asia. A study of the protein and amino acid content of NZ grown *U. pinnatifida* found a protein content of 13.1 g/100 g dry weight (Zhou et al., 2015), which is a lower value than the 16.7 ± 0.9 g/100 g dry weight seen in European samples (Jurkovic, et al., 1995) and the 16.3 ± 1.4% seen in Asian samples (Kolb, et al., 2004). This study also showed significant variation in the amino acid content of organisms between different sites along the NZ coast (Zhou, et al., 2015). However, sensory analysis has shown that these chemical differences in *U. pinnatifida* sourced from different geographical locations are not obvious to consumers, with the only detectable difference between NZ and Korean or Japanese products being in texture (Balbas, et al., 2015).

Fermentation of seaweed by the gut microbiota of herbivorous fish

It was long thought that the herbivorous fish gut did not have the anatomical complexity required to support microbial fermentation (Rimmer & Wiebe, 1986). However, it has since been revealed that digestion of algae by herbivorous fish has similarities to terrestrial ruminant digestion, with larger guts and longer evacuation times in herbivorous fish than their carnivorous equivalents (Hofer & Scheimer, 1981; Bitterlich, 1985). The *Kyphosus* gut was found to have a unique hindgut chamber and abundant diverse microflora (Rimmer & Wiebe, 1986). Then the discovery of volatile fatty acids (VFAs) in the *Kyphosus* gut confirmed occurrence of fermentation in the hindgut of these fish (Rimmer & Wiebe, 1986). These VFA values were just slightly lower than those reported in the sheep and cattle rumen (Wolin, 1979), demonstrating the importance of microbial fermentation to the *Kyphosidae*.

Marine herbivorous fish have been shown to have an increasing concentration of bacteria towards the distal portion of the gut (Fishelson, et al., 1985; Clements, et al., 1989; Anderson, 1991; Rimmer & Wiebe, 1986; Clements, 1991; Gleeson, 1992), suggesting that this is where fermentation occurs. Mannitol has been proven to decrease along the fish gut, in correlation with increasing fermentative action of the fish gut microbiota (White, et al., 2010). This has led to the suggestion that mannitol is an important dietary constituent for some herbivorous fish, utilised indirectly through fermentation by gut microorganisms (White, et al., 2010). The concentration of short chain fatty acids (SCFA's) is highest in the hind gut, as shown by Clements, et al., (1994) in studies on the herbivorous fish, *Odax cyanomelas*, which feeds on the brown algae *Ecklonia radiata*, and *Crinodus lophodon*, which feeds on red and green algae (Clements, et al., 1994). All these findings taken together are suggestive of the high intensity of microbial fermentation occurring in the distal portion of the fish gut, where the fish gut microbiota salvage indigestible nutrients for provision of energy to the host, in the form of SCFAs.

Much of the digestion process of the marine fish has been detailed in previous studies, with Clements and Choat (1995) producing a wide-ranging study on the production of VFAs through fermentation of algal carbohydrates in the hindgut of 32 fish species, representing 5 families. After the initial food intake and processing, including breaching of the algal cell wall, gut microbiota and host fish enzymes perform hydrolytic processing to fully digest cell contents (Anderson, 1991). These digestion products are then absorbed across the gut lumen by the host fish (Anderson, 1991).

It was suggested by Hungate (1975) that microbial protein may be taken up as an energy source by the ruminant. A later study by Clements and Choat (1995) revealed a presence of

SCFAs and proteins in the host fish blood, suggesting that these nutrients produced by gut microbiota are taken up by the host fish as an energy source (Clements and Choat, 1995). The concentration of SCFA along the gut of a range of fish species were found to be highest in the distal half of the intestine (Clements, et al., 1994; Montfort, et al., 2002). With SCFA turnover rate at its highest in gut Section IV (Montfort, et al., 2002). These results show that, for the marine herbivorous fish *K. sydneyanus*, microbial activity is highest in Section IV, despite this section preceding the specialised hindgut chamber of Section V, which is separated by a sphincter (Clements & Choat, 1993; Rimmer & Wiebe, 1987).

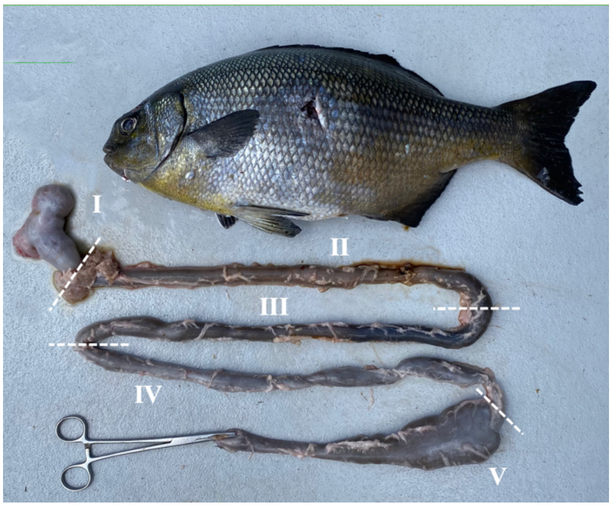


Figure 1: Anatomy and gut section I, II, III, IV, and V allocation of *K. sydneyanus*

Benefits of gut microbiota to herbivorous fish

The gut endosymbiosis found in terrestrial herbivores is well documented and functions as part of the plant material digestion process, generating important macronutrients which provide a great energy benefit to the host organism (Hungate, 1975). The study of fish gut microbiota has lagged that of terrestrial vertebrates, despite fish containing the greatest taxonomic and ecological diversity among all vertebrates (Clements, et al., 2014). After ingestion and mastication, herbivorous organisms optimise digestion of the refractory and biochemically complex nutrients from their diet using fermentation by bacteria in their gut (Hungate, 1975). Most herbivores do not have an intrinsic ability to digest plant cell structures such as cellulose and lignin by enzymatic hydrolysis (Rimmer & Wiebe,1986). These plants contain complex fibre that cannot be digested by the herbivore but can be fermented by the gut microbiota (Hungate, 1975).

In terrestrial vertebrates, both endogenous enzymes and a consortia of intestinal microbiota process large amounts of indigestible carbohydrate to energy and waste products such as carbon dioxide, methane and volatile fatty acids (VFAs) including short chain fatty acids (SCFAs) (Hungate, 1975; Stevens, 1988; Wolin, 1979). The anaerobic bacterial fermentation process releases wastes such as the acetic, propionic and butyric volatile fatty acids that can provide energy to the host via the Krebs cycle (Hungate, 1975; Troyer, 1982). A large permanent microbiota is indicative of fermentation and production of SCFAs, predominantly acetate, with propionate and butyrate also produced (Stevens, 1988).

Early work by Fishelson, et al., (1985) first detailed the presence of a protist closely associated with the gut lining of the surgeonfish (*Acanthurus* sp.), although no evidence of fermentation was presented in this study. Work by Rimmer and Wiebe (1986) was the first to demonstrate fermentative digestion in herbivorous fish with the identification of substantial VFA production. Later work by Clements, et al., (1989) demonstrated consistent microbiological consortia present in the gut of surgeonfish on the Great Barrier Reef, with most protists present in the surgeonfish gut being from the *Acanthurida*e family. Contrasting these results to the absence of *Acanthurus* sp. from the herbivorous fish in the families *Pomacentridae, Scaridae, Siganidae* and *Blenniidae,* (Clements, et al., 1989) demonstrates the consistent gut microbiological consortia specific to herbivorous fish family. Clements et al, (1989) also found correlation between feeding behaviour of different *Acanthurid* species and protist composition and morphology, further demonstrating the importance of a microbiological consortia for specific digestion of host dietary substrates.

Kyphosus sydneyanus

The gut environment and digestive mechanisms of *Kyphosus sydneyanus*

The culture experiments of this study use bacteria isolated from the hindgut of *Kyphosus sydneyanus* (Silver drummer). *K. sydneyanus* are a common herbivorous fish in North-Eastern New Zealand and Australia (Knudsen & Clements, 2016; Mountfort, et al., 2002). The gut of this exclusively herbivorous marine fish contains microbiota with the ability to ferment dietary algal substrates, which are low in easily assimilable energy, producing adequate nutrition for fish growth (Clements & Choat, 1997).

Kyphosids developed the ability to digest algae as a primary energy source in the late Miocene in southern Australia (Knudsen, et al., 2019). The gut systems of herbivorous fish are adapted for algal cell digestion via four major mechanisms, first outlined by Lobel et al. (1981), and then refined by Horn et al. (1989, 1992). These mechanisms are a pharyngeal mill, a gizzard-like stomach, acid lysis, and microbial fermentation (Lobel, et al., 1981; Horn, et al., 1989; Horn, et al., 1992). The evolution of these gut types occurred alongside a contemporaneous diversification in macroalgae (Durrant, et al., 2015; Phillips, 2001), this suggests a level of co-evolution between algae and herbivorous fish.

To feed on algae, herbivorous fish often utilise both endogenous and exogenous digestion. General adaption for the procession of algae by herbivorous fish include short, blunt snouts containing closely set teeth or a beak with which algae can be cropped to a blunt edge (Ogden & Lobel, 1978). This contrasts with the gaping jaws of predator fish (Ogden & Lobel, 1978). Adaptions for endogenous digestion of algae include variation in gut morphology, such as folding of the intestinal mucosa, villi and microvilli (Johnson & Clements, 2020). These gut features slow the rate of passage and decrease the assimilation rate of gut contents (Johnson & Clements, 2020). In comparison to predator fish, herbivorous fish have a low assimilation rate, balanced by a high ingestion rate (Ogden & Lobel, 1978). This long retention time benefits herbivorous fish that utilise hindgut fermentation as a major source of energy (Karasov & Martinez del Rio, 2007). Adaptions for exogenous digestion of algae by hindgut bacteria includes gut elasticity, wall thickness, and caecal pouches (Johnson & Clements, 2020; Ogden & Lobel, 1978). These adaptions provide environments within which a hindgut endosymbiotic community can be fostered (Johnson & Clements, 2020). These exogenous adaptions enable a large proportion of the energy requirements of herbivorous fish to be provided by the microbial fermentation of algae (Mountfort, et al., 2002).

Fish species in the genera *Kyphosus* are present in tropical, sub-tropical and temperate waters, with variations in the temperature of their surrounding environment having no effect on energy production from diet, as measured by short chain fatty acid (SCFA) content of the hind gut (Clements & Choat, 1996). Kyphosids are strictly herbivorous (Choat, et al., 2002; Clements and Choat, 1997; Moran and Clements, 2002) and can significantly impact algal biomass in areas where they are prevalent (Bennet, et al., 2015; Vergés, et al., 2012), such as Western Australia (Howard, 1989). Kyphosids feed on macroalgae and often have specialised gut sections used for microbial fermentation (Fishelson, et al., 2014; Clements and Choat, 1997; Montfort, et al., 2002; Clements and Zemke-White, 2008; Rimmer and Wiebe, 1987). *K. sydneyanus* are a temperate *Kyphosus* species, restricted to Australasia (Knudsen &

Clements, 2016). However, as Kyphosids can disperse across large distances there is thought to be a high level of inter-ocean connectivity between populations (Knudsen & Clements, 2016). The two temperate species *K. sydneyanus* and *Kyphosus gladius* make up a sister group to the tropical and sub-tropical Kyphosis species (Knudsen & Clements, 2016).

K. sydneyanus have a general olivaceous colour above the lateral line (Figure 1) which lightens to grey towards the ventral profile, head with a lateral surface concolorous with flank, fins grey with blackish ray tips (Scott, 1975). The body form of this fish is mesomatic with an apex median (Scott, 1975). Herbivorous fish are either browsers or grazers (Ogden & Lobel, 1978). *K. sydneyanus* are browsers (Horn, 1989), so selectively tear or bite individually recognisable upright macroalgae, rarely accidentally ingesting inorganic material (Jones, 1968; Lobel, 1981; Choat, 1982). *K. sydneyanus* are diurnally active, as are most herbivorous fish (Ogden & Lobel, 1978), with most feeding taking place during twilight hours (Johnson & Clements 2020; Pankhurst, 1989; Vial, 1997; Horn, 1989). Herbivorous fish then seek hiding places in the reef during the night (Ogden & Lobel, 1978). Temperate Kyphosids browse in small to large schools, including some feeding on drifting seaweeds near the sea surface (Horn, 1989; Rimmer, 1986).

At the anterior of the intestine, sections I and II (Figure 1), the algal substrate cellular structure remains intact (Rimmer & Wiebe, 1986). By the time the substrate arrives at the posterior end, sections III and IV (Figure 1) leading into the ceacal pouch, this cellular structure has broken down to produce an amorphous brown paste (Rimmer & Wiebe, 1986). The stomach of *K. sydneyanus* is strongly acidic, at a pH of 2.8 to 3 (Zemke-White & Clements, 1999; Rimmer & Wiebe, 1986). Algal cells are lysed in the *K. sydneyanus* stomach before passing into the intestine (Zemke-White & Clements, 1999). The pH remains fairly neutral along the gut, at between 6 and 8 (Zemke-White & Clements, 1999; Rimmer & Wiebe, 1986). The gut transit time of *K. sydneyanus* is 21 hours, with a gut temperature of around 23 °C (Rimmer & Wiebe, 1987).

The hindgut caecal pouch of the *Kyphosidae*, first described by Rimmer and Wiebe (1986), is large enough to contain 1.5 to 2 times the stomach volume when extended (Figure 1; Section V). In *K. sydneyanus* this pouch is a single lobed structure including a well vascularised proximal and distal valve or sphincter to separate the hindgut from the rest of the intestinal canal (Rimmer & Wiebe, 1986). After the hindgut caecum there is a 15 to 20 cm long distal rectum leading to the anus (Rimmer & Wiebe, 1986). Diverse abundant microflora is present throughout the algal substrate in the hindgut sections, with increasing diversity and abundance towards the distal end greatest in the hindgut caecal pouch (Rimmer and Wiebe, 1986).

The K. sydneyanus diet

Studies have found that the adult *K. sydneyanus* diet is dominated by brown algae (Clements and Choat, 1997; Moran and Clements, 2002). The brown algae *U. pinnatifida* was used in investigations for *K. sydneyanus* gut bacterial growth factors in this study due to a variety of factors, detailed in this section.

Red and green algae dominate the juvenile *K. sydneyanus* diet, before a shift to brown algae by the time they reach adulthood (Moran & Clements, 2002). Red and green algae can be digested by endogenous enzymes (from the host fish), unlike brown algae which requires fermentation to release digestible nutrients (Montgomery & Gerking, 1980). Red and green algae are also found in the stomach of adult *K. sydneyanus*, however on average these algae are present at a much lower concentration than the browns (Clements and Choat, 1997; Moran and Clements, 2002). The *K. sydneyanus* retain a low-level ability to digest red and green algae using endogenous enzymes throughout their adult life (Moran & Clements, 2002). This allows a gradual shift in diet and may explain why some adult *K. sydneyanus* have been found with stomachs containing a wide variety of red algae and some green algae (Clements and Choat, 1997; Moran and Clements, 2002).

Although restricted to a mostly brown algae diet, there is thought to be greater variation in algae making up the diet of *K. sydneyanus* than other brown algae consuming fish (White, et al., 2010). This prediction is based on the wide variation in the mannitol content of the gut between individual *K. sydneyanus* fish (White, et al., 2010). Large phenotypic variation in the diet is usually enabled by the ability to pull upon a large pool of secondary gut bacteria, providing metabolic flexibility for host adaption to a wider variety of environmental conditions (Macke, et al., 2017). This flexibility was proven in the results of this PhD project, which demonstrated a wide range of digestive abilities amoung the *K. sydneyanus* hindgut bacteria. The gut bacteria isolates used in this study were able to utilise all four species of algae tested for growth.

In a study by Rimmer and Wiebe (1986) the diet and gut morphology of *K. sydneyanus* from coastal Western Australia were closely examined. The diet of *K. sydneyanus* was found to consist of mainly benthic macroalgae, with the main substrate being Phaeophyceae (brown algae), at 93.9% of total diet (Rimmer & Wiebe, 1986). The limestone high latitude coral reef habitats of these fish mainly consist of diverse abundant benthic macroalgal communities (Womersley, 1981; Wilson & Marsh, 1979). Subsequent studies have confirmed that brown algae also dominate the diet of *K. sydneyanus* caught off the coast of New Zealand (Clements

& Choat, 1997). The New Zealand native brown algae *Ecklonia radiata* is the most common dietary component of *K. sydneyanus* caught off the New Zealand coast (Parsons, 1994). The *K. sydneyanus* diet also includes the brown algae *Carpophyllum* spp., along with various filamentous rhodophytes (red algae) and chlorophytes (green algae) (Clements & Choat, 1997; Moran & Clements, 2002). Brown macroalgae are the dominant subtidal organisms across many temperate shores (Mann, 1973; Choat & Schiel, 1982), providing *K. sydneyanus* with a large pool of substrate. With these temperate brown algae including some of the most productive plant systems in the world (Mann, 1979). Although less than 10% of this algal energy production enters herbivorous food chains, with the rest lost to detritus food chains (Mann, 1979).

Preliminary experiments within this study showed that *Undaria pinnatifida* aqueous extract had the same growth effects on *K. sydneyanus* hindgut bacteria as *E. radiata* (**Chapter 4**). *U. pinnatifida* is prevalent along the New Zealand coast (Hay & Luckens, 1987). There is also a large industrial interest in this seaweed (Nayar & Bott, 2014). All these factors decided that *U. pinnatifida* would be the substrate of choice to investigate for *K. sydneyanus* gut bacterial growth factors in this study.

The *Kyphosus sydneyanus* gut microbiota

Juvenile *K. sydneyanus* have short gut throughput times and rely on endogenous enzymes to digest primarily green and red algae (Clements, et al., 2014; Moran & Clements, 2002). While adult *K. sydneyanus*, have long gut throughput times and rely on gut bacteria to ferment a brown alga dominated diet to digestible SCFAs (Clements, et al., 2014; Moran & Clements, 2002). While herbivorous marine fish vary in their reliance on gut bacteria for digestion, there is a positive correlation between the distribution of SCFAs and bacteria in the fish gut (Clements, 1994). The produced SCFAs are absorbed along an osmotic gradient from the intestine into the blood of the fish (Titus & Ahern, 1988). Bacteria in the gut play an important role in nutrition by aiding in digestion or by providing the host with nutrients lacking in the diet (Engel & Moran, 2013). Herbivores cannot digest plant cell walls or resistant starches using endogenous enzymes, so rely on their gut microbiota to convert certain indigestible polysaccharides into absorbable SCFAs (Feldhaar, 2011; Douglas, 2015; Amato, 2016). These gut microbes also play essential roles in regulating the host immune response to pathogens (Belkaid & Hand, 2014). Herbivorous fish have been found to harbour a complex and specialised indigenous community of gut bacteria (Liston, 1957; Fishelson, et al., 1985; Cahill, 1990), phylogenetically distinct from bacteria detected in the surrounding environment (Lindsay and Gooday, 1985).

Just being present in the immediate environment of a host is not enough for a bacterium to become part of the fish gut microbiota, these bacteria are selectively recruited. The gut microbiota is usually made up of two parts; a core group of species under direct genetic and immune control by the host, and a secondary flexible group modulated by environmental diversity and external conditions (Shapira, 2016). The core group of bacteria provide a greater contribution to essential functions of the gut microbiota and are more likely to be transmitted down the generations (Shapira, 2016). The variation in microbiota composition throughout the host life cycle and during periods of environmental variation provides a plasticity to host digestive abilities (Macke, et al., 2017). The composition of the secondary flexible group of gut bacteria can quickly adapt to digest varying dietary substrates, providing metabolic flexibility for host adaption to a wider variety of environmental conditions (Macke, et al., 2017). When there is an intergenerational transfer of gut microbiota, such as that seen in terrestrial herbivores, these mechanisms result in a co-evolution between host genes and gut microbiome composition (Macke, et al., 2017). It is not currently known if there is intergenerational transfer of gut bacteria in herbivorous fish.

Differences seen in the polysaccharide fermenting abilities of various fish species suggest that the hindgut bacterial communities of herbivorous fish are adapted to the nutrients present in the host fish diet (Seeto, et al., 1996). However, it is thought to be unlikely that there is a significant intergenerational transfer of herbivorous fish gut microbiota. This is due to the independent early life of a *K. sydneyanus*, and the difference in diet of juvenile and adult *K. sydneyanus*. Therefore, the extent to which the gut bacteria of *K. sydneyanus* effect population dynamics and genetic diversity of their hosts may be limited (Macke, et al., 2017).

Work by Stevenson, et al., (2020) showed that the gut bacteria of the herbivorous fish are not as closely associated with the host as that of terrestrial vertebrates. The selection of *K. sydneyanus* core microbiota is mostly based on function rather than taxonomy (Stevenson, et al., 2020). However, *K. sydneyanus* have evolved a dependency on gut bacteria for digestion, including the evolution of a specialised gut Section IV with a separating second sphincter, in which many bacteria can perform fermentation of brown algae for the nutritional benefit of the host (Clements & Choat, 1993; Rimmer & Wiebe, 1987). The host associated phase is likely predominant in the lifecycle of these symbionts, therefore the conditions of this phase may have an evolutionary effect (Macke, et al., 2017). Co-evolution is common in gut bacteria when nutrients are easily taken up from the gut environment instead of synthesised (Engel & Moran, 2013; Shapira, 2016).

Horizontal transfer of *K. sydneyanus* gut bacteria

Horizontal transfer of bacteria allows individuals and populations, usually of the same species, to affect the gut bacteria composition and digestive abilities of one another (Feldhaar, 2011). This horizontal transfer and recruitment of bacteria produces a large genetic and functional diversity of microbial metabolism, enabling adaption of the host organism to a wide range environmental niches (Shapira, 2016). It has been shown by Stevenson, et al., (2020) that there is no core taxonomic group of bacteria in the *K. sydneyanus* gut. The secondary group of environmentally modulated gut bacteria is dominant in *K. sydneyanus* due to the highly variable diet of this herbivorous fish (Stevenson, et al., 2020). This dominant pool of secondary bacteria allows the host organism to be highly flexible and interactive with their environment (Macke, et al., 2017).

Transferred gut bacteria modify the internal environment of the host, with various outcomes (Macke, et al., 2017). The short generation times and fast evolution of gut bacteria increase the adaptiveness of the gut microbiota to different environments (Dillon & Dillon, 2004). The flexibility provided by adaption of the gut microbiota supports host organism dispersal and survival in new habitats (Macke, et al., 2017). The relative abundance of the various bacteria within the pools can also be modulated by host genes (Bordenstein & Theis, 2015). The gut microbiota influence host reproduction and range, thereby affecting the genetic diversity and population dynamics of these host organisms (Macke, et al., 2017).

Co-evolution between *K. sydneyanus* and their gut bacteria

Co-evolution between host organisms and their gut microbiota requires selective forces to be exchanged between both parties, resulting in reciprocal evolutionary change (Macke, et al., 2017). Co-evolution can also occur when the pressures of unidirectional selection acts upon both parties, in this case host evolution effects selectivity and mechanisms of community assembly, again resulting in reciprocal evolutionary change between organisms and their gut microbiota (Macke, et al., 2017). This co-evolution is only possible because the host-associated phase is predominant in the gut life cycle of gut bacteria (Macke, et al., 2017). Gut microbiota have been found to co-evolve with the host fish, leading to a loss of gene function and loss of the ability to synthesise costly metabolites that may instead be taken up from the gut environment (Engel & Moran, 2013; Shapira, 2016).

In herbivorous organisms, gut bacteria are likely to mediate host evolution and adaptation due to their key role in host digestion, mediating host-plant specialisation (Gilbert, et al., 2015; Tsuchida, et al., 2004). A mutual co-evolution has been found to occur between host and symbiont when a shift in environmental factors causes a change to host diet (Khol, et al., 2016; Chu, et al., 2013). A shift in environmental conditions can prompt the evolution of genes, such as immune defence genes, that are in control of the acquisition and tolerance of gut bacteria, this genetic shift leads to the intergenerational co-evolution of host genes and gut bacteria (Vavre & Kremer, 2014; Bordenstein & Theis, 2015). The pressure for adaption to new environments often leads to a dramatic shift in gut microbiome composition over a short period of time, facilitating species diversification in new environments (Vavre & Kremer, 2014; Macke, et al., 2017).

Mutualism between the *K. sydneyanus* gut bacteria

This study uses growth rate experiments to investigate functional relationships between different gut bacteria species in the *K. sydneyanus* gut. In the rumen gut bacteria have been shown to co-evolve with the host and perform mutually beneficial digestive processes. The evolution of specialised gut morphology for bacterial fermentation of brown algae on the part of the host fish (Clements & Choat, 1993; Rimmer & Wiebe, 1987) proves a level of evolution in *K. sydneyanus* to accommodate their gut microbiota. This study contributes towards answering important questions around the extent to which commonly identified *K. sydneyanus* gut microbiota have co-evolved with their host. To answer this question, we used bacteria commonly isolated from *K. sydneyanus* gut samples in our *in vitro* experimental work. It is also not known the extent to which bacteria species in the herbivorous gut mutually digest dietary substrates. This study also uses *in vitro* co-culture experiments to investigate co-evolution and mutual digestion in the herbivorous fish gut.

Within the rumen microbiota mutualistic relationships between bacteria allow fermentation by-products such as lactate, ethanol and succinate to be used as an energy source by neighbouring bacteria (Hungate, et al., 1975). An early *in vitro* demonstration of the mutualisms in the herbivore gut microbiota was the co-fermentation of glucose by *Ruminococcus albus* and *Vibrio succinogenes*. Iannotti, et al., (1973) found that hydrogen released as a fermentation waste product by *R. albus* was taken up by *V. succinogenes* to reduce fumarate to succinate. This mutualistic relationship increased the growth rate of these two bacteria in a glucose-rich medium, as electrons released during oxidation by *R. albus* of fermentation substrates were re-purposed by *V. succinogenes*, a hydrogen-utilising and methane producing bacterium, increasing energy yield from substrate breakdown (Iannotti, et al., 1973).

Further *in vitro* demonstrations of rumen microbiota mutualisms have included the provision of methane (Hoover & Lipari, 1971) and sugar (Scheifinger & Wolin, 1973) by one bacterium to another during co-culture of rumen anaerobes. These studies show that the microbiological consortia are key to maximal energy yield from a herbivorous diet. This maximal energy yield allows microbiota to flourish at the highest density possible in the gut, releasing large amounts of useful fermentation by-products for host consumption. As an expected consequence of these processes, high density of gut microbiota favours host growth (Hungate, 1975). In fact, sudden changes to diet have a dramatic effect on microbiota composition, where the sudden feeding of grain or glucose shown to cause death, due to proliferation of *Streptococcus bovis* causing a fatal lactic acid build up (Hungate, 1975). In this case there is not enough time for an increase in mutualistic microbes, and harmful digestion by-products are not processed quickly enough. This can cause irreversible damage to host health and to the rumen tissues (Hungate, 1975). In this way the gut microbiota and host work together to digest substrate for the optimal energy production and transfer.

Digestion of seaweed in the *K. sydneyanus* gut

The brown algae (Phaeophyceae) are comparatively harder to digest than the chlorophytes green algae (chlorophytes) or red algae (rhodophytes). The main storage component of brown algae is laminarin, requiring the hydrolysis of this β-linked glucan, an uncommon endogenous enzymatic ability in vertebrates (Moran & Clements, 2002). Whereas the α-linkages of floridean starch and starch, the storage polysaccharides of red and green algae respectively, are digestible by most fish due to the endogenous production of carbohydrases (Moran & Clements, 2002; Zemke-White & Clements, 1999).

Based on total nutrient and energy content, green algae are the richest energy source, the second richest is brown, with red algae providing the least energy of the three (Montgomery & Gerking, 1980). Based on these endogenous factors, without the presence of gut bacteria green algae would be the preferred food source for marine herbivorous fish, while brown algae would be avoided due to a lack of digestible carbohydrates (Montgomery & Gerking, 1980).

Adult *K. sydneyanus* rely on exogenous digestion by bacterial fermentation of brown algae as their main food source (Moran & Clements, 2002). The difficulty in endogenous digestion of brown algae causes Kyphosids to go through ontogenetic changes in feeding behaviour and digestion (Moran & Clements, 2002). Adult *K. sydneyanus* develop a resident gut microbiota by the time they are 36 mm in length (Rimmer, 1986). Along with a fermentation chamber that makes up gut Section V (Figure 1) separated by a sphincter.

Digestion of brown algae in the *K. sydneyanus* gut

Brown algae is digested to laminarin, mannitol, glucose and alginic acid in the gut (Painter, 1983; Seeto, et al., 1996). Mannitol was found to be present in *E. radiata* at a much higher concentration than the other compounds in the group of carbohydrates and sugar alcohols, at about a 16 : 4 : 1 ratio of mannitol : glucose : other carbohydrates (Seeto, et al., 1996). Brown algae contain a large amount of glucose and mannitol just within laminarin chains. Between 40 and 75% of the laminarin chains of the brown algae species *Laminaria* and *Fucus* are glycosidically linked from the reducing terminal D-glucopyranose (a pyranose form of glucose) residue to a primary hydroxyl group of D-mannitol (Painter, 1983).

The cell walls of brown algae are mainly made up of alginate (the salts of alginic acid), carrageenan and agarose (Skea, et al., 2005). These polysaccharide products of algae digestion are resistant to endogenous fish enzymes, so cannot be taken up by *K. sydneyanus* through the gut (Zemke-White & Clements, 1999; Moran & Clements, 2002). Instead, these polysaccharides are used as substrates for bacteria fermentation, mainly to SCFAs that are taken up by the fish and contribute to fulfilling their energy requirements. (Mountfort, et al., 2002).

The microbiota of the *K. sydneyanus* hindgut were shown by Skea, et al., (2005) to be responsible for the digestion of a large portion of important and otherwise inaccessible nutrients present in the brown algae. Enzymatic hydrolysis of laminarin and alginate were shown to occur at the highest rate in the hindgut of *K. sydneyanus*, where microbial density and fermentation rate are at their most intense (Skea, et al., 2005). The activity of laminarin degrading enzymes was also found to be higher in the hindgut fluid than the hindgut wall (Skea, et al., 2005). These results suggest that enzymatic hydrolysis of laminarin is mainly carried out by exogenous action by microbiota of the fish hindgut (Skea, et al., 2005). Therefore, laminarin, alginate and the associated polysaccharides mannitol and glucose can be expected to be digested by *K. sydneyanus* hindgut bacteria *in vitro*, providing energy, so acting as a growth factor for these bacteria.

Enzymatic hydrolysis of laminarin is highest in the hindgut of herbivorous fish, where microbial density and fermentation rate are greatest (Skea, et al., 2005). The activity of laminarin degrading enzymes is higher in the gut fluid than the gut wall (Skea, et al., 2005). These results suggest that enzymatic hydrolysis of laminarin is mainly carried out by exogenous action by microbiota of the fish hindgut. Enzymatic degradation of carrageenan and alginate were also limited to the hindgut, suggesting that these aspects of brown algae digestion are also carried out by the gut microbiota in *K. sydneyanus* (Skea, et al., 2005). Overall, the microbiota of the *K. sydneyanus* hindgut were shown by Skea, et al., (2005) to be responsible for the digestion of a large portion of important and otherwise inaccessible nutrients present in the brown algae. These differences in herbivorous fish and terrestrial herbivore substrate nutritional composition are reflected their gut morphology and microbiota composition.

As *K. sydneyanus* grow the activity of the laminarin hydrolysing enzymes drop, while exogenous digestion increases with an increasing volume of bacteria in the *K. sydneyanus* gut (Moran & Clements, 2002). Adult fish have a specialised hindgut section (Section V) for fermentation of brown algae, separated with a sphincter, and have a high volume of microbiota in the gut (Clements & Choat, 1993; Rimmer & Wiebe, 1987). The main storage carbohydrates of brown algae are fermented by *K. sydneyanus* hindgut bacteria to SCFAs that are taken up by the host fish through the gut wall (Moran & Clements, 2002; White, et al., 2010).

Digestion of red and green algae in the *K. sydneyanus* gut

Juvenile *K. sydneyanus* have no fermentation abilities, instead there are high levels of starch-degrading endogenous enzymes active in the guts of these fish for digestion of red and green algae (Moran & Clements, 2002). Juvenile *K. sydneyanus* also have relatively high levels of endogenous laminarin hydrolysing enzymes, for digestion of brown algae (Moran & Clements, 2002).

Red and green algae contain four main storage carbohydrates: starch, floridean starch, carrageenan and agar (Clements & Choat, 1997; Rimmer & Wiebe, 1987; Painter, 1983). After ingestion these carbohydrates are digested to glucose then SCFAs by endogenous enzymes in the fish gut (Moran & Clements, 2002; Mountfort, et al., 2002). The cell walls of green algae contain polymers of glucose, mannose and xylose that are resistant to breakdown by endogenous enzymes (Craigie, 1974; Mackie & Preston, 1974). In contrast, the extracellular polysaccharides of red algae are made up of galactose and other diverse polysaccharides joined by alternative α and β linkages (Montgomery & Gerking, 1980). The α-linked polysaccharides can by endogenously digested (Montgomery & Gerking, 1980). In a similarity to terrestrial plants, red algae also utilise starch for energy storage (Hentati, et al., 2020; Skea, et al., 2005). The highest overall enzyme activity in the *K. sydneyanus* gut is performed by endogenous amylolytic enzymes, mostly in the gut wall (Skea, et al., 2005).

Fermentation of simple carbohydrates (and sugar alcohols) by the gut microbiota of herbivorous fish

Proteins and lipids from algae provide a more easily accessible nutrient source than polysaccharides for endogenous fish enzymes (Montgomery & Gerking, 1980). However, the storage polysaccharides of algae are a greater energy reservoir than either proteins or lipids (Montgomery & Gerking, 1980). Therefore, polysaccharide digestion is prioritised by adult herbivorous fish if they host a gut microbiota community with the ability to hydrolyse these polysaccharides (Montgomery & Gerking, 1980). Bacteria in the gut of marine herbivorous fish ferment non-digestible algae carbohydrates such as cell wall and storage polysaccharides to produce SCFAs that are taken up by the host fish (Seeto, et al., 1996). These SCFAs are thought to be an important energy source for brown algae consuming marine herbivorous fish, such as *K. sydneyanus* (Clements, 1997).

It would be expected that the fish hindgut bacteria isolates used in these experiments would be able to utilise laminarin, mannitol, glucose and alginic acid as energy for growth. These are polysaccharide products of brown algae digestion that these organisms would in the natural environment of the *K. sydenyanus* gut.

SCFA production in the *K. sydneyanus* gut

Acetate is produced in the fish gut by microbial fermentation of glucose, fructose and mannitol from seaweed by gut microbiota (Seeto, et al., 1996). A study by Mountfort, et al., (2002) found that acetate was the SCFA present at the highest concentration in all *K. sydneyanus* gut sections, then propionate, then butyrate. This finding is in agreement with that seen in terrestrial ruminants (Iannotti, et al., 1973; Stevens & Hume, 2004). In a study by Clements, et al., (1994) acetate was by far the major SCFA present in fish hind gut samples across all fish species tested, with the ratio of acetate : propionate : butyrate : valerate, at 83 : 8 : 9 : 1 in *O. cyanomelas* and 74 : 17 : 9 : 0 in *C. lophodon* (Clements, et al., 1994).

In sections IV and V most of the acetate produced was not degraded to CO_2 or methane by bacterial fermentation in the gut, but instead absorbed across the gut wall (Mountfort, et al., 2002). This acetate is then processed to energy by the enzyme acetyl CoA synthetase, which has been found in the major tissues of marine herbivorous fish (Clements, et al., 1994). The detection of radioactively labelled acetate from the gut in the *K. sydneyanus* bloodstream further proves the presence of a gut to fish acetate digestion pathway (Mountfort, et al., 2002).

These findings show that SCFAs produced by bacterial fermentation of seaweed in the *K. sydneyanus* gut are taken up by the fish and contribute to fulfilling their energy requirements. This acetate production and digestion pathway completes a mutually beneficial symbiotic relationship between the hindgut bacteria and *K. sydneyanus*.

Laminarin

Laminarin-derived glucose makes up 97.5% of the total glucose in *E. radiata*, with only 2.5% being free glucose (Seeto, et al., 1996). Combined enzyme action by laminarinase and β-glucosidase releases glucose from laminarin (Seeto, et al., 1996). Activity from these two enzymes was mostly found in the distal gut portion (Russo, 1993). However, free mannitol makes up 73.5% of total mannitol available in *E. radiata*, meaning that enzymatic release of mannitol from laminarin is significantly less important than that seen for glucose (Seeto, et al., 1996). Polysaccharides are moved into the bacterial cells by permease transporters are then phosphorylated, or by a phosphotransferase system (Seeto, et al., 1996). These molecules are then glycosylated, the maximum rate of glycolysis is similar to the combined maximum rates of mannitol and glucose phosphorylation (Seeto, et al., 1996).

Glucose and mannitol are released from laminarin and utilised by gut bacteria fermentation to SCFAs (Percival and McDowell, 1967; Percival, 1979). Laminarin, the main storage molecule of brown algae, is a beta (β) linked polymer of glucose and uronic acids (Craigie, 1974; Mackie & Preston, 1974). It is rare for endogenous enzymes to be capable of digesting the β-links between monosaccharide subunits of laminarin (Kapoor, et al., 1975; Barrington, 1957; Al-Hussaini, 1947). Instead, digestion of this complex algae carbohydrate in the herbivorous fish gut is likely achieved by bacterial fermentation (Stickney & Shumway, 1974; Trust & Sparrow, 1974). The domination of brown algae in the *K. sydneyanus* diet is therefore a reflection of the benefits of a large intestinal gut microbiota, which allows a high level of exogenous digestion of laminarin to SCFAs, ultimately providing energy to the host fish.

There are 20-25 glucose units present in laminarin chains, these chains are either terminated by another glucose or a mannitol molecule (Percival, 1979). Laminarin-derived glucose makes up 97.5% of the total glucose in *E. radiata*, with only 2.5% being free glucose (Seeto, et al., 1996). Combined enzyme action by laminarinase and β-glucosidase releases glucose from laminarin (Seeto, et al., 1996). Activity from these two enzymes in mostly found in the distal portion of the fish gut (Russo, 1993). However, free mannitol makes up 73.5% of total mannitol available in *E. radiata*, meaning that enzymatic release of mannitol from laminarin is significantly less important to herbivorous fish than that seen for glucose (Seeto, et al., 1996).

Mannitol

There are large amounts of mannitol in brown algae, at up to 50% of dry weight (Meeuse, 1962; Painter, 1983). Mannitol is released from the cells of brown algae into the gut fluid during acid lysis in the stomach (White, et al., 2010). Findings by White, et al., (2010) that mannitol from brown algae is an important dietary constituent for *K. sydneyanus*, where it is utilised as an indirect nutrient after fermentation by the gut microbiota. These findings were based on a decreasing mannitol concentration towards the distal end the *K. sydneyanus* gut which contains the highest concentration of bacteria, indicating bacterial fermentation of mannitol (White, et al., 2010).

The *K. sydneyanus* stomach has a pH of 3.5 (Zemke-White, et al., 1999), this acidity increases the porosity of algal cells allowing molecules up to 6 nm to diffuse out (Zemke-White, et al., 2000). Therefore, mannitol, at a diameter of 0.8 nm (Schep, et al., 1997), will diffuse out of the ingested brown algae cells and into the surrounding fluid during digestion in the stomach by acid lysis. This movement is reflected in the mannitol concentration in the

K. sydneyanus gut, as White, et al., (2010) found that in Section I mannitol concentrations were equal in between gut contents and gut fluid (White, et al., 2010). The concentration of mannitol in both the gut contents and fluid then dropped dramatically between K. sydneyanus anterior gut Sections I and II and posterior Sections IV and V (White, et al., 2010), as this molecule was removed by an increasing level of microbial fermentation along the gut.

There are no specific transporters for mannitol amoung the vertebrates (Saunders & Wiggins, 1981; Morishita, 1994). Mannitol absorption across the gut epithelium only occurs via passive diffusion or solvent drag (White, et al., 2010). Mannitol is not directly taken up by the fish in any significant way, but instead fermented to SCFAs by bacteria in the hindgut (White, et al., 2010). These SCFAs are an important energy source for the host fish. In this way, fermentation by gut bacteria salvages energy from mannitol which can then be to the host fish (Seeto, et al., 1996; Choat & Clements, 1998). Findings by White, et al., (2010) showed that mannitol from brown algae is an important dietary constituent for *K. sydneyanus*, where it is utilised as an indirect nutrient after fermentation by the gut microbiota. These findings were based on a decreasing mannitol concentration towards the distal end the *K. sydneyanus* gut which contains the highest concentration of bacteria, indicating bacterial fermentation of mannitol (White, et al., 2010). Mannitol fermented by *K. sydneyanus* gut microbiota provides an indirect source of nutrients for the host, as products of microbial metabolism are assimilated for energy and bioactivity (White, et al., 2010).

The decrease of mannitol along the gut is concurrent with findings by Ferraris & Ahearn (1984) that the anterior portion of the herbivorous gut has the greatest nutrient absorption capacity. This heightened nutrient transfer in the anterior gut is also partially explained by an increase in general luminal concentration towards the posterior end, creating a strong osmotic gradient for movement of nutrients across the gut epithelial walls (Ferraris & Ahearn, 1984). Mannitol is not likely to be absorbed through the *K. sydneyanus* gut epithelium by diffusion or solvent drag as mannitol removal suddenly increases between the anterior to the posterior gut sections, with mannitol concentration being highest in the first gut sections (White, et al., 2010). An increase in mannitol concentration towards the posterior is necessary for diffusion and solvent drag to dominate mannitol movement across the epithelium (Krugliak, et al., 1994; Loretz, 1995). Therefore, removal of mannitol in the hindgut is instead performed by fermentation of this substrate by gut bacteria to SCFAs (White, et al., 2010). Mannitol is fermented to acetate in the *Olisthops cyanomelas* gut (Seeto, et al., 1996).

Alginic acid

Alginic acid is the most prevalent polysaccharide in brown algae (Arijón, et al., 2021; Painter, 1983; Percival, 1979). Alginic acid is the main component of brown algae cell walls, giving these cell walls their semirigid form (Percival, 1979). This polysaccharide is made up of unbranched chains in blocks of contiguous β-D-mannuronic acid or α-L-guluronic acid (Percival, 1979). Seeto, et al., (1996) predicted that that catabolism of structural polysaccharides of macroalgae was not important for microbial metabolism. This prediction was based on there being no remaining energy in the rate limiting glycolysis conversion to acetate for additional substrates such as alginic acid, which would be prepared for glycolysis by alginase activity (Seeto, et al., 1996).

Glucose

The major storage components of green and red algae are starch and floridean starch respectively. Starch is an alpha (α) linked polymer of glucose (Craigie, 1974; Mackie & Preston, 1974). Floridean starch is also an α-linked polymer of glucose (Craigie, 1974;

Mackie & Preston, 1974). The α-links between monosaccharide subunits of these starch polysaccharides can be digested by endogenous fish enzymes because these bonds are susceptible to endogenous amylase (Montgomery & Gerking, 1980). The intestines of marine herbivorous fish contain high levels of this amylase activity (Barrington, 1957; Kapoor, et al., 1975). These endogenous enzymes allow breakdown of intracellular polysaccharides from green and red algae to monomers for use in bioactivities and as energy for growth by the host fish.

The amount of energy these bacteria can salvage from glucose from ingested green and red algae in adult *K. sydneyanus* gut is limited by the action of endogenous enzymes. Endogenous *K. sydneyanus* enzymes such as amylase allow breakdown of the intracellular polysaccharides from green and red algae to monomers for use in bioactivities and as energy for growth by the host fish (Montgomery & Gerking, 1980; Barrington, 1957; Kapoor, et al., 1975).

It would be expected that fish hindgut bacteria would be able to utilise glucose as an energy source for growth, as glucose is the major component of laminarin, the main storage carbohydrate of brown algae (Craigie, 1974; Mackie & Preston, 1974). This laminarin derived glucose is only released by microbial fermentation in the hindgut (Stickney & Shumway, 1974; Trust & Sparrow, 1974).

A previously studied fish microbiota metabolome: *Odax cyanomelas*

The herbivorous fish *Odax cyanomelas* (Herring Cale) feeds primarily on the brown algae *E. radiata* (Andrew and Jones, 1990). The fermentation of simple carbohydrates (and sugar alcohols) from *E. radiata* to SCFAs by gut bacteria in the gut of *O. cyanomelas* has been closely studied by Seeto, et al., (1996), revealing the complex digestive pathway for *E. radiata* in the fish gut for the first time. Interestingly, the ratio of enzyme activity in both the glucose and mannitol pathways in the gut of *O. cyanomelas* were found to be similar to the ratio between glucose and mannitol in *E. radiata*, proving that these two compounds dominate nutritional uptake by the *O. cyanomelas* gut bacteria (Seeto, et al., 1996). These findings suggest that the gut bacteria are specifically adapted to the brown algal substrate most common in the *O. cyanomelas* gut environment, with a level of co-evolution between these two organisms.

Work by Seeto, et al., (1996) found that uptake of glucose and mannitol by gut bacteria is facilitated by both a permease mechanism, where the polysaccharide is not broken down before transport, and a phosphotransferase system, where these molecules are phosphorylated during transportation across the bacterial cell wall (Seeto, et al., 1996). Polysaccharides that are moved into the bacterial cells by permease transporters are then phosphorylated (Seeto, et al., 1996). Once these polysaccharides have been phosphorylated through either transport pathway, they go through a glycolytic sequence of three reactions for conversion to pyruvate then acetate (Seeto, et al., 1996). These glycolytic enzymes must include the rate controlling step of overall carbohydrate metabolism, as the maximum rate of glycolysis is similar to the combined maximum rates of mannitol and glucose phosphorylation (Seeto, et al., 1996). Eventually *E. radiata* polysaccharides are fermented to acetate for release outside the cell and uptake by the host fish (Seeto, et al., 1996). Acetate is found in the blood of the herbivorous fish *O. cyanomelas* and the enzyme acetyl CoA synthetase is found in all major tissues of *O. cyanomelas*, proving this acetate is then quickly activated and utilised by the host fish as an energy source (Clements, et al., 1994).

Seeto, et al., (1996) also found that *O. cyanomelas* gut bacteria possessed phosphotransferase systems for galactose and fructose, despite neither of these polysaccharides being present in the *E. radiata* dominated diet of these fish. Other enzymes in the fructose digestion pathway are also present among the gut bacteria of *O. cyanomelas,* and these enzymes may be utilised during the phosphorylation of the fructose produced during mannitol processing (Seeto, et al., 1996). This finding is evidence of the importance of culture dependent experimental work on these isolates. Despite possessing the ability to digest galactose and fructose, these pathways are not active in the natural environment of these bacteria. This finding also reveals a wide metabolic scope for these bacteria, suggesting these organisms enhance the phenotype of their host with their ability to adapt to shifting nutritional environments. This could also indicate a lack of specialisation to the environment of the host fish gut, due to still possessing pathways surplus to nutritional requirements. This relates to the work of this PhD, which found that *K. sydneyanus* gut bacteria was able to utilise nutrients extracted from four different native New Zealand algae. Growth rates on three of these seaweed extracts was significantly faster than that seen on media with *K. sydneyanus* gut fluid, the natural nutritional environment of these fish.

The *in vitro* culture of gut bacteria

The importance of *in vitro* bacterial studies

This study includes improvements to *in vitro* culture of fish gut bacteria. The bacteria present in the *K. sydneyanus* gut are chemoorganotrophic, so derive their energy from the degradation of organic molecules (Monod, 1949). Many chemoorganotrophic bacteria can be grown in culture, in media containing a carbon and energy source, a hydrogen acceptor, inorganic ions, and carbon dioxide (Monod, 1949). However, some of these bacteria are symbiotic or parasitic organisms missing certain pathways and processes essential for growth. These bacteria require the addition of certain intermediate nutrients for successful growth *in vitro* (Monod, 1949). When a bacterial growth media is lacking in one of these essential compounds it becomes a limiting factor to bacterial growth rate (Monod, 1949). Discovering the identity of these limiting compounds allows successful growth of previously unculturable organisms. These additional nutrients can be referred to as bacterial growth factors (GFs) and are required for optimal growth.

Culturing bacteria is the only way to reveal factors contributing to host health and ecological balance, and the only way to discover and produce natural products (Stewart, 2012). The market for these products is huge, half of all commercially available pharmaceuticals are derived from natural bacterial products (Damain & Sanchez, 2009). These natural products are not only antibiotics but also secondary metabolites that can be used in medicine during organ transplantation, as a cancer treatment, or as a method of cholesterol control (Demain & Sanchez, 2009). These products may also be used in industry as fungicides, insecticides, and antiparasitics (Demain & Sanchez, 2009). Recent work on culturing uncultured bacteria has focussed on tailoring cultivation techniques utilizing antibiotics, nutrient dilutions, and cocultivation with other organisms (Stingl, et al., 2007; D'Onofrio, et al. 2010; Kaeberlein, et al., 2002).

The diversity of cultured bacteria is limited by the great plate count anomaly, which has led to a lack of novel natural products (Stewart, 2012). Over the last 30 years cultivation-independent molecular techniques have revealed the huge variety of bacteria present in the natural environment, but the cultivated portion of this diversity is orders of magnitude smaller (Keller and Zengler, 2004; Connon and Giovannoni, 2002). This 'great plate count anomaly' is a decades old problem in microbiological research, as originally described by Stanley and Konopka (1985). It has been estimated that 60% of oceanic marine bacteria are viable (Button, et al., 1993), however multiple studies have found that only 0.01 to 0.1% of the bacteria present in a marine sample could be successfully cultured using standard plating techniques (Kogure, et al., 1979; Amann, et al., 1995). Alternative approaches to instead screen synthetic compound libraries have not yielded any novel molecules, only produced modifications to known product core structures (Baltz, 2008). It is therefore in the interests of the scientific community to attempt a different approach to accessing the uncultured majority of bacteria; re-visiting the search for novel growth factors (GFs).

In fact, it is commonly cited that less than 1% of extant bacterial species have ever been grown in the laboratory, and even fewer have been metabolically assessed for function (Bomar, et al., 2011; Amann, et al., 1995; Stott, 2008; Torsvik, et al., 2002; Rappé and Giovannoni, 2003; Keller and Zengler, 2004; Janssen, 2006). These as-yet uncultivated microorganisms are a large unexplored reservoir of biological compounds, with a great potential for structural diversity and uses in novel drug development (Demain & Sanchez, 2009; Stott, et al., 2008). These natural microbial products include antibiotics, along with various secondary metabolites that can be used in drugs for organ transplantation, cancer treatment, and cholesterol control (Demain & Sanchez, 2009).

History of bacterial growth factors

There is a long history to bacterial growth factor discovery. The study of microbiology as it is understood today began with the advent of microscopy, which led to the revelation in 1837 by Theodor Schwann that yeast was a living organism involved in alcoholic fermentation (Schwann, 1837). Between 1850 and 1880 biologists began to make advances in microbiology, particularly microbial physiology, as different kinds of yeast and some bacteria were described. These discoveries were led by Louis Pasteur, who began his work on this area in 1860 and shed much light on the processes of sugar fermentation to alcohol by yeast and also the process of lactic acid fermentation (Tanner, 1925). He proved that microorganisms were able to convert the inorganic to the organic by demonstrating that yeast cells can convert inorganic nitrogen into organic material (Tanner, 1925).

However, other researchers struggled to reproduce Pasteur's earliest result; in 1860 he reported successful growth of yeast in a mineral salt-sugar solution (Pasteur, 1860; Pasteur, 1874), this claim was contested in 1871 by Liebig when he could not repeat this growth success despite using what he believed to be the same media composition and experimental conditions (Tanner, 1925; Liebig, 1871). These discrepancies drove investigations into the specific ingredients required for successful growth of yeast in the laboratory. This led to the earliest discovery of an important microbial growth factor in 1901, when Wildiers demonstrated that filtrate from boiled yeast cells caused a greatly improved rate of growth for yeast inoculum in a mineral salt-sugar medium (Wildiers, 1901; Tanner, 1925).

Wilders named this yeast-derived extract of unknown composition 'bios' (Wildiers, 1901). Wilders suggested that production of this yeast-derived GF only occurred when a large enough inoculum of yeast was used, explaining the failure of Liebig's experiments, as Liebig used a much smaller inoculum than Pasteur (Tanner, 1925). Further work showed that two factors present in the yeast extract were also necessary for vigorous growth of some propionic acid bacteria (Wood, et al., 1937). This 'bios' yeast extract was later found to be high in B vitamins (Burkholder, 1943). This 'yeast extract' GF is now used in many fields of scientific research, commonly added to culture media to ensure the successful growth of many bacteria.

Various vitamins have since been identified as bacterial GFs (Burkholder, 1943). Vitamins are taken up by bacteria to be used in the generation of co-factors and functional groups of certain enzymes (Webb, et al. 2007; Gruber, et al., 2011; Rodionova, et al., 2015). Bacteria can scavenge purines and pyrimidines from nucleotides for bacterial synthesis of nucleic acids and nucleotide cofactors (Moffatt, 2002). In the human gut nucleic acids cannot be fully digested, instead their catabolism ends with uric acid (Jurkovic, et al., 1995), which is then metabolised for energy by gut microbiota (Zang, et al., 2022). Some of these essential compounds cannot be produced by the bacteria themselves, but must be taken up from their immediate environment, a common characteristic of symbiotic bacteria (Monod, 1949). These growth inducing compounds that are usually taken up from the environment are prime targets for GF investigation.

It is important to point out that bacterial growth factors are not always required for growth but are added to culture media to stimulate *vigorous* growth, allowing microorganisms to carry out more of their usual processes. For example, in Wilders' experiments he demonstrated that yeast could grow in media without the 'bios' yeast extract, but that these cells are very slow growing and unhealthy, with a great reduction in fermentation abilities (Tanner, 1925; Wildiers, 1901).

Most bacterial GFs in use today such as yeast extract, volatile fatty acids, cysteine, tryptophan, and haemin were discovered between 1900 and 1970 (Wildiers, 1901; Wood, et al., 1937; Burkholder, 1943; Allison et al. 1962; Burrows, 1934; Fildes, 1921). Despite these discoveries the bacterial growth media used today is still highly selective. This selective pressure is so strong that when comparing colony forming units to total cell count Amann, et al. (1995) found between 99 and 99.9% of bacteria present in environmental samples cannot be cultured in the laboratory. These unculturable organisms can be the most abundant and metabolically active species in a community (Bomar, et al., 2011). Therefore, developing a media that enables the culture of these organisms is a priority. Since the advent of metagenomics large scale sequencing projects have frequently been used to discover more about these unculturable bacteria. These studies are used to reveal the metabolic potential of microorganisms, but this has not led to any major improvements in bacteria culture practices (Bomar, et al., 2011).

The importance of novel media development

An area of intense interest currently is the structure and function of the gut microbiome of various host organisms, particularly in relation to human health and disease (Hooper, et al., 2012; Sharon, et al., 2016; Holmes, et al., 2012). Much progress has been made using a metagenomic cultivation-independent approach (Amann, et al., 1995; Bomar, et al., 2011; Quin, et al., 2010; Tyson et al., 2005). However, to determine the metabolic pathways, present in these organisms, and the compounds they degrade and produce, they must be cultured in the lab (Stewart, et al., 2012). Discovery of these pathways will help to determine metabolic function of these organisms in the gut, and impact on the host. Difficulties in the *in vitro* culture of these organisms has held back research progress, with one problem being the lack of defined or minimal media in which a range of isolates will grow (Neidhardt, et al., 1974; Sebald, et al., 1975; Lopes, et al., 1976; Larsbrink, et al., 2014). Currently only certain important bacteria species can be isolated from the human gut in various defined media (Tramontano, et al., 2018). These limitations reduce the ability to investigate the metabolic interactions bacteria in the gut community, and their likely function (Ponomarova & Patil, 2015).

The development of specialist media to culture fish gut microbiota *in vitro*

Culture of gut microbiota in the lab represents a unique set of challenges. These obligately anaerobic bacteria must always be in an anaerobic environment, as they cannot tolerate any exposure to oxygen (Hungate, 1975). Anaerobic culture conditions and specialist media must be developed to provide the nutrients that these microorganisms would usually take up from their natural environment. Due to the complexity of the gut environment, these bacteria are difficult to culture *in vitro*. A common approach to encourage vigorous growth of gut bacteria is to supplement media with various nutrient rich substances such as egg yolk and sheep's blood (Lister, et al., 2014), or beef heart and cow brains as used in brain heart infusion broth (Tramontano, et al., 2018), or to add samples of total gut contents to media to produce a liquid fermentation (Olano-Martin, et al., 2000). However, when using this complex media, the exact nutrient load is not known, and different bacteria species from the same host gut have highly variable nutrient preferences. A study by Tramontano, et al., (2018) found that contrasting 4 rich and 15 defined media demonstrated a great variation the survivability of human gut bacteria species between media types. The search for a media that enables successful growth of most bacteria species from a gut sample is still ongoing. Discovering the growth factor compounds present in gut contents, and adding these to media in known quantities, will greatly improve the accuracy of *in vitro* experiments on these microorganisms.

To successfully culture previously unculturable bacteria their precise *in vitro* nutritional needs must be understood. This study is concerned with bacteria isolated from the gut of the fish *K. sydneyanus*. Novel media and culture methodologies have been developed in the Clements laboratory to successfully culture these organisms and assess their growth rate (Wornell, et al., 2022). These methods were then used to test the growth effects of various nutrients of interest to discover limiting factors to growth. These studies reveal more about the nutritional preferences of fish gut bacteria. Along with media improvements these discoveries allow more bacteria to be isolated from the *K. sydneyanus* gut environment, reducing great plate count anomaly effects in this environment.

This study searches for a novel bacterial GF in brown algae, to enrich culture media and allow the isolation of previously uncultured or unculturable bacteria from the hindgut of marine herbivorous fish. The Clements laboratory has successfully cultured a range of fish gut bacteria species using the complex, broad-range bacteriological medium **Y**east extract-**c**asein hydrolysate-volatile **f**atty **a**cids (YCFA), developed by Flint, et al. and later outlined in their paper; Duncan, et al., (2002). The recipe and preparation methods of YCFA medium was then adapted specifically for fish gut bacteria culture by Roberton, et al in the Clements laboratory at the University of Auckland (Wornell, et al., 2022).

Successful growth in broth media is necessary to perform many experiments, including precise growth rate analysis, as it allows the change in bacteria density over time to be measured using spectrophotometric methods. However, attempts to culture many of these same species in a liquid version of this YCFA medium were unsuccessful (Wornell, et al., 2022). The sub-par growth of fish hindgut bacteria in liquid broth in contrast to the more successful growth seen on agar plates is an issue that has also been seen in anaerobic bacteria isolated from various other sources (Speers, et al., 2009). Agar plates may generate this more successful growth as they more closely resemble the intestinal environment, with a solid surface for bacteria to grow in stable colonies. Novel methodologies were developed within this study to accurately measure the effect of various nutrients on the growth of fastidiously anaerobic bacteria cultured on a solid media.

Growth factor discovery

The search for novel bacterial GFs to improve culture media has become a rare approach to solving the unculturable bacteria issue. This current study provides the opportunity to resume this work after a forty-year lull, which could be the key to a renaissance in the discovery of novel bacterial products. Any superior culture media developed using these methodologies will also enable the isolation of novel bacteria from a range of other environments, with many research and industrial applications.

To search for bacterial growth factors, present in *U. pinnatifida*, the vitamin and nucleotide (nucleic acid, purine, pyrimidine) components were separated from the aqueous extract by fractionation. These compound types can then be tested for their growth effects in culture with fish hindgut bacteria. A growth rate similar to that caused by whole extract suggests the presence of a growth factor in that fraction. These compound types have been chosen for study based on the strength of their presence in brown algae, their water solubility, their published history of use as a bacterial GF, and their known functions in essential bacterial processes.

Literature Review Summary

In any symbiotic relationship between bacteria and the host, there are many factors affecting the nutrient environment, making identification of precise growth factors for *K. sydneyanus* gut bacteria a difficult task. This literature review details these factors, including the variable nutrient content of *U. pinnatifida*, diversity of *K. sydneyanus* gut bacteria, anaerobic bacterial culture methods, and the process of growth factor discovery.

The following is a brief overview of the literature reviewed in this study, discussed in the context of this PhD. First, there is a well-known energetic benefit provided by the digestive activities of gut bacteria to the host fish. The diet of *K. sydneyanus* is mainly made up of brown algae (Rimmer & Wiebe, 1986), the main storage component of this substrate, laminarin, cannot be broken down endogenously by most vertebrates (Moran & Clements, 2002). Therefore, the morphology and function of the *K. sydneyanus* gut has evolved to accommodate large scale bacterial fermentation of brown algae (Clements & Choat, 1993; Rimmer & Wiebe, 1987). These gut bacteria can hydrolyse the β-linked glucans of laminarin and make use of this substrate for energy (Moran & Clements, 2002). The host fish then gain an energetic benefit from this process as short chain fatty acids (SCFAs) are released by the gut microbiota and taken up by *K. sydneyanus* as an energy source (Clements and Choat, 1995).

The literature reviewed in this study provided the background knowledge necessary when choosing twelve nutrients to be tested for growth promoting activities. A focus of these chosen nutrients was the storage polysaccharides of algae, as these carbohydrates make up a greater energy reservoir in algae than either proteins or lipids (Montgomery & Gerking, 1980). The major compounds available for bacterial fermentation in all brown algae are glucose and mannitol (Seeto, et al., 1996). Glucose and mannitol are released from laminarin and fermented to SCFAs by bacteria in the fish gut (Percival and McDowell, 1967; Percival, 1979). Acetate is the major SCFA present in the average fish hind gut (Clements, et al., 1994). This SCFA is produced in the fish gut by microbial fermentation of glucose, fructose and mannitol (Seeto, et al., 1996). Therefore, glucose, mannitol and laminarin were all tested for growth factor potential in this study. The polysaccharide present in the highest quantities in brown algae is alginic acid, this structural polysaccharide forms part of the algal cell wall, giving these cell walls their semirigid form (Percival, 1979). Therefore, this carbohydrate was also tested for growth factor potential with *K. sydneyanus* gut bacteria in this study, despite the prediction by Seeto, et al., (1996) that catabolism of the structural polysaccharides of macroalgae is not important for microbial metabolism. Therefore, based on a review of the literature, four carbohydrates (and a sugar alcohol) common in brown algae; glucose, mannitol, laminarin, and alginic acid, were tested for growth factor potential in this study.

This study also uses the results of growth rate experiments to investigate functional relationships between different gut bacteria species in the *K. sydneyanus* gut. As *K. sydneyanus* age their diet slowly shifts from green and red algae to brown algae as their gut develops (Moran & Clements, 2002). This large phenotypic variation in *K. sydneyanus* diet is likely enabled by the ability to pull upon the metabolic abilities of a large pool of secondary gut bacteria, allowing digestion of a wide range of substrates (Macke, et al., 2017). As adults, when the diet of these fish shifts to majority brown algae, they develop a separate distal gut chamber that contains a large number of bacteria, enabling fermentation of brown algae to SCFAs for the nutritional benefit of the host (Clements & Choat, 1993; Rimmer & Wiebe, 1987). Despite the gut bacteria of the herbivorous fish not being as closely associated with the host as that of terrestrial vertebrates, *K. sydneyanus* have evolved a dependency on gut bacteria for digestion (Clements & Choat, 1993; Rimmer & Wiebe, 1987; Clements, 1994). This anatomical specialisation for microbial fermentation suggests that there is also some level of co-evolution on the part of the core microbiota. As this is known to be the case when a host associated phase is the predominant phase in the lifecycle of a symbiont (Macke, et al., 2017). This bacterial co-evolution with the host usually correlates with a loss of gene function and ability to synthesise costly metabolites if they can easily be taken up from the gut environment of the host fish instead (Engel & Moran, 2013; Shapira, 2016). Based on this literature review, analysis of the results of this study was used to better understand the level of co-evolution and the consortial nature of bacteria in the *K. sydneyanus* gut.

A major topic of this study was the development of novel methodology to improve the speed and reliability of the *in vitro* investigation of strictly anaerobic bacteria. Briefly, the 'great plate count anomaly', a decades old problem in microbiological research (Stanley & Konopka, 1985), it is estimated that only 0.01 to 0.1% of environmentally isolated bacteria can be cultured using standard plating techniques (Kogure, et al., 1979; Amann, et al., 1995). Much progress has been made in making all environmental bacteria viable *in vitro* using a metagenomic and cultivation-independent approach (Amann, et al., 1995; Bomar, et al., 2011; Quin, et al., 2010; Tyson et al., 2005). However, in order to determine the metabolic pathways present in these organisms, and the compounds they degrade and produce, they must be cultured in the lab (Stewart, et al., 2012). This is necessary as only *in vitro* culture allows investigation into the metabolic interactions bacteria in the gut community, and their likely function (Ponomarova & Patil, 2015). As-yet uncultivated microorganisms are a large unexplored reservoir of biological compounds, with a great potential for structural diversity and uses in novel drug development (Demain & Sanchez, 2009; Stott, et al., 2008). Due to the complexity of the anaerobic gut environment, gut bacteria are difficult to culture *in vitro*. In order to successfully culture these organisms their precise *in vitro* nutritional requirements must be understood. These obligately anaerobic bacteria cannot tolerate any exposure to oxygen (Hungate, 1975). A high-throughput method to measure and directly compare the growth rates of anaerobic bacteria was developed and published within this PhD. The development process took into account the fastidious nature of these anaerobic organisms, along with their preference for a solid media over a liquid media (Speers, et al., 2009). This development process is detailed in **Chapter 4,** the published methodology (Wornell, et al., 2022 **Appendix 1**) makes up **Chapter 3**.

Another topic of this study was improvements made to media recipes. Nutrient additions to YCFA media were used to improve the performance of fish gut bacterial isolates *in vitro*. Culture of gut microbiota *in vitro* represents a unique set of challenges. The nutritional complexity of the gut environment is difficult to replicate *in vitro*, hindering the study of these organisms. Development of simple and replicable growth media for *in vitro* culture of *K. sydneyanus* gut bacteria will greatly increase culture success and ease the metabolomic and functional studies of these organisms.

This study is concerned with bacteria isolated from the gut of the fish *K. sydneyanus*. The methods published in this study (Wornell, et al., 2022) were used to test the growth effects of various nutrients of interest on these *K. sydneyanus* gut bacteria. The nutritional preferences of these isolates reveal novel growth factors which were used in improved culture media recipes. Most bacterial growth factors in use today such as yeast extract, volatile fatty acids, cysteine, tryptophan, and haemin were discovered between 1900 and 1970 (Wildiers, 1901; Wood, et al., 1937; Burkholder, 1943; Allison et al. 1962; Burrows, 1934; Fildes, 1921). Despite these discoveries, the bacterial growth media used today is still highly selective. Developing media that enables the culture of unculturable bacteria is a priority as these organisms can be the most abundant and metabolically active species in a community (Bomar, et al., 2011). The improved media developed within this PhD will allow more bacteria to be isolated from the *K. sydneyanus* gut environment, reducing the effects of the great plate count anomaly.

Understanding the *in vitro* nutrient requirements of gut bacteria helps in understanding their function and relationship with the host fish. This study produced novel methodology that then led to the development of simple and replicable growth media for *in vitro* culture of *K. sydneyanus* gut bacteria, along with investigation into substrate preference and co-culture effects of these bacteria. This literature review provides a wider context to the search for novel growth factors for the *in vitro* culture of *K. sydenyanus* gut bacteria. With the presence of novel growth factors in an aqueous extract of *U. pinnatifida* explored in this study. This work has also provided answers to questions about co-evolution and mutually beneficial digestion in the *K. sydneyanus* gut. For the first time these important questions have been investigated using *in vitro* and culture-based work with these gut bacteria.

Chapter 3: Methods

Chapter 3 Section A: Multi-well growth rate measurement method

In the publication for this PhD, Wornell, et al., (2022) (Appendix 1) we presented a high-throughput protocol for comparing growth rates of fastidiously anaerobic bacteria on different media. These protocols can be used to develop a solid media made up of commercially sourced ingredients, providing replicable growth conditions for previously uncultured anaerobic bacteria. Detailed investigation of these organisms requires *in vitro* culture, this can be a challenge as the gut environment they inhabit is nutritionally complex. Use of complex media containing nutritionally rich but undefined gut fluid reduces accuracy of physiological and metabolomic studies. As many fastidious bacteria grow poorly in a liquid broth, these protocols measure bacterial growth rate on solid media. These protocols speed up and simplify the growth rate measurement process with use of a multi-well format and of equations in place of physical McFarland standards to calculate approximate cell density. Bacterial strains belonging to the families Erysipelotrichaceae and Lachnospiraceae (Phylum Firmicutes) isolated from the hindgut of *Kyphosus sydneyanus* were used to demonstrate the efficacy of these protocols. Bacterial growth rates were compared between a nutritionally rich medium with gut fluid, versus a novel replicable medium with mannitol. These protocols (Figure 27) are robust, reproducible and easily adaptable.

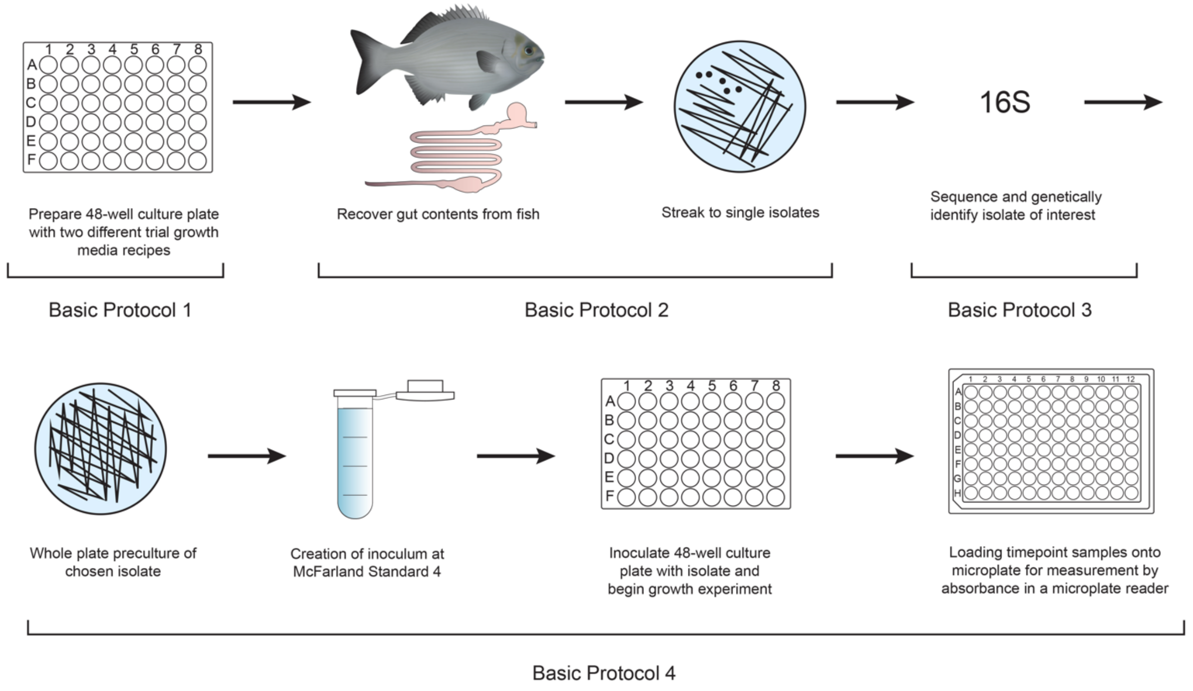


Figure 27: Basic protocols for high throughput assessment of growth effects of different media ingredients on fish gut bacteria. Culture plates of 48 wells are prepared, containing two trial growth media recipes with controls (Basic Protocol 1). Bacteria is recovered from the fish gut and streaked to single isolates (Basic Protocol 2). The 16S rRNA marker gene is amplified from the genomic DNA of each isolate, then sequenced and genetically identified (Basic Protocol 3). The chosen isolate is streaked across a whole plate to create a preculture, the culture is adjusted to McFarland Standard 4 in water-gel and inoculated onto the culture plates. Timepoints taken by absorbance in a microplate reader, timepoint samples are culture taken from a well by washing in water-gel (Basic Protocol 4).

Basic Protocol 1: Preparing solid YCFA growth media

Basic Protocol 2: Sample collection from the fish gut and plating to single isolates

Basic Protocol 3: Genetic identification of single isolates with colony PCR and 16S rRNA gene sequencing

Basic Protocol 4: Measurement of bacterial growth rates on solid media

These protocols describe how to isolate and identify bacteria from the fish gut while maintaining anaerobic culture conditions. They also set out a high-throughput and replicable method of measuring the effect of various media additions on bacterial growth rate. Results of these protocols may be used to develop an optimal replicable medium for gut bacteria isolates of interest from the fish gut, or other organisms including humans. This replicable media may then be used during metabolomic analysis of the isolate. These protocols can be used to improve the *in vitro* culture conditions of both previously isolated and novel gut bacteria.

Strategic planning

Equipment

Many bacteria isolated from the gut environment are strictly anaerobic and cannot tolerate any exposure to oxygen (Hungate, 1975). Excluding all oxygen from an *in vitro* culture system requires specialized equipment and techniques. Detailed information on how to set up the Hungate gassing apparatus, used in Basic Protocols 1, 2 and 4, is outlined in Hungate (1975) and Bryant (1972). This method requires the use of Hungate gassing apparatus with the ability to deliver nitrogen, carbon dioxide, and hydrogen gas to the system. The technique, first introduced by Hungate (1969), enables aseptic and anaerobic transfer of ingredients between containers for generation of sterile anaerobic bacterial culture media (Hungate, 1969). Gas is passed via a pressure reducing valve through a hot reduced copper wire column, removing all trace oxygen present in the source gas (Moore, 1966). The gas passes out of this column through a glass Y tube, then through rubber tubing and out of 16-18 gauge bent gassing needles through 2 mL sterile cotton-plugged Luer-Lok syringe barrels

(Bryant, 1972). A reducing agent (e.g. cysteine-HCl) is also added to the media to ensure all oxygen is removed (Bryant, 1972).

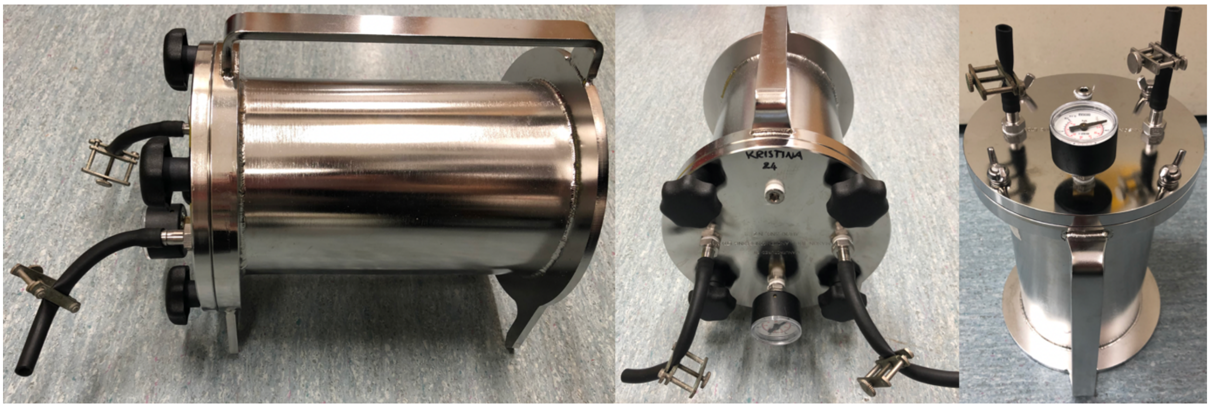


Figure 28: Anaerobic jars custom built by Mason Tool and Engineering Limited (Auckland, New Zealand). Has the option of agar plate vertical mode of operation as well as microwell plate horizontal mode of operation. Input and output valves allow gassing cylinder input through the Hungate gassing apparatus. Pressure gauge enables maintenance of a specific pressure during incubation.

Other specialized equipment used throughout the culture process are required to maintain *in vitro* culture of strictly anaerobic bacteria, including anaerobic jars, an anaerobic hood, and an incubator. The anaerobic jar, first introduced by McIntosh, et al., (1916), has long been used for the cultivation of anaerobic microorganisms. The anaerobic jar used in this protocol (Figure 28) was custom built for us by Mason Tool and Engineering Limited (Auckland, New Zealand). This anaerobic jar is filled with N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) to 5 psi using the Hungate gassing apparatus. Anaerobic hoods and incubators can be purchased from many suppliers and will interchangeably perform the same function. The anaerobic hood must be large enough to fit the anaerobic jar through the air lock. To ensure maintenance of anaerobic conditions in the hood, a catalyst (palladium) and a desiccant (calcium chloride) are used. To ensure maintenance of anaerobic conditions in the jars a catalyst (palladium) and activated charcoal are present. Activated charcoal absorbs hydrogen sulfide produced by the bacteria, preventing it from affecting catalyst function. The catalyst, desiccant, and charcoal are activated by heating at 150^{0}C for 1 hour. An anaerobic monitor allows quick detection of any oxygen entering the system. The incubator must also be large enough to fit the anaerobic jar and must have an adjustable temperature control that can be adjusted to the body temperature of the host organism from which the gut bacteria were isolated. In our protocol the gut bacteria are isolated from the gut of a silver drummer (*Kyphosus sydneyanus*), and incubated at 20°C, within the 17-23°C range of ambient seawater temperature used as *K. sydneyanus* body temperature in Mountfort, et al., (2002).

Bacteria of interest

Proteobacteria, Firmicutes and Bacteroidetes comprise 90% of the fish intestinal microbiota across species (Ghanbari et al., 2015; Egerton, et al., 2018). These protocols were verified using two bacterial isolates derived from section IV the *K. sydneyanus* gut (Figure 1).

The isolate designated strain BP52G, named *Tannockella kyphosi,* is a novel genus and novel species, in the Erysipelotrichaceae family, from the Firmicutes phylum (Pardesi, et al., 2022). Isolate BP52G is described as a gram-negative, non-spore-forming, rod-shaped, obligate anaerobe (Pardesi, et al., 2022). Isolate BP52G 16S rRNA gene sequence accessed from: NCBI prokaryotic genome annotation pipeline (Tatusova, et al., 2016). Accession number: MG827409.

The isolate designated strain BP5G, named *Chakrabartyella piscis*, is a novel genus and novel species, in the Lachnospiraceae family, from the Firmicutes phylum (Pardesi, et al., 2023). Isolate BP5G is described as a gram-positive, non-spore-forming, rod-shaped, obligate anaerobe (Pardesi, et al., 2022). Isolate BP5G 16S rRNA gene sequence accessed from: NCBI prokaryotic genome annotation pipeline (Tatusova, et al., 2016). Accession number: CP092441.

Both strains used were isolated from the *K. sydneyanus* gut by the Clements laboratory at the University of Auckland, and described by Pardesi, et al., (2022, 2023).

Media

YCFA (**Y**east extract-**c**asein hydrolysate-volatile **f**atty **a**cids) medium was designed by Dr Flint's lab, the specific recipe for this medium was later outlined in Duncan, et al. (2002). The work by Duncan, et al. (2002) proved the effectiveness of YCFA as a base medium for the culture of anaerobic bacteria. Here we use an adapted version of YCFA medium with reduced yeast extract and tryptone in comparison to Duncan, et al., (2002) (see Reagents and Solutions). Reducing nutrient richness reduces confounding variables during bacterial growth rates experiments, allowing the effect of nutrient additions to be seen clearly for all isolates. Also, this ensures that any novel media developed is as minimal as possible.

The addition of gut fluid to this medium provides an unknown quantity and unknown variety of nutrients. Using the older anaerobic M2GSC medium recipe (Miyazaki et al., 1997), Barcenilla *et al.* (2000) found gut fluid was necessary to cultivate the obligately anaerobic bacteria *Fusobacterium prausnitzii* from human feces (Duncan, et al., 2002). However, Duncan, et al., (2002) found that the nutrients present in a YCFA medium with added glucose contained all the nutrients required to culture *F. prausnitzii in vitro*. YCFA medium with *K. sydneyanus* gut fluid is used in these protocols as a standard against which the growth effects of alternative nutrient additions can be compared. However, our research showed that YCFA with glucose did not support the growth of strains BP5G and BP52G isolated from the hindgut of the herbivorous (seaweed eating) fish *Kyphosus sydneyanus*. This was not unexpected given the environment from which it was isolated. Members of the gut microbiota of *K. sydneyanus* are likely under selection to use the abundant carbon substrates available in the gut of the host. Mannitol is a common sugar alcohol present in brown algae, which is the main food source of *K. sydneyanus* (Clements and Choat, 1997), the host organism of our isolates. Also, mannitol concentration decreases along the gut of *K. sydneyanus,* correlating with an increase in gut microbiota density (White, et al. 2010). This suggests that mannitol is fermented by the gut microbiota. For these reasons mannitol was chosen as the trial growth factor in these protocols. Mannitol was used at an overall 0.2% (w/v) or ~10 mM concentration in YCFA-M. This is based on recommendations by Holdeman, et al., (1977), and Browne, et al., (2016), who used a 0.2% (w/v) concentration of sugars in their anaerobic media.

The growth rate of the chosen isolates (Erysipelotrichaceae and Lachnospiraceae) were measured on both YCFA medium with mannitol (YCFA-M) and YCFA medium with gut fluid (YCFA-GF) in controlled conditions to allow comparison. Apart from gut fluid, the remainder of YCFA medium ingredients were commercially sourced. These protocols provide a pathway to develop a commercially sourced alternative to YCFA-GF for any gut bacteria isolate of interest.

Reproducibility

All parameters in this experiment, qualitative and quantitative, were be reported to ensure reproducibility. Each timepoint for one isolate in two conditions is replicated in triplicate on a single 48-well plate. Triplicate repeat timepoints are separately converted from absorbance readings to growth rates. These growth rates are averaged to generate a reliable average growth rate of each isolate on each media.

Data analysis

Reproducible and efficient data analysis methods are required for the analysis of growth measurements from high-throughput methods. This protocol details the equations required to convert microplate reader absorbance measurements over time to approximate growth rates in the units of colony forming units (CFU) over time (hours), using McFarland standards (Casciato, et al., 1975; McFarland, 1907). Analysis was conducted in R v.4.1.2 (R Core Team, 2014) producing figures using the package ggplot2 v.3.3.2 (Wickham, 2016).

Basic Protocol 1: Preparing Solid YCFA Growth Media

Introduction

This protocol (Figure 29) has two parts, with Part A detailing the method used to adapt YCFA medium from Duncan, et al., (2002) (see Reagents and Solutions). Part B uses this method to create 48-well plates with two different media types. Example layouts are provided (Figure 31) that allow trial of possible bacterial growth factors, for example mannitol in YCFA-M medium, against a standard of growth rate on YCFA medium with gut fluid (YCFA-GF). Throughout these protocols, strict adherence to Hungate technique is required to maintain anaerobic conditions (Hungate, 1969).

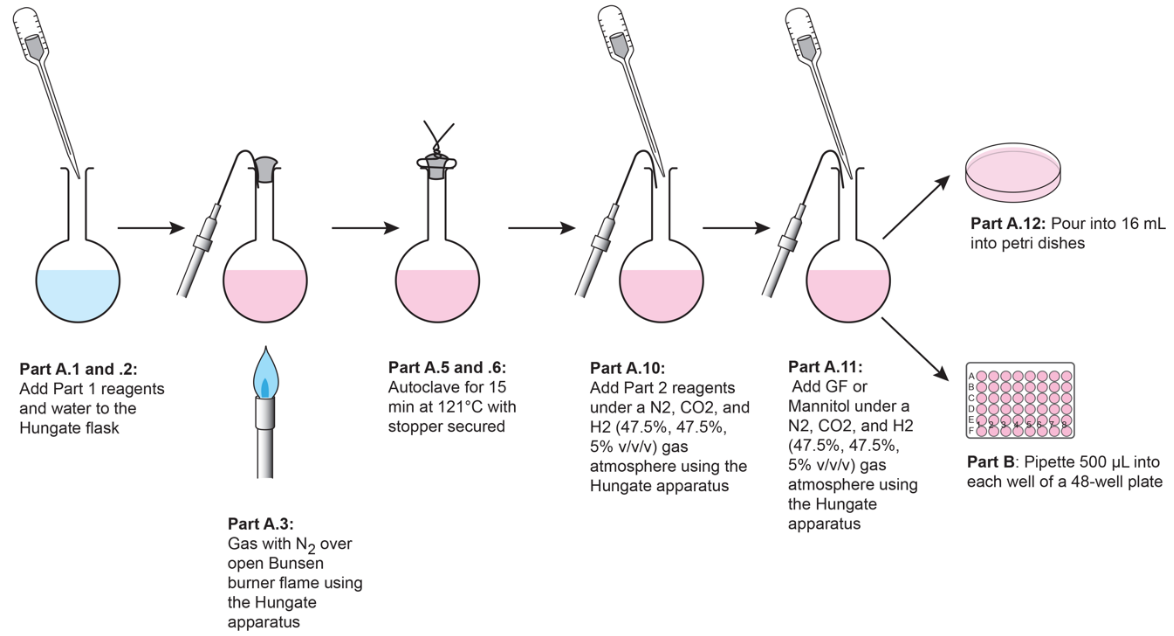


Figure 29: Basic Protocol 1, generating solid anaerobic media for the culture of obligately anaerobic gut bacteria. Includes steps from Basic Protocol 1 Part A, generating petri dishes containing a version of YCFA media. Also including steps from Basic Protocol 1 Part B, generating 48-well plates containing anaerobic media, for use in growth rate measurement experiments (Basic Protocol 4).

Materials

Reagents for media (see Reagents and Solutions)

Stak-Pak, Catalyst (Palladium) (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6501000)

Stak-Pak, Dessicant (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6502000)

Charcoal, Technical, ActIVated for Gas Adsorption (Fisher Chemical, cat no. 7440-44-0)

Mass scale

Weigh paper or trays

Spatula (for weighing out reagents)

Hungate flasks (100 ml) (Custom made by Mr. Alistair Mead, Senior Technician, Faculty of Science, University of Auckland)

Butyl rubber stoppers for Hungate flasks (Thomas Scientific, cat no. 1208Z83).

Bunsen burner

Autoclave, autoclave tape

Water bath (at 60 °C)

Sterile 90 mm petri dishes

Sterile 48 well culture plates with lids

Serological pipette controller

Serological pipettes (10 ml)

Laminar flow hood

Anaerobic jar with pressure gauge (Custom made by Mason Tool and Engineering Ltd, Auckland, New Zealand)

Hungate gassing apparatus delivering N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere

Materate Flowmeters for Air – Bench standing (Glass Precision Engineering Ltd., England, cat no. MFA1001-BS)

Sterile water (37 ml) (deionized or autoclaved)

Protocol steps

Part A Creating 100 ml of anaerobic YCFA medium

1. Measure the 'Part 1' ingredients from 'Reagents and Solutions' to quantities specified.

   These reagents are prepared in advance as individual stock solutions of minerals to speed up the process of media making.

2. Add to a Hungate flask with 74 ml sterile water.

Deionized or autoclaved water can be used.

3. Gas flask with N_2 over an open flame until medium turns a pink colour, and agar has melted. The gas delivered to flask by a gassing needle from the Hungate gassing apparatus, held in place with butyl rubber stopper using the Hungate technique.

   Hungate flask should be constantly swirled while held with the base one inch away from the top of the Bunsen burner flame.

   Hungate gassing apparatus, developed by Hungate (1969) and described by Bryant (1972), enables the aseptic and anaerobic transfer of ingredients between containers outside of an anaerobic hood. This allows generation and manipulation of sterile anaerobic bacterial culture media without the restrictions associated with the use of an anaerobic hood (Bryant, 1972; Hungate, 1969). The details of setting up and using the Hungate apparatus are outlined in Bryant (1972).

4. Once gassed, remove the gassing needle and stopper the flask using the Hungate technique (Hungate, 1969; Bryant, 1972).

   The Hungate technique of aseptic removal of the Hungate gassing needle and replacement of the stopper into the flask is outlined in Bryant (1972). This technique enables aseptic and anaerobic conditions to be maintained throughout the culture or media making processes.

5. Secure stopper in place with using two pieces of aluminum wire twisted around the stopper and the neck of the Hungate flask.

6. Autoclave for 15 min at 121°C.

   Move onto the next step immediately before medium cools and agar solidifies.

7. Secure the 100 ml Hungate flask in a 60°C water bath. Remove the aluminium wire.

8. Adjust the gas canisters of the Hungate apparatus to deliver a N_2, CO_2 and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere.

   Three pure gas cylinders separately containing N_2*,* CO_2 *and* H_2 *are attached to the Hungate apparatus prior to a copper column, as outlined in Bryant (1972). The ratio of gasses provided through the Hungate apparatus to the gassing needles can be adjusted using the dials on the cylinders, these adjust the pounds per square inch (psi) released from each cylinder. The volume delivery of each gas to the Hungate gassing apparatus can further be checked using flow meters. The flow meters are used to measure the standard cubic centimeter per meter (sccm) for each gas. This allows a precise* N_2, CO_2 and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere to be provided by the gassing needles.

9. Insert a needle of the Hungate gassing apparatus, held in place with a butyl rubber stopper using the Hungate technique (Hungate, 1969; Bryant, 1972).

   Remove wire, then insert needle of Hungate gassing apparatus into the flask alongside the bung using the Hungate technique.

10. Add ‘Part 2’ ingredients from ‘Reagents and Solutions’ to quantities specified, these are added anaerobically under a N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere using the Hungate apparatus.

    Between new ingredient additions return the bung into place alongside the needle, maintaining anaerobic conditions.

    These reagents are prepared as stock solutions of minerals to speed up the process of media making.

11. For YCFA-GF medium add Gut fluid; volumes required are detailed in Part 3A from ‘Reagents and Solutions’ . For YCFA-M medium add Mannitol; volumes required are

detailed in Part 3B from ‘Reagents and Solutions’. For YCFA negative control medium add nothing at this stage.

Swirl after all ingredients added to ensure even distribution of ingredients, keep in the 60°C water bath until ready to add to plates.

12. For 90 mm petri dishes, pour 16 mL media into each petri dish inside a sterile hood.

    Leave in the sterile hood to solidify and cool for 30 mins.

    This step of the process is performed in aerobic conditions. Any dissolved oxygen is removed from the media during Step 13.

    These petri dishes are used for plating to single isolates, and for whole plate streaking for the growth rate experiments that are outlined later in this paper.

13. Store anaerobically at 3 - 5°C for up to 90 days in sealed anaerobic jars, gassed with N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure. Storage of the petri-dishes in the anaerobic jar removes any dissolved oxygen from the media, to completely deplete the media of oxygen.

    Gas jars using Hungate apparatus with tube attachment that can be inserted into jar (Figure 30). Copper tube can be inserted into one of the clampable rubber tubes attached to the jar.

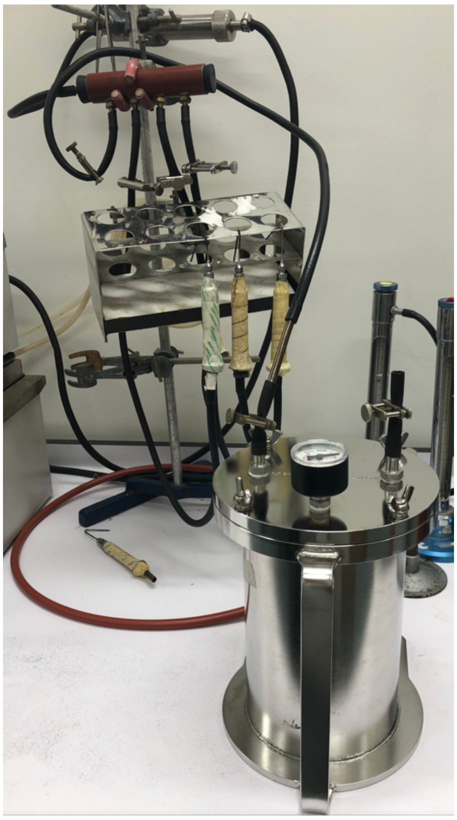


Figure 30: Copper tube attachment to Hungate apparatus allowing anaerobic delivery of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas from cylinders, through the Hungate apparatus, to the anaerobic jar.

Each jar should contain one autoclave tape-sealed petri-dish full of Stak-Pak Catalyst, and one full of activated charcoal. These petri-dishes should be placed at the bottom of the petri-dish stack inside each jar. This helps maintain anaerobic conditions inside the jar. Ensure catalyst and charcoal are freshly activated by heating at 150^{0}C for 1 hour.

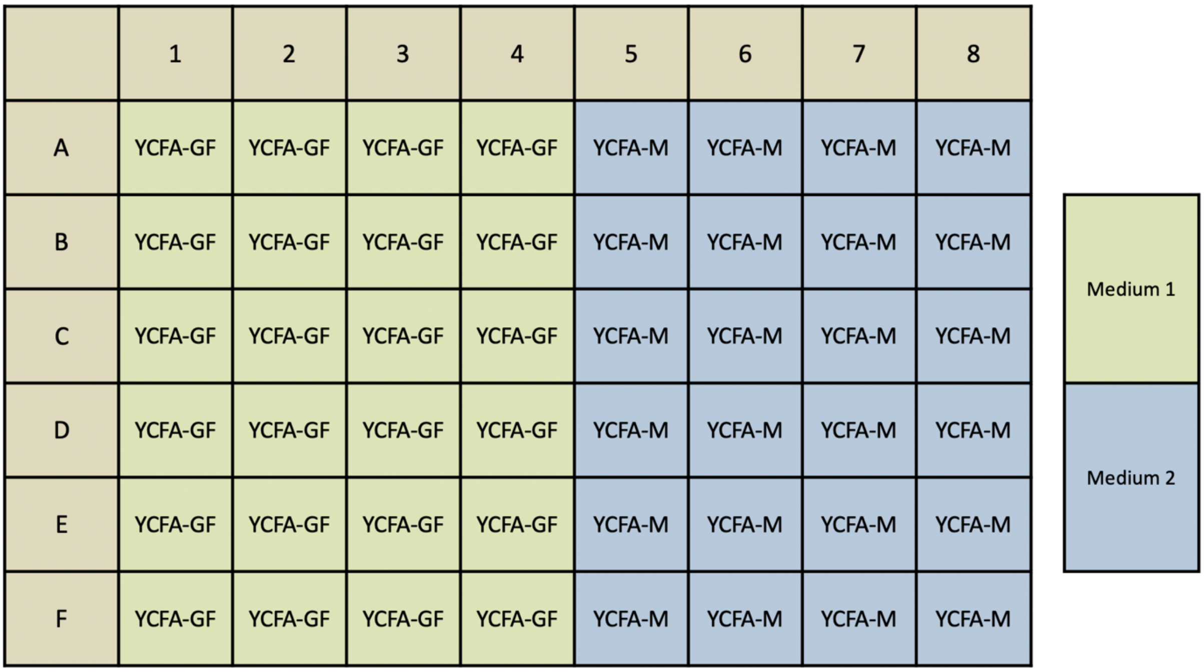
14. For 48 well plates, refer to Part B of this protocol.

These plates are used for growth rate experiments, outlined later in this protocol.

Part B: Creating 48-well plates containing different possible growth factors for use in growth rate analysis

The purpose of this protocol is to discover the optimal medium for *in vitro* growth of a specific anaerobic bacteria of interest. When culturing bacterial species in the lab for the first time the optimal medium must be found by trial and error. For fastidious anaerobic bacteria, this process can take a long time. This protocol outlines a high-throughput method of optimizing *in vitro* growth of anaerobic bacteria. In this protocol each 48-well plate is loaded with two different solid agar media, YCFA-GF and YCFA-M, allowing direct comparison of isolate growth rate. These plates are poured in aerobic conditions, then depleted of any dissolved oxygen by storage in sealed anaerobic jars, gassed with N2, CO2, and H2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure, stored at 3 – 5 °C. To ensure plates are completely depleted of oxygen, store new plates in the anaerobic jar for at least two days.

1. Prepare 48-well plates in a sterile hood, ready for media addition
2. Take the prepared YCFA media variants, contained in Hungate flasks, from the 60°C water bath and into the sterile hood
3. Decide layout of media in 48-well plates. An example, used in our work, is Figure 31.



	1	2	3	4	5	6	7	8
A	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-M	YCFA-M	YCFA-M	YCFA-M
B	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-M	YCFA-M	YCFA-M	YCFA-M
C	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-M	YCFA-M	YCFA-M	YCFA-M
D	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-M	YCFA-M	YCFA-M	YCFA-M
E	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-M	YCFA-M	YCFA-M	YCFA-M
F	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-GF	YCFA-M	YCFA-M	YCFA-M	YCFA-M

Medium 1

Medium 2

Figure 31: Example of media layout in a 48-well plate for comparison of bacterial growth on two trial media. In our experiment Medium 1 is YCFA-GF (Yeast extract-casein hydrolysate-volatile fatty acids with gut fluid), and Medium 2 is YCFA-M (Yeast extract-casein hydrolysate-volatile fatty acids with mannitol).

Growth rate experiment requires three replicates of each condition at each timepoint, with a no isolate control. A minimum of four timepoints during the growth phase are required to calculate the bacterial growth rate (Hall, et al., 2013). A zero-hour timepoint may also be useful to check the inoculum concentration is consistent across wells. There may also be an optional stationary phase timepoint, taken after an isolate has reached maximum growth, this informs study of optimal culture conditions, as it suggests when growth stopped, and the stationary phase began.

4. Use sterile serological pipettes to add 500 µl of prepared medium into each well of the 48-well plates inside the sterile hood.

This step must be performed quickly as soon as the medium leaves the 60°C water bath it begins solidifying. Leave the poured 48-well plates in the sterile hood to solidify and cool for 30 mins.

Use of a serological pipette allows a large amount of medium to be taken up at once in a sterile fashion, then quickly pipetted into many wells. A multichannel pipette or a 1000 μl pipette may be used instead, as long as this method can still be performed quickly and in sterile conditions.

5. Store anaerobically at 3 – 5 °C for up to 90 days in sealed anaerobic jars, gassed with N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure. Storage in sealed anaerobic jars for two days or more guarantees reduction, for completely anaerobic plates.

   Gas jars using Hungate apparatus with copper tube attachment (Figure 30). Copper tube can be inserted into one of the clampable rubber tubes attached to the jar.

   Each jar should contain one autoclave tape-sealed petri-dish full Stak-Pak Catalyst, and one full of activated charcoal. These petri-dishes should be placed at the bottom of the petri-dish stack inside each jar. This helps maintain anaerobic conditions inside the jar. Ensure catalyst and charcoal are freshly activated by heating at 150°C for 1 hour.

Basic Protocol 2: Sample Collection from the Fish Gut and Plating to Single Isolates

Introduction

This protocol consists of two parts (A) sample collection and (B) culture to single isolates. For our experiment, bacteria were isolated from the hindgut of *K. sydneyanus* (Silver drummer). Specimens were collected off the Northeastern coast on New Zealand, where *K. sydneyanus* is a common herbivorous fish (Mountfort, et al., 2002). The gut was removed by dissection and allocated into five sections (Figure 1) following the guidelines of Johnson and Clements (2022). Microbial collection can take place from segments III, IV and V, as microbial activity is highest in these hindgut sections (Clements, et al., 1994). This was shown by Clements, et al., (1994), where three herbivorous fish were found to have significantly higher concentrations of short chain fatty acids, a microbial fermentation product, in gut segments III, IV and V than in the earlier segments I and II. Increased short chain fatty acid concentration is a sign of bacterial fermentation (Clements, et al., 1994). We sampled from gut sections IV and V in this protocol. We then went on to use novel bacteria species isolated from gut section IV in our example results from Basic Protocols 3 and 4. YCFA media containing fish hindgut fluid (YCFA-GF) was used to culture these bacteria to single isolates. The bacteria isolated in this protocol can be used in the following Basic Protocols 3 and 4.

Materials

Solid media petri dishes

Dissection equipment

Falcon tubes (50 ml) (Fisher Scientific, cat no. 14-432-22)

Disposable inoculating loops (disposable loops recommended as it is difficult and tedious to sterilize loops in the anaerobic hood using the incandescent flaming device) (Thomas Scientific, cat no. 1230Z30)

Anaerobic jar with pressure gauge (Custom made by Mason Tool and Engineering Ltd, Auckland, New Zealand)

Vinyl Anaerobic Chamber Type A Glove Box (Coy Laboratory Products Inc., Grass Lake, Mich., cat no. 7000000)

Stak-Pak, Catalyst (Palladium) (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6501000)

Stak-Pak, Dessicant (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6502000)

Charcoal, Technical, ActIVated for Gas Adsorption (Fisher Chemical, cat no. 7440-44-0)

CAM-12 Anaerobic monitor (COY Laboratory Products Inc., Grass Lake, Mich., cat no. 6250000)

Hungate gassing apparatus delivering N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere

Hungate tubes (10 ml glass test tubes)

Butyl rubber stoppers for Hungate tubes (Thomas Scientific, cat no. 1208Z83)

Incubator

Protocol steps

Part A: Sample Collection

1. Remove gut from fish, divide gut into five sections, numbered I-V, as in Figure 1.

   This step is performed at the collection site.

2. Remove gut contents from gut sections by squeezing contents from the gut into a Hungate tube

3. Transport the contents of gut sections IV and V to the laboratory in Hungate tubes, stoppered under anaerobic conditions.

   A transportable version of the Hungate apparatus can be used on the boat at the collection site. Hungate gassing apparatus is connected to a small cylinder containing

90% N_2 and 10% CO_2 to purge the Hungate tubes of oxygen before sealing with a sterile butyl rubber stopper.

Contents of gut sections IV and V to be stored separately so as to later enable precise in vivo location information to be found for a bacteria isolate of interest

4. Place the tube of gut contents inside the anaerobic chamber

   Anaerobic chamber maintained at an atmosphere of N_2:H_2:CO_2 (87.5:7.5:5) (H_2 levels at 3% inside the chamber) using gas cylinders. The absence of oxygen inside the anaerobic chamber is confirmed throughout the process using the CAM-12 anaerobic monitor. Stak-Pak Catalyst and Dessicant should be present inside the anaerobic chamber. These paks are made from wire mesh to allow air to be circulated through the material.

   Allow solids to settle before moving onto the next part of this protocol. As the gut contents are collected anaerobically, they can be streaked straight away after the solids have settled (5 minutes standing time).

   Everything is done at room temperature until this stage, but if there is a need to store these gut contents until streaking can be done, they need to be stored at 19 – 20 ^{o}C.

Part B: Culture to Single Isolates

1. Streak 100 µl crude gut contents onto a YCFA-GF petri dish inside the anaerobic chamber.

   Streak using a sterile disposable inoculating loop.

   This is 'culture one' of the isolate of interest.

2. Bring anaerobic jar into the anaerobic chamber, place streaked plates inside jar and seal.

Anaerobic chamber maintained at an atmosphere of N_2:H_2:CO_2 *(87.5:7.5:5) (*H_2 *levels at 3% inside the chamber) using gas cylinders. The absence of oxygen inside the anaerobic chamber is confirmed throughout the process using the CAM-12 anaerobic monitor. Stak-Pak Catalyst and Dessicant should be present inside the anaerobic chamber. These paks are made from wire mesh to allow air to be circulated through the material.*

Each jar should contain one autoclave tape-sealed petri-dish full of Stak-Pak Catalyst, and one full of activated charcoal. These petri-dishes should be placed at the bottom of the petri-dish stack inside each jar. This helps maintain anaerobic conditions inside the jar. Ensure catalyst and charcoal are freshly activated by heating at 150^0*C for 1 hour.*

3. Remove anaerobic jar from anaerobic chamber and gas with gas mix of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure, using the Hungate gassing apparatus.
4. Incubate anaerobic jar at 20 ^{o}C. Incubation period of 3 days, or until single colonies are visible.

    Place sealed anaerobic jar inside incubator. As in Mountfort, et al., (2002), bacterial incubation should be maintained at the ambient seawater temperature of 17 to 23 ^{o}*C, because the gut environment of K. sydneyanus will also be within this temperature range.*

5. Pick a well isolated colony and re-streak on YCFA-GF once they grow to maximum cell number (about 3 days).

    This is 'subculture one' of the isolate of interest.

6. Re-streak two more sub-cultures of each colony of interest to produce pure single isolate cultures, ready for identification using colony PCR followed by Sanger sequencing using 16S V3-V4 primers.

   Label cultures after each streak to ensure consistency in colony picking.

Basic Protocol 3: Genetic Identification of Single Isolates with Colony PCR and 16S Sequencing

Introduction

This protocol describes how to genetically identify the single isolates that were cultured in Basic Protocol 2. This protocol consists of two parts: (A) colony PCR amplification with purification, and (B) 16S sequencing with analysis. Genetic identification of these isolates enables the results of Basic Protocol 4 to be analyzed in relation to previous studies on the same species, or close genetic relatives.

Materials

PCR tubes and lids

PCR SuperMix (Invitrogen, cat no. 10572014)

Vinyl Anaerobic Chamber Type A Glove Box (Coy Laboratory, cat no. 7000000)

Toothpics (sterilised)

16S V3-V4 region primers: e.g. 341F forward primer and 785R reverse primer (Klindworth, et al., 2013)

PCR hood (Grant Instruments, cat no. 7.970 866)

Thermal cycler (Thermo Fisher Scientific, cat no. 4375305)

QIAquick PCR Purification Kit (Qiagen, cat no. 28104)

Water, PCR grade (Roche, cat no. 03315843001)

IMPLEN NanoPhotometer N60 Micro-Volume UV-VIS Spectrophotometer (Fisher Scientific, cat no., 15442203)

Eppendorf Safe-Lock Tubes, 1.5 mL (Eppendorf, cat no. 0030120086)

Geneious Prime 2019.2.1 bioinformatics software (Kearse, et al., 2012) (https://www.geneious.com) (see 'Internet Resources')

NCBI BLAST: Basic Local Alignment Search Tool) (nih.gov) (Altschul, et al., 1990) (see 'Internet Resources')

Permanent marker pen

Protocol steps

Part A: Colony PCR

1. Prepare the following PCR master mix to amplify each sample:

Primer F341	1 µl	x no. of samples
Primer R806	1 µl	x no. of samples
2x concentrated PCR SuperMix	12.5 µl	x no. of samples
H_2O	10.5 µl	x no. of samples

Forward primer (F341) sequence: 5' CCTACGGGNGGCWGCAG 3'. Reverse primer (R806) sequence: 5' GACTACHVGGGTATCTAATCC 3'. These primers cover the V3 – V4 region. Primer concentration of 25 µM. The volume of each reaction will be 25 µl.

2. Take the anaerobic jar containing the third sub-culture of single isolates from the incubator into the anaerobic chamber.

These culture plates from Basic Protocol 2 have been incubating in an anaerobic jar at 20 °C N2, CO2, and H2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure for a period of 3 days, or until single colonies were visible

Anaerobic chamber maintained at an atmosphere of N_2:H_2:CO_2 (87.5:7.5:5) (H_2 levels at 3% inside the chamber) using gas cylinders. The absence of oxygen inside the anaerobic chamber is confirmed throughout the process using the CAM-12 anaerobic monitor. Stak-Pak Catalyst and Dessicant should be present inside the anaerobic chamber. These paks are made from wire mesh to allow air to be circulated through the material.

3. Take the PCR tubes, lids, and toothpicks into the anaerobic chamber.
4. Touch a single colony with a toothpick and rub onto the wall of a PCR tube. Circle colony on plate using a permanent marker pen. Using the marker pen, label the circle on the plate to match the PCR tube. The culture of this isolate can then remain linked to the genomic identification during subsequent re-streaking.
5. Attach PCR lids and take out of anaerobic hood and into PCR hood. Add 25 µl PCR mix into each PCR tube, pipette up and down to mix.
6. Perform PCR with the following thermal cycler settings:

1 cycle:	95^{0}C	5 min
25 cycles:	95^{0}C	30 sec
	55^{0}C	30 sec
	72^{0}C	30 sec
1 cycle:	72^{0}C	30 min
Final step:	12^{0}C	(hold)

7. Use the QIAquick PCR Purification Kit to purify DNA and elute with 30 µl PCR grade water.
8. Measure DNA concentration using the NanoPhotometer then send for sequencing.

Part B: 16S Sequencing

1. Use the NanoPhotometer to quantify the DNA.

   Optimum DNA concentration is 5-20 ng/µl, can adjust concentration using PCR grade water.

   Optimum purity (A260/280) of 1.8 or above.

2. Send purified samples, along with appropriate V3-V4 primers, for sequencing of the 16S rDNA gene at a sequencing facility, using Sanger sequencing.

   Forward primer (F341) sequence: 5' CCTACGGGNGGCWGCAG 3'. Reverse primer (R806) sequence: 5' GACTACHVGGGTATCTAATCC 3'. These primers cover the V3 – V4 region. Primer concentration of 25 µM. The volume of each reaction will be 25 µl.

3. Perform species identification using BLAST computer algorithm, accessed on the NCBI website. BLAST is used to compare and align these 16S sequences to the BLAST public database (Altschul, et al., 1990) (see 'Internet Resources').

   If the isolate is a known species, then there will be a 16S rRNA genetic sequence match in the NBCI database. In this case further experimental work will add to the current knowledge of this species.

If the isolate is a novel genus or species, then relatedness to known species can be found using BLAST and Geneious analysis. Currently, the cut-off for a novel species and genus is a sequence identity of 98.7% and 94.5% respectively (Yarza, et al., 2014). Further experimental work on these novel isolates will provide new information on these previously uncultured species.

4. Perform computational analysis of 16S sequencing data using Geneious bioinformatics software. This software is used to generate phylogenetic trees (Kearse, et al., 2012) (see ‘Internet Resources’).

   Phylogenetic trees should include 16S sequences from the public NBCI database, along with any unpublished sequences that have previously been identified using these methods.

   Generation of a phylogenetic tree informs future work on the isolate. If close relatives are found within the NBCI database, then previously published studies will inform media choice for culture of these bacteria.

   Phylogenetic trees generated from many bacteria isolated from the same environment will give insight to the microbiome of this environment.

Basic Protocol 4: Measurement of Bacterial Growth Rates on Solid Media

Introduction

This is a high-throughput protocol measuring the growth rate of fastidious anaerobic gut bacteria on different solid media. Growth effects of various nutrient additions can be compared to develop an optimal medium. In this protocol YCFA medium (Duncan, et al., 2002), with the addition of gut fluid (YCFA-GF), is used as a standard against which novel media recipes are compared. YCFA-GF often produces optimal growth rates of bacteria *in vitro* as gut fluid replicates the natural nutrient environment of gut bacteria in vivo. However, as the nutrient content of fish gut fluid is variable (Egerton, et al., 2018), this is an un-replicable media recipe. This protocol allows a preferable replicable medium, with commercially sourced ingredients, to be developed for any gut bacteria of interest. Importantly, strict anaerobic conditions are maintained at all times. Fish hindgut bacteria, cultured to single isolates and genetically identified using Basic Protocols 2 and 3, were tested for growth rate on two solid media: YCFA-GF and YCFA-M. Here YCFA-M is being tested as an alternative to YCFA-GF media. Mannitol was chosen as a media ingredient of interest for reasons explained in Basic Protocol 1.

Determination of bacterial growth rate on solid media in this protocol is based on methods developed for anaerobic bacteria culture by Casciato, et al., (1975). Casciato, et al., (1975) used water containing 0.1% gelatin (water-gel) to wash bacteria from the surface of solid media in a petri dish. Approximate cell density in this water-gel suspension was measured by conversion of the optical density at 600 nm to approximate colony forming units per ml (CFU/ml) with the widely used McFarland standards (Casciato, et al., 1975; McFarland, 1907). In this protocol we adjusted the inoculum to the cell density of McFarland standard 4 (approximately 12.4 x 10^8 CFU/ml) for inoculation into each well of the 48-well plates created in Basic Protocol 1 (Figure 31). Any of the four McFarland standards can be chosen, this choice is based on the growth success of the isolate of interest in the preculture stage. If the isolate grows poorly a lower McFarland standard can be used. The purpose of the choice of a single McFarland standard for all inoculums within an experiment is to maintain consistency, producing directly comparable results between isolates and conditions.

This protocol applies principles from Casciato, et al., (1975) to a high-throughput method, with 48-well plates allowing triplicate replicates and testing of multiple factors under the same conditions. Another novel development in this protocol is that there is no need for physical McFarland standards. These have been replaced with Equations 1 and 3, which we generated by graphing McFarland standards on an exponential curve against their approximate CFU/ml concentrations. These equations speed up and simplify the bacterial growth rate calculation process.

As explained in the introduction, these previously uncultured anaerobic bacteria are often slow growing, in which case the usual exponential growth profile does not apply. If bacterial growth is linear, as with fish gut bacteria isolates BP5G and BP52G, equations are provided within this protocol to calculate bacterial growth rate as an increase in colony forming units over time (CFU/hour). If bacterial growth is exponential, this protocol provides equations to calculate growth rates as either a doubling time in units of hours, or a growth rate constant (α) in units of minutes^{-1}, both of which are long established ways to express bacterial growth rate, outlined in Hall, et al., (2013). All these growth rates allow the direct comparison of the growth effects of various media ingredients in order to develop an optimal medium.

Materials

Gelatin from Porcine Skin (Sigma-Aldrich, cat no. G2625)

Sterile water (50 ml) (deionized or autoclaved)

Eppendorf Safe-Lock Tubes, 2 mL (Sigma-Aldrich, cat no. EP022363344)

96-well plate, flat bottom (Sigma-Aldrich, cat no. CLS3340)

Absorbance microplate reader (Parkin Elmer, EnVision 2104 Multilabel Plate Reader)

Centrifuge (that can accommodate 2 ml tubes)

Disposable sterile inoculating loops (Thomas Scientific, cat no. 1230Z30)

Disposable sterile delta cell spreader (Fisher Scientific, cat no. 50-751-5036)

Falcon tubes (50 ml) (Fisher Scientific, cat no. 14-432-22)

Mass scale

Weigh paper or trays

Spatula (for weighing out reagents)

Pipette controller

Vortex (that can accommodate 50 ml tubes)

Vinyl Anaerobic Chamber Type A Glove Box (Coy Laboratory Products Inc., Grass Lake, Mich., cat no. 7000000)

CAM-12 Anaerobic monitor (COY Laboratory Products Inc., Grass Lake, Mich., cat no. 6250000)

Stak-Pak, Catalyst (Palladium) (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6501000)

Stak-Pak, Dessicant (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6502000)

Charcoal, Technical, ActIVated for Gas Adsorption (Fisher Chemical, cat no. 7440-44-0)

Hungate gassing apparatus delivering N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere

Hungate tubes (10 ml glass test tubes)

Butyl rubber stoppers for Hungate tubes (Thomas Scientific, cat no. 1208Z83)

Protocol steps

Part A: Prepare the primary growth culture

1. Streak chosen bacteria from single colony isolates onto individual 90 mm petri dishes containing solid YCFA-GF medium (generated in Basic Protocol 1). Perform this step inside the anaerobic hood using disposable inoculating loops.

    One isolate of interest per petri dish. Streak over entire plate.

2. Place inoculated petri dishes into an anaerobic jar inside the anaerobic chamber. Remove sealed jar from anaerobic chamber and gas jar with gas mix of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) to 5 psi using the Hungate apparatus with the copper tube attachment to insert into jar (Figure 30).

   Anaerobic chamber kept at 20 °C and maintained at N_2:H_2:CO_2 *(87.5:7.5:5) (*H_2 *levels at 3% inside the chamber). The absence of oxygen inside the anaerobic chamber is confirmed throughout the process using the CAM-12 anaerobic monitor. Stak-Pak Catalyst and Dessicant should be present inside the anaerobic chamber. These paks are made from wire mesh to allow air to be circulated through the material.*

   Each jar should contain one autoclave tape-sealed petri-dish full of Stak-Pak Catalyst, and one full of activated charcoal. These petri-dishes should be placed at the bottom of the petri-dish stack inside each jar. This helps maintain anaerobic conditions inside the jar. Ensure catalyst and charcoal are freshly activated by heating at 150^0*C for 1 hour.*

3. Incubate anaerobic jar containing the preculture at 20 °C for 3 days, or to peak growth phase of chosen isolate under these conditions.

   Place sealed anaerobic jar inside incubator. Incubation temperature chosen as in Mountfort, et al., (2002): bacterial incubation should be maintained at the ambient seawater temperature of 17 to 23°C because the gut environment of K. sydneyanus will also be within this temperature range.

4. Inside the anaerobic chamber, pipette 2000 μl water-gel (see Reagents and Solutions) onto petri dish with isolate culture. Resuspend bacteria into the water-gel using a disposable cell spreader, or alternatively using a disposable inoculating loop.

   Gently rub colonies with disposable cell spreader or disposable inoculating loop to resuspend them into the water-gel.

5. Transfer preculture water-gel suspension from petri dish to 2 ml Eppendorf tube.

   Tip petri dish to gather all water-gel at one side. Pipette water-gel suspension from just above the agar layer into 2 ml Eppendorf tube.

6. Transfer 100 µl sample of this preculture water-gel suspension into a 96-well plate. Remove from the anaerobic chamber and measure absorbance at 600 nm in an absorbance microplate reader. In addition, transfer 100 µl of sterile water-gel to another well to serve as a blank reference for the absorbance reading.

   The volume of sample in the 96-well plate must remain consistent as the distance the light has to travel affects the resulting absorbance readings. The same volume is used for absorbance readings in the 96-well plate at all stages of the protocol.

7. Calculate preculture cell concentration (x 10^8 CFU/ml) in the 100 µl sample from its absorbance at 600nm using Equation 1.

$$\text{Cell density in sample } (x\ 10^8 \text{ CFU/ml}) = 1.1449\ e^{3.5633\ x\ (\text{Absorbance at } 600\ nm)}$$

Equation 1

   Equation 1 explains the exponential relationship between approximate cell density and absorbance at 600 nm among the widely used McFarland standards (Casciato, et al., 1975; McFarland, 1907). Equation 1 is the equation of the exponential trendline from a graph plotting these two variables against one another for each of the four McFarland Standards.

8. Calculate amount of water-gel needed to reconstitute a cell pellet from the 2 ml water-gel suspension sample to McFarland standard 4 (cell density approximately 12.4 x 10^8

CFU/ml) using Equation 3. Equation 3 finds the numerical ratio between the cell density in the sample versus the desired cell density of McFarland standard 4. It then multiplies this by the suspension sample volume (2 ml). This produces the volume of water-gel required to reconstitute the pellet at a cell density of McFarland standard 4.

$$\textbf{Volume water-gel for McFarland standard 4 (ml)}$$

$$= 2 \text{ ml } x \; \frac{\text{Cell density in sample } (x \; 10^{8} \text{ CFU/ml})}{\text{Cell density of McFarland standard 4 } (12.4 \; x \; 10^{8} \text{ CFU/ml})}$$

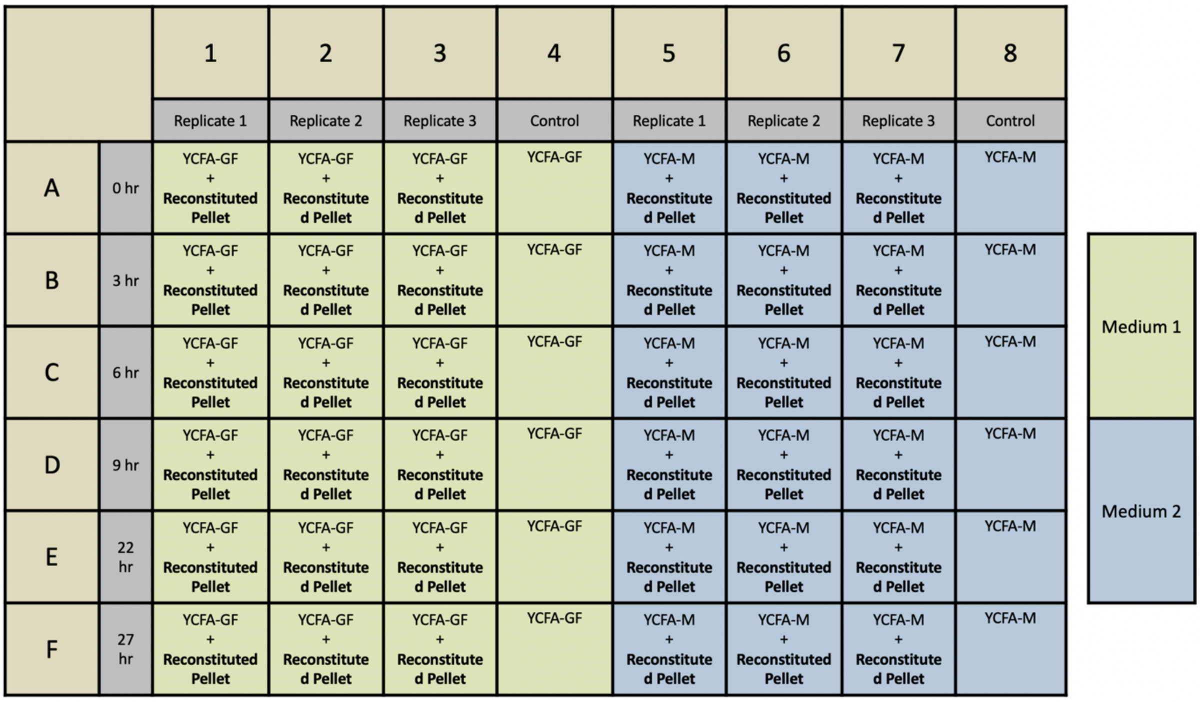
Equation 3

9. Inside the anaerobic chamber, centrifuge the 2 ml Eppendorf of water-gel suspension at 1500 rcf for 90 seconds, generating a pellet of the bacteria in this sample
10. Inside the anaerobic chamber, reconstitute the preculture pellet at the cell density of McFarland standard 4 by removing the supernatant, adding the calculated volume of water-gel (from Step 8), and gently pipetting up and down to resuspend the pellet.

    Gentle pipetting is important to minimize sheer stress effects on the bacterial cells.

Part B: Prepare 48-well plate and perform growth rate experiment

1. Inoculate 10 µl of the reconstituted pellet onto the solid media in individual wells. Following the new example plate layout (Figure 32), reconstituted pellet is inoculated into Columns 1 – 3 and 5 – 7 of a 48-well plate. The plate, containing two different solid media, was prepared in Basic Protocol 1 (Figure 31).



		1	2	3	4	5	6	7	8
		Replicate 1	Replicate 2	Replicate 3	Control	Replicate 1	Replicate 2	Replicate 3	Control
A	0 hr	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M
B	3 hr	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M
C	6 hr	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M
D	9 hr	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M
E	22 hr	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M
F	27 hr	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF + Reconstituted Pellet	YCFA-GF	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M + Reconstituted Pellet	YCFA-M

Medium 1

Medium 2

Figure 32: Example plate layout for growth rate experiment in the 48-well plate prepared in Basic Protocol 1. Six timepoints are possible (rows A - F), on two trial media (YCFA-GF and YCFA-M). One timepoint at zero hours, four timepoints during the growth period, and a timepoint at stationary phase. Reconstituted pellet at McFarland Standard 4 pipetted onto plate in columns 1 - 3, and 5 – 7 for three replicates of each condition at each timepoint. No isolate controls are placed in columns 4 and 8.

Each well is inoculated with the same volume of bacteria at the same concentration. This allows direct comparison between growth rates and analysis of the growth effects of different media ingredients.

Columns 4 and 8 are used as no isolate controls in this plate layout.

2. Take the time zero timepoint by pipetting 100 µl water-gel onto the zero-hour timepoint replicate wells. In the example plate layout (Figure 32) these wells are Row A, Columns 1

– 3 and 5 – 7. Resuspend bacteria into the water-gel by lightly scraping the plate with a disposable inoculating loop.

3. Pipette the 100 µl water-gel suspension from each of the three replicate zero-hour wells into a 96-well plate for absorbance measurement at 600 nm in an absorbance microplate reader. This absorbance reading can be converted into cell concentration using McFarland standards with Equation 1. In addition, transfer 100 µl of sterile water-gel to another well to serve as a blank reference for the absorbance reading.

   Use of three replicates reduces the influence of any "edge effects" that can cause differences in absorption between wells.

   These zero-hour readings are used for analysis purposes only, not growth rate calculations, as they are outside of the growth period.

4. Calculate at least four further timepoints during growth phase using these methods. Growth rates are measured using a window of at least four timepoints over the growth period, as recommended by Hall, et al., (2013).

   These four timepoints must occur after lag phase, and before stationary phase. These vary among bacteria and must be discovered with preliminary experiments on a chosen isolate. For our fish gut bacteria isolates the growth phase lasted from 3 to 26 hours and timepoints were taken within this range.

Part C: Calculating bacterial growth rate for linear growth

Calculate linear growth rates by increase in colony forming units (CFU) per well over time (hours).

1. Use Equation 1 to convert the absorbance readings of at least four timepoints to a value of approximate cell density in each well in the units of colony forming units per 1 ml (x 10^8 CFU/ml).
2. Take ten percent of these values, this converts the cell density into the approximate cell number in each well (x 10^8 CFU/well), as 100 µl samples are taken of all cells in each well to be measured for absorbance at O.D. 600.
3. Plot CFU/well against time, apply a linear trend line. The gradient of this line is the growth rate of bacteria in units of CFU/hour.

> *Comparison of these bacterial growth rates (CFU/hour) on different media allows the development of an optimal media recipe for any anaerobic bacteria of interest. Trialing various nutrient additions allows novel growth factors to be discovered.*

Part D: Calculating bacterial growth rate for exponential growth

Calculate exponential growth rates as a first order growth rate constant (α) (minutes^{-1}), or as a doubling time (hours).

1. The first order growth rate constant (α) is expressed in Equation 4 from Hall, et al., (2013), where α is the gradient of a linear trendline applied to a logarithmic graph of OD_{600} versus time. To calculate this growth rate, plot the natural logarithmic value of the mean O.D. values ($\ln N$) versus time (t) in hours using a graphing program. Equation 4 is generated from the gradient of a straight line drawn between two points (at time t and time 0) on this graph during the exponential growth phase, further explained in Hall, et al., (2013).

$$\ln\frac{N_t}{N_0} = \alpha(t - t_0)$$

Equation 4

Where N is the number of cells at time t and time 0, and α *is the first-order growth rate constant. When time (t) is measured in minutes,* α *is reported in reciprocal minutes (minutes*$^{-1}$*) (Hall, et al., 2013).*

2. The doubling time (hours) is calculated using the Equation 2 from Hall, et al., (2013) by finding the κ coefficient associated with exponential bacterial growth. The κ coefficient for this data can be found by plotting mean O.D. values against time (hours) and applying an exponential trendline to the graph. The equation associated with this exponential trendline contains the κ coefficient as the exponential index. This is used in Equation 2 to calculate the doubling time.

$$\text{Doubling time (hours)} = \frac{ln_2}{\kappa\ coefficient}$$

Equation 2

Growth rates are estimates and interpreting them requires some judgement, many confounding factors can affect absorbance readings and therefore growth rate calculations (Hall, et al., 2013). Hall, et al., (2013) recommends that if the standard error is >3% of the growth, or if the correlation coefficient is <0.995, then the results can be considered unreliable.

Reagents and solutions

100 ml YCFA media (adapted from Duncan, et al., 2002)

Part 1

Tryptone (Bacto) (BD, cat no. 211705) (0.2 g)

Yeast extract (BBL) (BD, cat no. 211929) (0.05 g)

Agar (Difco) (BD, cat no. 21430) (1.7 g)

K_2HPO_4 at 0.6% w/v in distilled water (3800 µl)

> *Make 100 ml stock at a time. This stock can be stored in a Schott bottle for up to 30 days at 3 – 5 ^oC.*

Mineral mix from stock (3800 µl)

> *Make up stock in advance: KH_2PO_4 phosphate (0.6% w/v), $(NH_4)_2SO_4$ (0.6% w/v), NaCl (1.2% w/v), $MgSO_4.7H_2O$ 0.245% w/v), $CaCl_2.2H_2O$ (0.15% w/v). Autoclave for 15 minutes at 15 psi on a liquid cycle. Make 100 ml stock at a time. This stock can be stored in a Schott bottle for up to 30 days at 3 – 5 ^oC*

Trace elements from stock (10 µl)

> *Make up stock in advance: $CuSO_4.4H_2O$ (4 mg), $MnSO_4.4H_2O$ (50 mg), $Ni(II)Cl_2$ (0.2 mg), $FeSO_4$ (10 mg), $Co(II)(NO_3)_2.6H2O$ (0.2 mg), H_2MoO_4 (0.2 mg), and bring to 100 mL with distilled water. Autoclave for 15 minutes at 15 psi on a liquid cycle. Make 100 ml stock at a time. This stock can be stored in a Schott bottle for up to 30 days at 3 – 5 ^oC*

Resazurin at 0.1% w/v in distilled water (100 µl)

Make 100 ml stock at a time. This stock can be stored in a foil covered schott bottle for up to 30 days at room temperature.

Part 2

NaHCO$_3$ at 4% w/v in distilled water (10000 µl)

Sterilise 4% w/v sodium carbonate by autoclaving in an open flask, this turns to sodium hydroxide during heating. While hot, bubble with CO$_2$ gas until cool, using a serological pipette attached to the Hungate apparatus, delivering N$_2$ gas. Solution will has turned into sodium bicarbonate and carbonic acid when white flecks vivible on the end of the serological pipette. Make 100 ml stock at a time. Stopper in 10 ml aliquots under CO$_2$ gas. This stock can be stored for up to 30 days at 3 – 5 ^{o}C

Cysteine-HCl at 2.5% w/v in distilled water (2000 µl)

Dissolve 1 g Cystine-HCL in 40 ml distilled water. Bring to boil under N$_2$ atmosphere by gassing stock in Hungate vial with N$_2$ using the Hungate apparatus. Stopper vial, secure using aluminium wire, and autoclave. Make 100 ml stock at a time. Store in 10 ml aliquots in Hungate tubes under 100% N$_2$ by gassing tubes using the Hungate technique. This stock can be stored for up to 30 days at 3 – 5 ^{o}C

Vitamin K$_3$ (0.005% w/v) + Hemin (1% w/v) mix in distilled water (1000 µl)

For Hemin dissolve 10 mg hemin in 0.2 ml 1M NaOH, add 20 ml distilled water and autoclave. For vitamin K$_3$ add 100 mg sterile vitamin K$_1$ or vitamin K$_3$ to 20 ml 95% ethyl alcohol. For mixed stock add 0.2 ml sterile vitamin K$_3$ to 20 ml hemin. Make 100 ml stock at a time. Store in 10 ml aliquots in Hungate tubes under 100% N$_2$ by gassing tubes using the Hungate technique. This stock can be stored for up to 30 days at 3 – 5 ^{o}C

Volatile fatty acids mixture from stock (1000 µl)

> *Make up stock in advance: Acetic acid (17 mL), propionic acid (6 mL), N-valeric acid (1 mL), isovaleric acid (1 mL), and isobutyric acid (1 mL). Make up to ~50 mL with distilled water, neutralize with 5M NaOH to pH 6.5, and make up to 100 mL with water. Autoclave under a* N_2 *atmosphere by gassing stock in Hungate vial with* N_2 *using the Hungate apparatus. Stopper vial, secure using aluminium wire, and autoclave. Make 100 ml stock at a time. Store in 10 ml aliquots in Hungate tubes under 100%* N_2 *by gassing tubes using the Hungate technique. This stock can be stored for up to 30 days at 3 – 5 °C*

Part 3

Part 3A (GF): Autoclaved and filtered gut fluid supernatant from stock (10000 µl)

> *Make up stock in advance: Kyphosus sydneyanus hindgut lumen contents from sections IV and V (Figure 1) are filtered through a cheese cloth and the filtrate is centrifuged at 9000 g for 20 min at 4°C. Collect supernatant and autoclave. Centrifuge supernatant at 13500 g for 20 min at 4°C. Make 100 ml stock at a time. Freeze in two 50 ml Falcon tubes at -20°C to store for up to 9 months. Alternatively, store in 10 ml aliquots in Hungate tubes under 100%* N_2 *using the Hungate technique. These stocks can be stored at 3 – 5 °C for up to 30 days.*

Part 3B (M): Mannitol (Sigma, cat no. 63H01621) at 5% w/v (4000 µl)

> *This stock can be stored for up to 30 days at 3 – 5 °C*

YCFA media can be stored up to 90 days at 3 – 5 °C.

50 ml Water-gel containing 0.1% gelatin (adapted from Casciato, et al., 1975)

0.05 g Gelatin (Gelatin from Porcine Skin, Sigma-Aldrich, cat no. G2625)

50 ml Sterile water (deionized or autoclaved)

1. Add gelatin and water to a Falcon tube, vortex for 30 seconds to mix.
2. Autoclave the solution for 15 minutes at 15 psi on a liquid cycle.

   Twist Falcon lid only half way on, to allow pressure equilisation inside autoclave.

3. Transfer 10 ml water-gel into each of five Hungate tubes using the Hungate technique.

   A serological pipette can be used to aliquof from the Falcon tube into Hungate tubes in the Hungate apparatus. Hungate tubes should have Hungate needles inserted throught aliquoting. Butyl rubber stoppers are insterted into the Hungate tubes using the Hungate technique (Bryant, 1972; Hungate, 1969). This ensures the headspace is completely free of oxygen.

4. Gas these tubes with anaerobic gas mix of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) using the gassing needles of the Hungate apparatus.

   Gas tubes using the Hungate technique (Bryant, 1972; Hungate, 1969). Secure with a butyl rubber stopper.

5. Leave to become anaerobic overnight inside the anaerobic chamber with bungs loose to allow gas exchange.

   When moving stoppered Hungate tubes into the anaerobic hood, secure stopper into the tube with tape. This prevents gas purging in the airlock from removing the butyl rubber stoppers.

Water-gel can be stored up to 90 days at 3 – 5 ^{0}C with bungs secure.

Commentary

Background Information

Why the procedure is performed and how the technique has evolved

The starting point of developing a replicable media for the culture of a novel bacterium is to include the essential elements for growth in sufficient amounts. Some environmentally isolated bacteria grow in simple media with just a single carbon source (Plugge, 2005). Others require addition of macro elements C, H, O, N, P and S, and metals Ca, Cl, Fe, K, Mg and Na, which together make up 95% of average microorganism dry weight (Plugge, 2005). However, many bacteria remain unculturable even when all common essential elements have been added in sufficient amounts (Plugge, 2005). These species require further nutrients to be added, these nutrients are classed as growth factors and are typically one of either amino acids, vitamins, or pyrimidines and purines (Plugge, 2005). For example, many *Bacteroides* strains require the presence of vitamin K_1 and hemin for growth (Gibbons & MacDonald, 1960).

Investigation of anaerobic bacteria *in vitro* requires specialized media containing all essential nutrients for growth. To improve the accuracy of bacterial function analysis, a medium of reduced complexity with minimal undefined ingredients should be used (Tramonatano, et al., 2018). We present a high-throughput method of developing an optimal medium. This protocol uses solid media because fastidious anaerobic bacteria often do not grow in a liquid media (Speers, et al., 2009). Use of a solid media allows this protocol to be used on a broader range of gut bacteria species.

The central advantages (and disadvantages) of the technique chosen

The key advantage of this technique is the use of a high-throughput set up. Anaerobic bacteria require long incubation times; for routine clinical microbiology anaerobic cultures should be incubated for a minimum of five days (Lagier, et al., 2015; Chapin & Murray, 2007). Also, as the gut environment is highly nutritionally complex, bacterial species from the same host often have highly variable nutrient preferences (Egerton, et al., 2018). These complications make developing an optimal medium for previously uncultured bacteria a long process that is nevertheless essential for the successful culture of many species. This high-throughput experimental set up allows three replicates of a potential growth factor over up to six times points on a single 48-well plate. If the anaerobic jar is large enough two plates can be tested at one time. One experimental run using this jar can produce average growth rates of two isolates on two media. This protocol is to our knowledge the quickest way to find the precise nutrient requirements of a species of interest.

Another advantage of this technique is that the novel media recipe developed in these protocols can be exactly replicated in future work, reducing the effect of confounding variables on results. Most human gut bacteria have no growth characterization data available in replicable media (Tramonatano, et al., 2018). These bacteria have only been cultured in media with gut fluid or other complex and undefined nutrient sources. A replicable media recipe has the advantage of allowing metabolomic and proteomic studies to be carried out on bacteria *in vitro*. Gut bacteria must be cultured in a nutritionally defined media to study the link between host diet, microbiota composition and dynamics, and interspecies interactions (Tramonatano, et al., 2018).

Comparison of Basic and Alternate protocols or comparison with other methods currently in use

Other available protocols for measuring growth rate in a high-throughput fashion are not suitable for strictly anaerobic bacteria. A high-throughput method developed by Hall, et al., (2013) uses microtiter plates to measure absorbance over the growth period aerobically in a plate reader. However, this method is not appropriate for strictly anaerobic species, as an anaerobic environment cannot be maintained in most plate readers.

Another method by Tramontano, et al., (2018) uses a microwell plate inside an anaerobic chamber to measure bacterial growth rates anaerobically. This method was designed specifically for anaerobic bacteria. However, this method requires culture in liquid media (Tramontano, et al., 2018), whereas many fastidious gut bacteria only survive on a solid media (Speers, et al., 2009).

There is a need for our method, the first for high-throughput growth rate analysis of fastidious anaerobic bacteria on solid media. These protocols will enable quick development of novel media recipes for a broad range of fastidious anaerobic bacteria.

Uses and applications of methods

The structure and function of gut microbiome communities and their relation to the host is an area of great research interest. However, few microorganisms isolated from environmental samples survive *in vitro* culture (Barer & Harwood, 1999; Giovannoni, et al., 1990; Wayne, et al., 1987; Torsvik, et al., 1990; Ward, et al., 1990; Barns, et al., 1994; Kaeberlein, et al., 2002). As culture methods were found to be inadequate for the study of microbial community composition, there was an increased interest in sequence-based genetic techniques that bypassed the need for *in vitro* culture (Ward, et al., 1990; Barns, et al., 1994). These genetic techniques greatly expanded knowledge of microbial biodiversity and related evolutionary processes (Barns, et al., 1994). While much progress has been made using a metagenomic cultivation-independent approach, the only secure and operational proof of organism viability is culturability (Barer & Harwood, 1999). While genomic 16S and rRNA studies of environmentally isolated bacteria can suggest the presence of metabolic pathways (Béjà, et al., 2000; Béjà, et al., 2002), cultivation is required in order to understand the physiology of novel microbes and the functional processes they engage in (Zengler, et al., 2002; Stewart, 2012). Our protocol can be used to successfully culture anaerobic bacteria from the environment for the first time. This addresses a current challenge for microbiologists of improving *in vitro* culture methods, leading to greater understanding of the metabolic properties and potentials of these organisms (Zengler, et al., 2002).

These protocols allow the trial of novel nutrient additions to media in a high-throughput fashion. The growth effects of media ingredient removal can also be tested with these protocols. When using a nutrient media, SCFAs and amino acids that act as bacterial growth promotors for some species can inhibit the growth of other species (Tramonatano, et al., 2018). Removal of these inhibitors allows previously unculturable bacteria to be cultured *in vitro* for the first time. The result of these experiments is the development of an optimal media for the *in vitro* growth of a chosen anaerobic isolate.

Critical parameters

There are several critical parameters influencing bacterial growth that can be individually addressed to improve the performance of chosen isolates in these protocols. Maintenance of anaerobic conditions is crucial; hence oxygen must be completely excluded from the system. The Hungate technique must be strictly applied when making media to keep media ingredients anaerobic. The 48-well plates must be stored in an anaerobic jar gassed with standard gas mix of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) at 5 psi at 3 - 5 ^{0}C for at least two days before use. This ensures the solid media is completely reduced. When gassing anaerobic jars, the pressure must not drop below 1 psi at any point. The gas must only move in one direction, away from the gas cylinders, through the jar and out. The seals on the anaerobic jars are verified by minimal loss of pressure over time. Water-gel must be reduced in the anaerobic hood overnight before use. Oxygen levels in the anaerobic hood are monitored with an anaerobic monitor to ensure no oxygen has entered the system.

The amount of trial growth factor added to YCFA media to test growth effects must be decided based on previous work with similar compounds. Basic Protocol 1 explains that the concentration of mannitol in the YCFA-M media used in our study was chosen based on recommendations by Holdeman, et al., (1977), and Browne, et al., (2016), who used a 0.2% (w/v) concentration of sugars in their anaerobic media.

Troubleshooting

Table 6: Troubleshooting Guide for Measurement of Bacterial Growth Rates on Solid Media

Problem	**Possible causes**	**Potential solutions**
Not enough bacteria CFU collected from one petri dish	Fastidious or slow growing isolate	Create two petri dishes for this isolate, transfer bacteria suspended in water-gel from the first petri dish to the second to collect bacteria from both
Bacteria adhere tightly to solid media and cannot be removed by rubbing with disposable inoculating loop	Isolate strongly adheres to nutrient source	Incubate at 20 ^{0}C for a shortened time period, to harvest before strong adherence occurs
Unsuccessful growth of inoculum on 48-well plate	Bacteria was dead before inoculation	Needs a shorter incubation time on petri dishes Less spin time in centrifuge required to reduce sheer

	stress on cells. A range of 60 to 120 seconds recommended at 1500 rcf
	Pipette gently to reduce sheer stress
	Warm plates in hood for at least 20 minutes before streaking to reduce temperature shock effects on bacteria
Bacteria did not pellet	More spin time in centrifuge required to pellet cells. A range of 60 to 120 seconds recommended at 1500 rcf
Bacteria growth was interrupted	Maintain strict anaerobic conditions at all points during growth
	Store anaerobic jars containing plates at $3 – 5\ ^{o}C$ to prevent media drying
	Ensure palladium and charcoal is freshly baked before use inside the anaerobic jar. Activate by heating at $150^{0}C$ for 1 hour.
	Increase gaps between timepoints for longer uninterrupted incubation time
	Increase the length of the growth period, as some bacteria have long lag phases

Understanding the results

As an example of anticipated results, we conducted growth rate experiments on two *Kyphosus sydneyanus* gut section IV (Figure 1) derived bacterial isolates from families Erysipelotrichaceae and Lachnospiraceae. Growth was measured by optical density at 600 nm in two different conditions: YCFA medium with gut fluid (YCFA-GF), and YCFA medium with mannitol (YCFA-M). Standard YCFA medium with gut fluid (Duncan, et al., 2002) is used when culturing previously uncultured gut bacteria *in vitro* for the first time because it contains many of the nutrients present in the natural environment of these organisms. Mannitol is present at decreasing levels along the fish gut (White, et al. 2010). As this decrease could be caused by fermentation by gut bacteria (White, et al. 2010), this sugar alcohol is a possible growth factor for these organisms. In these experiments the effect of mannitol on growth rate was tested on both the Erysipelotrichaceae and Lachnospiraceae isolates. The medium YCFA-M is a defined alternative to YCFA-GF, as all ingredients are industrially sourced. Therefore, YCFA-M is preferable to the complex and nutritionally undefined YCFA-GF for certain metabolic analyses of bacteria *in vitro* (Tramonatano, et al., 2018).

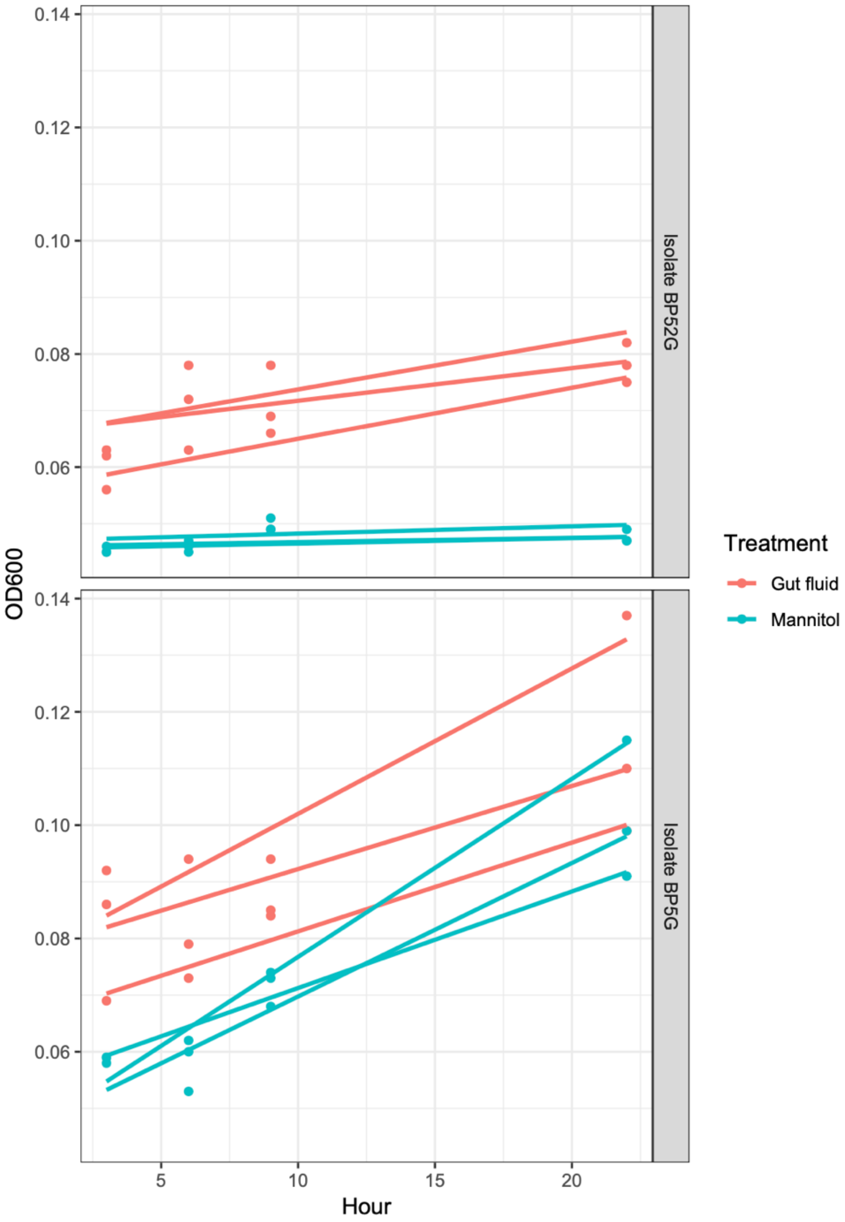


Figure 33: Representative data of bacterial growth by absorbance at OD_{600} on two different media. **Top:** Growth comparison of strain BP5G in YCFA + GF and YCFA + M media. **Bottom:** Growth comparison of strain BP52G in YCFA + GF and YCFA + M media. Four timepoints taken over the growth period for three replicates in each treatment (timepoints: 3, 6, 9 and 22 hours). Each replicate fitted to a linear regression line. YCFA+GF, Yeast extract-casein hydrolysate-volatile fatty acids media with gut fluid. YCFA+M, Yeast extract-casein

hydrolysate-volatile fatty acids media with mannitol. Analysis was conducted in R (R Core Team, 2014) producing figures using the ggplot2 package (v3.3.3; Wickham, 2016).

For representative data, absorbance at wavelength 600 nm was measured at four timepoints during the growth phase of two isolates, BP5G and BP52G (Figure 33). Growth phase for these isolates was found in preliminary work to be between 3 and 27 hours. Three replicates were produced for each of two treatments of YCFA media: addition of gut fluid, or addition of mannitol. Gradients of the linear regression lines applied to the representative data in Figure 33 can be used as approximate growth rates in units of colony forming units per hour (CFU/hour), using the method in Basic Protocol 4, Part C. Alternatively, growth rates of bacteria with exponential growth would be calculated in minutes^{-1}, or hours, as in Basic Protocol 4, Part D. The individual growth rates of the three replicates in this study were averaged to generate Table 7: average approximate growth rates.

Table 7: Average approximate growth rate in CFU/hour for fish gut bacteria isolate BP52G in the Erysipelotrichaceae family and isolate BP5G in the Lachnospiraceae family, cultured on two different media: YCFA-GF, and YCFA-M. Average calculated from three replicates of each condition, with standard deviation. (Expanded table with all replicates in appendix).

Isolate	**Media**	**Average approximate growth rate from three replicates (± standard deviation) (x 10^5 CFU/hour)**
BP5G (Lachnospiraceae)	YCFA-GF	1.10 (± 0.44)
	YCFA-M	1.30 (± 0.45)
BP52G (Erysipelotrichaceae)	YCFA-GF	0.40 (± 0.10)
	YCFA-M	0.05 (± 0.01)

For the Lachnospiraceae isolate BP5G, YCFA-GF medium produced an average growth a rate of 1.1 x 10^5 (± 0.44) CFU/hour (Table 7). This result is used as the desired growth rate against which the alternative media recipe of YCFA-M can be compared. The average growth rate on YCFA-M was found to be 1.3 x 10^5 (± 0.45) CFU/hour (Table 7). For a linear regression model of OD_{600} against time for this isolate, growth on both YCFA-GF ($R^2 = 0.5574$, $p = 0.001386$) and YCFA-M were significant ($R^2 = 0.9302$, $p = 6.82 \times 10^{-9}$) compared to no growth. Also, the interaction effect (Hour:Media) is significant ($p = 0.0302842$), indicating a significant difference in the growth rate of BP5G on YCFA-GF and YCFA-M. These results show that, for Lachnospiraceae isolate BP5G, YCFA-M produces a significantly faster growth rate than YCFA-GF. Based on these results, mannitol can replace fish gut fluid in YCFA medium for culture of Lachnospiraceae isolate BP5G as it is a more effective growth factor. This creates YCFA-M, a defined and replicable alternative to YCFA-GF for fish gut bacteria isolate BP5G.

For the Erysipelotrichaceae isolate BP52G, YCFA-GF medium produced an average growth rate of 0.4 x 10^5 (± 0.10) CFU/ml (Table 7). This result is used as the desired growth rate against which the alternative media recipe of YCFA-M can be compared. The average growth rate on YCFA-M was found to be 0.05 x 10^5 (± 0.01) CFU/ml (Table 7). For a linear regression model of OD_{600} against time for this isolate, growth on YCFA-GF was significant compared to no growth ($R^2 = 0.3678$, $p = 0.01653$), whereas YCFA-M was not significant ($p > 0.05$). Although there was some growth of BP52G on YCFA-M (Supplementary material) this growth was poor and not statistically significant. Based on these results, mannitol cannot replace fish gut fluid in YCFA media for culture of Erysipelotrichaceae isolate BP52G as it produces significantly inferior growth.

These results demonstrate the unique nutritional requirements of isolates from different families isolated from the same fish gut environment, even from the same gut section (IV) (Figure 1). These results demonstrate that within the fish gut microbiota there is family level variation in nutrient requirements. These same protocols can be used to search for other essential growth factors present in nutritionally complex media additions sourced from the natural environment. By evaluating the growth rate of anaerobic bacteria on different media, these protocols can be used to discover novel defined alternatives to YCFA-GF for *in vitro* gut bacteria culture.

Time considerations

The preparation of multi-well anaerobic plates (Basic Protocol 1) takes three days. The initial isolation of fish gut bacteria from an environmental sample takes approximately 4 days (Basic Protocol 2). Though this step, along with all other steps involving bacteria culture, may vary depending on the growth profile of the isolate used. Time taken to reach saturation phase should be identified for an isolate in preliminary work before it is used in this protocol. The saturation phase should be reached by the end of incubation for all steps in Basic Protocol 2. Identification of isolates takes 1 day of laboratory work; further time depends on the sequencing facility used. Preculture preparation (Basic Protocol 4) takes 4 days. Performing growth rate experiments (Basic Protocol 4) takes at least 2 days. This may take more time if isolates take more than 48 hours to reach saturation phase.

Chapter 3 Section B: Final nutrient extraction methods from brown algae

Isolating the growth factor from seaweed

Carbohydrates, lipids, proteins and nucleic acids are the four major biomolecules which make up the living system (Wells and Hart, 2013). We are interested in isolating the nucleotides and vitamins from *Undaria pinnatifida* aqueous extract, to test them for bacterial growth rate abilities. We must separate the nutrients of interest, vitamins and nucleotides, from all other water-soluble nutrients such as some carbohydrates, lipids and proteins.

DNA and RNA are highly soluble in water, nucleic acids are readily soluble in water, and nucleosides and bases also have some solubility in water. Nucleotides can be defined as any *N*-glycoside phosphate that contains a purine or pyrimidine base and includes a phosphoric acid esterified to one of the hydroxyl groups of the sugar (Henderson & LePage, 1958). Nucleotides touch all phases of metabolism, including carbohydrates, lipids, proteins, nucleic acids, and the transfer of energy (Henderson & LePage, 1958). There are many processes, such as oxidation, reduction, dismutation and epimerization, that only take place when the substrate has been activated by combination with a nucleotide (Henderson & LePage, 1958). This is in addition to the vital role of nucleotides as nucleic acid precursors in organism genetics (Henderson & LePage, 1958). While all nucleotides are derived from just two compounds, purine and pyrimidine, the various combinations used have given rise to more than 110 nucleotides (Henderson & LePage, 1958). Acid is commonly used to precipitate polymers from a mixture as the insoluble fraction will contain most of the protein, nucleic acid and lipids, while the supernatant will contain low molecular weight components such as nucleotides (Henderson & LePage, 1958).

The water-soluble vitamins include Vitamin C and Vitamin B complex (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folate, and cobalamin) (Lykstad & Sharma, 2021). Water soluble vitamin main function in the diet is in the catabolism of food, where they act as coenzymes to produce energy (Gentili, et al., 2008), particularly some vitamins in the B-group (Santos, et al., 2012).

In a review by Bocanegra, et al. (2009), *U. pinnatifida* carbohydrate contents were reported at 47.8 g/100 g d.w. As carbohydrates are poorly soluble in almost all solvents except water (Zakrzewska, et al., 2010), desired nutrients such as nucleotides and vitamins may be removed from carbohydrates in a solution by their dissolution into a solvent other than water. Lipids make up between 1.5 and 2.7 g/100 g d.w. (Bocanegra, et al., 2009). Some of these lipids are soluble in water e.g. phospholipids with short chain fatty acyl groups. Proteins make up between 12.7 and 14.1 g/100 g d.w. (Bocanegra, et al., 2009). Most of these proteins are water soluble up to a certain concentration.

Treatment of seaweed samples

Fresh samples of *U. pinnatifida* blade were collected from mussel farming lines in the Marlborough Sounds, New Zealand. These samples were stored at – 18 ºC for two days, then 60 g *U. pinnatifida* blade was crushed under liquid nitrogen to a powder. This 60 g sample of

U. pinnatifida is enough to carry out both the vitamin and the nucleotide extractions detailed in these protocols.

Seaweed extraction methods

1. Cool a 100 mL compatible centrifuge with one run at 10,000 g for 15 minutes at 4 °C
2. Thaw *U. pinnatifida* from frozen storage, by leaving at room temperature for 30 minutes
3. Warm 60 mL of autoclaved water in a measuring cylinder in a water bath at 60 °C for 30 minutes
4. Weigh 60 g of *U. pinnatifida*, after removing and disposing of stipes
5. Grind *U. pinnatifida* to a powder with a mortar and pestle in liquid nitrogen
6. Move *U. pinnatifida* powder into a 200 mL glass flask
7. Wait 10 – 20 minutes for seaweed to warm to RT
8. Add the 60 mL warm autoclaved water to the flask and mix
9. Warm mixture in the 200 mL glass flask in a water bath at 60°C for 1 hour
10. Transfer mixture to a 100 mL centrifuge tube
11. Centrifuge at 10,000 g for 15 min at 4°C
12. Transfer the supernatant into a 500 mL Schott bottle and autoclave at 121°C for 15 minutes

    Schott bottle size must leave large amount of headspace during autoclaving in case of bubbling

    Schott bottle lid loosened before autoclaving to allow release of pressure throughout process
13. Re-centrifuge the supernatant at 13,523 g for 20 min at room temperature in 1 ml aliquots in Eppendorfs

14. Pour off bacteria free supernatant into 50 mL Falcons in aliquots of 10 ml per tube
15. Store supernatant aliquots at – 20 °C for future use in media making
16. Aliquots of 10 ml can also be dry-frozen in 50 mL Falcons and stored at – 20 °C for future use in nutrient extraction or media making

Extract Weight Conversion

In the process we use for *U. pinnatifida* aqueous extraction and preparation for fractionation, frozen seaweed is extracted with water, forming an aqueous extract. This extract is then freeze dried before the vitamins or nucleotides can be separated and purified by fractionation methods.

Preliminary experiments within this PhD found that when added to YCFA media, the freeze-dried aqueous extract of *U. pinnatifida* produced growth on agar plates that was visually indistinct from the growth achieved by the liquid aqueous extract. These results demonstrated that freeze drying had no effect on the growth enhancing effects of this extract, therefore the freeze-dried version of this extract was used in all future work to further refine the nutrient additions to the YCFA media.

A final volume of 28.5 mL of seaweed extract was obtained from 60 g seaweed by these methods, in a ~55% weight conversion. Freeze dried extract weight is ~4% of Liquid extract weight. Therefore, Freeze dried extract weight is ~2% of *U. pinnatifida* wet weight. Oven dried *U. pinnatifida* weight is ~9% of wet weight.

Further Nutrient Fractionation

Unlike other protocols for nucleotide extraction, these protocols are not designed to analyse and measure the nucleotide content of algae, but to extract these nutrient sources in a way that they can be utilised in further experimental work.

Solvent extraction was used to isolate the vitamin and nucleotide fractions from an aqueous *U. pinnatifida* extract. These are compounds likely to be bacterial growth factors. These fractions were then used in culture experiments to test bacterial growth factor abilities. A growth rate similar to that caused by whole extract suggests the presence of a growth factor in that fraction.

Nucleotides were fractionated from whole *U. pinnatifida* aqueous extract using Trichloroacetic acid and potassium hydroxide in methanol, method adapted from Brown, et al., 1982. The end product was a purified nucleotide extract suspended in ultrapure water. This extract can be placed directly into bacteria culture experiments to see the effect on growth.

Vitamins were fractionated using ammonium acetate in methanol, method adapted from Santos, et al., 2012. The end product was a pure vitamin extract, created by evaporating the extracting solvent of methanol in a steady stream of nitrogen gas. This dry extract can be re-constituted into a bacteria media for use in culture experiments

Further Extract Weight Conversion

The nucleotide and vitamin extraction methods used in this thesis are adapted from previously published work where a 5 g starting sample was used. If these methods were exactly replicated with our seaweed sample we would use 5 g wet weight seaweed, this is the equivalent to 2.8 mL of aqueous extract, and 0.1 g of freeze-dried extract. Instead, we used around double this amount in these methods. A 5 mL sample of aqueous extract was freeze dried, producing 0.2 g of sample. This decision was made due to the extensive processing the seaweed has been through before fractionation, which may have reduced nutrient content. This nutrient decrease needs to be compensated for during the extraction process. The use of a two times concentration of starting sample will increase nutrient yield from the starting sample.

Vitamin extraction

The following methodology for vitamin extraction, used in this thesis, was drawn from Santos, et al., (2012). A 5 mL sample seaweed aqueous extract freeze dried then mixed with 16 mL 10 mM ammonium acetate/methanol 50:50 v/v with 0.1% BHT, to extract all exchangeable compounds from the mixture by ion exchange and complexation (Slukovskaya, et al., 2020). Methanol was used to better extract the less polar water-soluble vitamins (Gentili, et al., 2008; Santos, et al., 2012). BHT was used to prevent oxidation of some vitamins such as vitamin C or b-carotene and a-tocopherol (Gentili, et al., 2008; Santos, et al., 2012). Good sample dispersion is achieved by shaking for 15 minutes, then sonication for 15 minutes in ultrasound bath. The ultrasound bath was temperature controlled due to the liable nature of most vitamins (Santos, et al., 2012). The non-soluble fraction is then removed by centrifugation for 15 minutes at 14000 g. The supernatant is filtered through a 0.45 µm nylon filter, then methanol is evaporated from the final sample by a stream of nitrogen gas.

Vitamin extraction protocol

1. Add 16 ml 10 mM ammonium acetate/methanol 50:50 v/v with 0.1% BHT to a freeze dried 5 ml sample of brown algae (equivalent to 0.25 g) in 50 ml Falcon

2. Shake for 15 minutes
3. Place in an ultrasound bath for 15 minutes.

   Keep ultrasound bath at <25 ^{o}C by the addition of ice

4. Centrifuge for 15 minutes at 14000 g
5. Remove supernatant

   Supernatant totals at ~ 8 mL

6. Filter supernatant through 0.45 um nylon filter
7. Evaporate the methanol from 1 mL aliquot in an N_2 stream from a 10 mL Universal tube

   The vitamins are left behind as a powder

   The vitamins can be stored wrapped in foil and frozen at – 18 to – 24 ^{o}C

Reagents and solutions

The 16 mL 10 mM ammonium acetate/methanol 50:50 v/v is made up of 8 mL 0.01 M ammonium acetate containing 0.1 % BHT, mixed with 8 mL methanol

Analysis

Measure the Vitamin C and Vitamin E content using a Nanodrop (a UV-vis spectrophotometer) by absorbance at 265 nm for Vitamin C and 295 nm for Vitamin E.

Quantity Conversion

During this vitamin extraction protocol, a 5 ml sample of *U. pinnatifida* aqueous extract is more than three times diluted by an extraction process that uses 16 mL of solvent. In the normal media making process, detailed in Chapter 4 Section A, 2.1 mL of *U. pinnatifida* aqueous extract is added to every 50 mL of YCFA-Undaria media. Therefore, three times as much vitamin extract was used in media making in order to maintain vitamin concentration in the media. The end result is that 6.3 mL worth of vitamin extract is used per 50 mL of YCFA-Vitamin media.

Nucleotide extraction

The following methodology for vitamin extraction, used in this thesis, was adapted from Brown, et al. (1982) and Newton et al. (1995), with most chemicals changed from those used in these published methods. A 5 mL sample of seaweed aqueous extract freeze dried then mixed with 2.5 mL 0.6 M trichloroacetic acid to precipitate all protein from the sample. This step is performed on ice to prevent heating of the mixture. This is an adjustment from the perchloric acid used in Newton, et al. (1995), as both chemicals have been used interchangeably in the literature (Moughan, et al., 1990). Acid is a commonly used to precipitate polymers from a mixture, with the soluble fraction only containing low molecular weight compounds, such as nucleotides (Henderson & LePage, 1958). Stirred for 3 minutes at 0 – 4 ºC. Centrifuged for 3 minutes, 14000 g, supernatant mixed with 5.5 mL 0.5 M potassium hydroxide in methanol to neutralise the homogenate after acid treatment then vortexed. This is an adjustment from the tri-n-octylamine in Freon used in Newton, et al., (1995) and Brown, et al., (1982). Newton used Trioctylamine + Freon in preference to Potassium hydroxide (KOH) to reduce positive potassium ion (K^+) content of the samples for mass spectrometric analysis (Newton, et al., 1995), but both chemicals are appropriate for this step. The mixture was centrifuged for 3 minutes at 14000 g, 8 mL supernatant removed to sixteen 500 uL aliquots in 2 mL Eppendorfs. The nucleotides are in this aqueous phase.

The nucleotides were then precipitated from this supernatant to remove impurities. This was performed on 500 µL aliquots of seaweed nucleotide extract. For precipitation 50 µL 3 M acetate, 1.7 µL glycogen and 1.25 mL 100% ethanol were added to each 500 µL aliquot and the mixture was inverted then incubated at – 80 °C overnight. The mixture was then centrifuged, 14000 g, 10 minutes, at 4 °C. Supernatant is removed, pellet resuspended in 300 µL 80% ethanol and centrifuged 14000 g, 15 minutes, at 4 °C. Supernatant discarded; remaining ethanol dried in a fume hood for 3 – 5 minutes. Pellet washed with 300 µL ultrapure H_2O. Purity tested using the nanodrop, at absorbance A260/280 and A260/230, both should give ≥1.8 purity.

Nucleotide extraction protocol

1. Add 2.5 mL 0.6 M trichloroacetic acid in H_2O to a freeze dried 5 ml sample of brown algae (equivalent to 0.25 g) in a 50 mL Falcon.

   Keep mixture at 0 - 4 °C throughout by securing Falcon in an ice bath

2. Stir 3 for minutes

   Keep mixture at 0 - 4 °C throughout by securing Falcon in an ice bath

3. Centrifuge for 3 minutes at 14000 g, aliquoted into four 1.5 mL Eppendorfs
4. Remove supernatant into a 50 mL Falcon
5. Add 5.5 mL 0.5 M potassium hydroxide in methanol

   Mixture totals at ~ 8.25 mL

6. Vortex for 30 seconds
7. Centrifuge for 3 min at 14000 g
8. Split 8 mL supernatant into 16 x 500 uL aliquots in 2 mL Eppendorfs

   The nucleotides are in this aqueous phase

Nucleotide precipitation protocol

1. Add to each 500 uL aliquot of seaweed nucleotide extract in a 2 mL Eppendorf:
   a. 10% of the total sample volume of 3 M acetate

    b. 0.3% of the total sample volume of 20 ng/ml glycogen
    c. 250% of the total sample volume of 100% ethanol
2. Invert Eppendorf three times
3. Leave Mixture overnight at – 80 °C
4. Centrifuge at maximum speed for 10 minutes at 4 °C
5. Remove supernatant,

   Do not invert the Eppendorf

   Circle the pellet with a marker pen

   A little EtOH can be left behind so as not to disturb the pellet
6. Add 300 uL of 80% EtOH, invert the tube and flick to mix with pellet
7. Centrifuge at max speed for 15 minutes at 4 °C
8. Remove and discard supernatant
9. Dry any leftover ethanol from the pellet in a fume hood for 3 to 5 minutes
10. Wash the pellet with 300 uL ultrapure H_2O once, then add 300 uL ultrapure H_2O to keep the nucleotides suspended for storage

    Pellet consists of pure precipitated nucleotides

    The nucleotides in H_2O can be stored frozen at – 18 to – 24 °C

Reagents and solutions

For 0.6 M trichloroacetic acid 9.8 g of 1M stock is made up to 100 mL with H_2O. For 0.5 M potassium hydroxide 2.8055 g of 1M stock is made up to 100 mL with methanol.

Analysis

On the nanodrop on the nucleic acid setting. This also gives a purity ratio, where A260/280 ratio should be ≥1.8 and A260/230 should be ≥2.0.

Quantity conversion

The extraction and precipitation process cause a two times increase in nucleotide concentration. A 10 mL sample of aqueous *U. pinnatifida* extract converts to 8 mL of initial nucleotide extract, which is then precipitated to 5 mL of final nucleotide extract. The final product of these extraction methods are Eppendorfs containing 300 uL of precipitated nucleotides in H_2O, at two times the concentration at which they are found in aqueous *U. pinnatifida* extract.

In preliminary experiments to test for growth factor effects of Undaria nucleotides on a fish gut bacteria isolate; a six times concentration of nucleic acids was used compared to that in Undaria aqueous extract. This increase in nutrient concentration would emphasise any growth effects these nucleotides have on these bacteria. While making the YCFA-nucleotide media, one 300 uL aliquots of precipitated nucleotides was used per 2.5 mL well of media in a 12-well experiment to measure the growth rate effects of this extract. As usually 105 uL SWU extract is used per well this is further increasing the vitamin concentration from two times concentrated to around six times concentrated, when compared to *U. pinnatifida*.

Chapter 4: Results Part 1 - Method Development

Method Development Introduction

This chapter details the experimental process that was carried out to arrive at the final methods shown in **Chapter 3**. A reliable and replicable method of growth rate measurement was required to trial candidate growth factors in a directly comparable manner. Used to build up a picture of the nutrient requirements of fish gut bacteria *in vitro*.

Reliable and replicable culture of fastidiously anaerobic fish gut bacteria proved to be a complex endeavour. Every stage of media making, culturing, isolate manipulation and growth was conducted in a way that completely excludes oxygen from the system. All growth rate measurement activities must also be conducted with the knowledge that these samples will be discarded after measurement, as once these samples are outside of these anaerobic systems and exposed to oxygen, they are no longer viable. To overcome these problems, the multi-well growth rate measurement method was developed, then published (Wornell, et al., 2022). The experimental work of this development process is detailed in this chapter.

Choosing Seaweed Species

The brown algae *U. pinnatifida* was chosen as addition of the extract of *Undaria pinnatifida* to culture media showed that this brown alga enabled vigorous growth of bacteria isolated from the hindgut of the herbivorous fish *Kyphosus sydneyanus*. Also, *U. pinnatifida* receives a large amount of international interest as part of the global food production industry (Nyar & Bott, 2014).

Bacterial Isolation

A round of *K. sydneyanus* hindgut bacterial isolation and identification was carried out using stool samples collected at sea from dissection of spear-caught fish. Gut contents of *K. sydneyanus* was spread onto YCFA media containing the aqueous extract of *U. pinnatifida,* referred to as YCFASWU. After three subcultures of single isolated colonies, pure cultures of nine isolates of hindgut bacteria were obtained, these new isolates were named KW190-198 and genetically identified by 16S rRNA gene sequencing.

Bacterial species identification

To identify the bacteria isolated from the fish hindgut, colony PCR was performed on single colonies of new isolates, followed by rapid Sanger sequencing of the ~400 bp V3-V4 region of the 16S rRNA gene, which is sufficient for species identification in most cases. The 16S rRNA gene sequence is commonly used for bacterial identification due to its ubiquity across bacterial species and the regional variation which allows bacterial genus identification in >90% of cases, and species identification in 65-83% of cases (Janda & Abbott, 2007). Sequencing was performed by Kristine Boxen at the DNA sequencing facility at the University of Auckland. Comparison of these sequences to those already present in the Clements laboratory was done using the program Geneious to construct phylogenetic trees to discover the percentage relatedness of these bacteria (https://www.geneious.com). The BLAST sequence analysis tool by NCBI was used to compare the 16S rRNA gene sequences to published sequences of known isolates, this shows if the bacteria isolated in this study is novel or has been previously cultured (Madden, 2002).

Protocol: Preparing isolates KW9/190 – KW17/198 for 16S sequencing

1. Inside the anaerobic hood crude *K. sydneyanus* gut contents were streaked directly onto YCFASWU agar plates and incubated for four days in an anaerobic jar, in an incubator at 20 ° *C*

   A temperature of 20 ° C is used for incubation of these fish gut bacterial isolates as this is the host K. sydneyanus body temperature (Wornell, et al., 2022)

2. Colonies of interest picked and streaked on fresh YCFASWU agar plates (this is culture 1 or C1)
3. Isolates re-streaked once colonies formed (this is Subculture 1 or SC1)
4. Two more sub-cultures performed

   This is done so that you certainly have "pure" cultures of each of the 9 isolates. Having a mixed culture will produce unreliable sequencing results.

5. Colony PCR performed, and product sent for Sanger sequencing using 16S V3-V4 primers (forward primer 5′-GCACCTAAYTGGGYDTAAAGNG-3′, reverse primer 5′-TACNVGGGTATCTAATCC-3′).

The BLAST computer algorithm, accessed on the NCBI website, was used to compare and align 16S sequences to the BLAST public database (Altschul, et al., 1990) (see 'Internet Resources'). Relatedness to known species can be found using BLAST and Geneious analysis, the cut-off for a novel species and genus is a sequence identity of 98.7% and 94.5% respectively (Yarza, et al., 2014). The bioinformatic software Geneious was used to analyse 16S sequencing data and generate phylogenetic trees (Kearse, et al., 2012) (see 'Internet Resources'). Phylogenetic trees were constructed including 16S sequences from the public NBCI database, along with unpublished sequences from the laboratory of Bikiran Pardesi, Kendall Clements, et al. at The University of Auckland. These trees were used to find if these isolates were novel species or identical to those already in stock in the Clements laboratory.

Of the new isolates, three were found to be *Erysipelotrichaceae* (190, 197, 198), all three had 100% similarity to an *Erysipelotrichaceae* isolate (BP52G) previously isolated by Pardesi, et al. (2022) named *Tannockella kyphosi,* is a novel genus and novel species, in the Erysipelotrichaceae family, from the Firmicutes phylum (Pardesi, et al., 2022). This was confirmed by BLAST results, as BP52G is in the BLAST database as part of an unpublished paper by Clements, et al. Therefore, these isolates are all likely to be the same species. Isolate BP52G 16S rRNA gene sequence accessed from: NCBI prokaryotic genome annotation pipeline (Tatusova, et al., 2016). Accession number: MG827409.

The six remaining isolates (KW191-196) were identified as *Oscillospiraceae* (Family Ruminococcaceae) and have > 98% similarity to isolate BP180, and > 97% similarity to the *Oscillospiraceae* isolate BP47G, previously isolated by Pardesi, et al., (2022). The isolates BP47G and BP180 also have a > 97% similarity to each other, so all these isolates may be the same species. In fact, a BLAST search suggests that these isolates are *Oscillospiraceae*, part of the family Ruminococcaceae, also in the in the class *Clostridia*. Including a 97% similarity to "NT4_C42 16S ribosomal RNA gene, partial sequence" which is an uncultured bacterium from a herbivorous fish gut, previously sequenced by Clements, et al., in 2012 and registered in the BLAST NBCI Database.

These sequencing results suggest that it is unlikely that new species of bacteria have been isolated by this present study. However, the isolates generated will be used in future experiments, as they are now confirmed as fermenting bacteria, with many showing a vigorous growth phenotype in laboratory conditions.

Liquid media growth rates by absorbance

Another challenge when working with fish gut bacterial isolates is the failure of traditional bacterial growth rate measurement methods. Commonly, samples of liquid culture are removed over a period and spectrophotometrically measured for absorbance, which is converted to bacterial cell count using standard equations. With these methods the growth rate of a bacterium cultured in a liquid media is efficiently measured over a period. However, the fish gut bacterial isolates used in our lab do not grow well in a liquid media (Table 1), this is a common issue seen with fastidiously anaerobic bacteria (Speers, et al., 2009).

Table 1: Four *K. sydneyanus* gut bacterial isolates were inoculated into 3 ml of YCFASWU. The isolates were identified at the Family level as: Lachnospiraceae (BP5G), Ruminococcaceae (BP47G), Erysipelotrichaceae (BP52G) and Ruminococcaceae (BP179). Test for growth was determined after three days by measuring absorbance at 600 nm wavelength using a spectrophotometer (ThermoFisher, Spectronic 20D+). To zero the spectrophotometer, blanks of each media without inoculum were measured before the samples.

	Absorbance at 600 nm wavelength in YCFASWU liquid media		
Isolate	**0 hr**	**3 days**	**10 days**
Lachnospiraceae - BP5G	0.063	0.029	0
Oscillospiraceae - BP47G	0.053	0.003	0.051
Erysipelotrichaceae - BP52G	0.037	0	0.062
Oscillospiraceae - BP179	0.002	0	0

The results of the liquid media growth rate tests were no growth of any of the four isolates in the YCFASWU liquid media (Table 1). As our fish gut bacterial isolates were found to have poor growth in a liquid media, growth rate calculations must be carried out on bacteria growing on a solid agar plate. Traditionally bacteria growth on a solid media is calculated by counting bacterial colony number increase over time. Unfortunately, many of the fish gut bacterial isolates we are interested in grow in continuous mats, or colonies invisible to the naked eye (Figure 2), which both prevent colony counting. Also, measurement of growth by colony counting is time consuming and inefficient, increasing experimental labour and error during data collection.

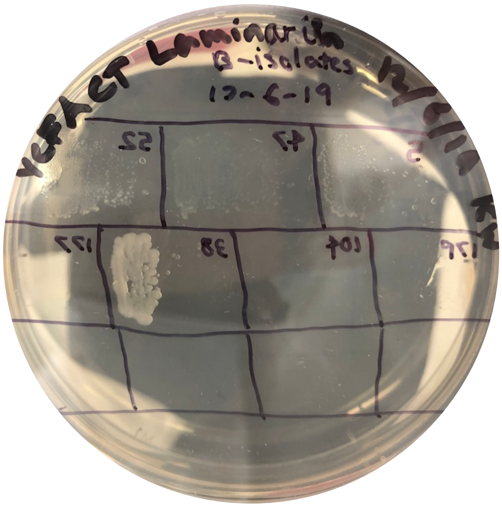


Figure 2: Demonstration of the mat growth phenotype preventing colony counting as a method of growth rate measurement. Growth of sequenced fish gut bacterial isolates, identified at the Family level as Lachnospiraceae (BP5G), Ruminococcaceae (BP47G), Erysipelotrichaceae (BP52G), Ruminococcaceae (BP179), Lachnospiraceae (BP104G), Rikenellaceae (BP38) and Rikenellaceae (BP177) growing on a solid agar media.

These complex requirements represent operational challenges which are overcome by the tactical use of anaerobic equipment in novel ways, to maintain continuous growth rate measurement from hour zero until the saturation phase. The development of this methodology, which has been published in Wornell, et al., (2022), occurred through much trial and error, and is detailed in this chapter.

Solid media growth rates by visual assessment

After the failure of growth in liquid media was established, the next effort to understand the growth requirements of the fish gut bacterial isolates was performed on solid media. Growth of these bacteria when subjected to various nutrient variations in the solid media was simply visually judged as either successful or unsuccessful.

Seven fish gut bacterial isolates in culture in the Clements lab and nine bacteria that were previously isolated within the present study were used. These isolates were tested for growth on YCFA media. The carbohydrate addition to YCFA of either mannitol or laminarin tested the ability of isolates to use these carbohydrates as an energy source. Laminarin is the most common storage carbohydrate across all brown algae. When this storage peaks in the autumn and winter months, laminarin may comprise up to 35 % of brown algae dry weight (Painter, 1983). Mannitol is also present in high quantities in all brown algae, as 40 – 75 % of laminarin chains are glycosidically linked to a D-mannitol molecule (Painter, 1983). This mannitol is released during brown algae digestion in the *K. sydneyanus* gut and has been seen to decrease along the gut as bacteria density increases, suggesting that this carbohydrate is used as an energy source by these bacteria (White, et al., 2010).

Due to their high concentration in brown algae, laminarin and mannitol are possible growth factors for *K. sydneyanus* gut bacteria *in vitro*. These nutrients were used as additions to *in vitro* culture media of fish gut bacteria to test for growth in these preliminary studies. For both YCFA-Laminarin and YCFA-Mannitol plates, the carbohydrate was added at 0.2 % (w/v), or ~10 mM concentration in YCFA, based on recommendations by Holdeman, et al., (1977) and Browne, et al., (2016), who used a 0.2 % (w/v) concentration of sugars in their anaerobic media.

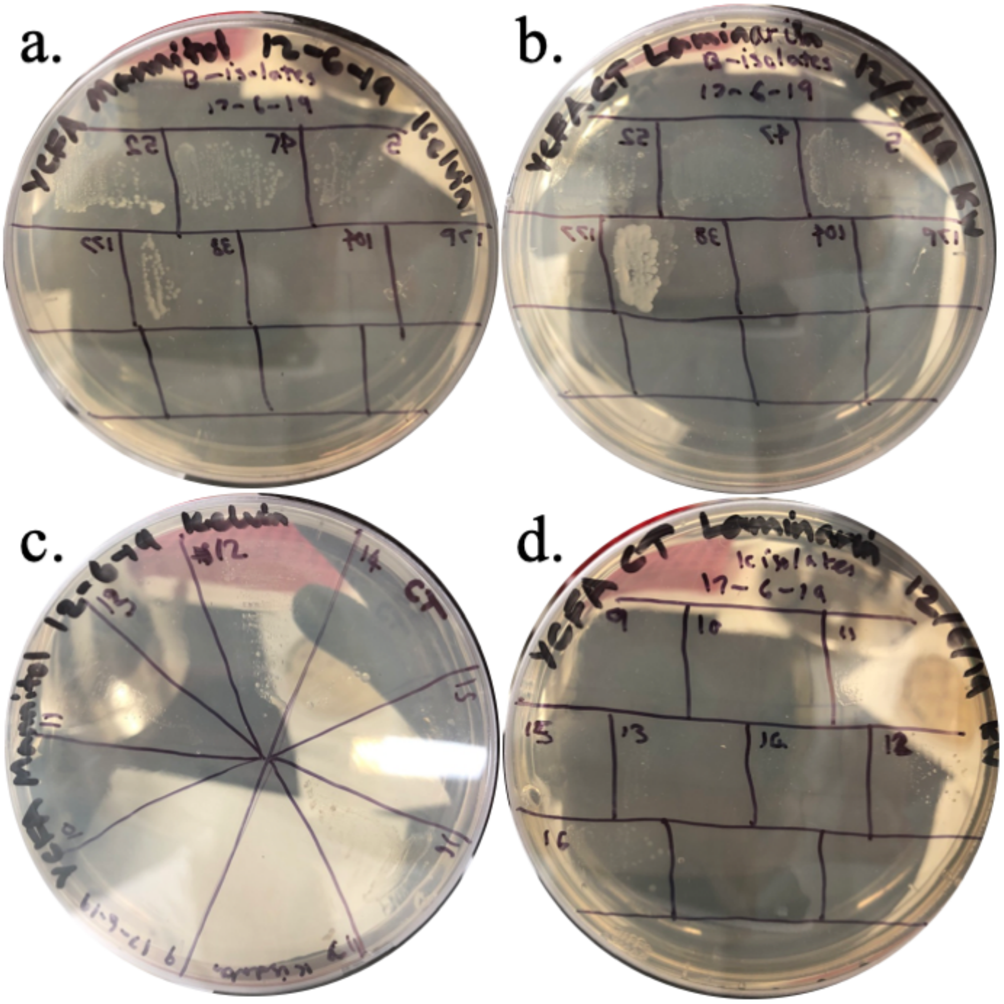


Figure 3: Growth of fish gut bacterial isolates on solid media. Successful growth identified visually by presence or absence of culture. **A)** Isolates BP5G, BP47G, BP52G, BP179, BP104G, BP38 and BP177 cultured on **YCFA-Mannitol** media. **B)** Isolates BP5G, BP47G, BP52G, BP179, BP104G, BP38 and BP177 cultured on **YCFA-Laminarin** media. **C)** Isolates KW9/190, KW10/191, KW11/192, KW12/193, KW13/194, KW14/195, KW15/196, KW16/197 and KW17/198 cultured on **YCFA-Mannitol** media. **D)** Isolates KW9/190,

KW10/191, KW11/192, KW12/193, KW13/194, KW14/195, KW15/196, KW16/197 and KW17/198 cultured on **YCFA-Laminarin** media.

After 14 days of incubation growth success was visually judged (Figure 3). If any significant growth was seen that isolate was assigned a 'yes' to growth on YCFA with the carbohydrate addition of either laminarin or mannitol (Table 2). All isolates were found to grow successfully on YCFASWU media (Table 2). YCFA only media does not produce growth for any of these isolates.

Table 2: Bacterial isolates previously obtained by the Clements lab (BP5G – 179) and obtained during this PhD (KW9/190 – 17/198) cultured on either YCFA-Mannitol, YCFA-Laminarin, or YCFASWU. Any significant growth seen by eye was assigned a 'yes' to growth success.

Isolate	**Growth on Mannitol**	**Growth on Laminarin**	**Growth on *U. Pinnatifida* extract**
Lachnospiraceae - BP5G	Yes	Yes	Yes
Rikenellaceae - BP38	Yes	Yes	Yes
Erysipelotrichaceae - BP52G	Yes	Yes	Yes
Oscillospiraceae - BP47G	Yes	Yes	Yes
Lachnospiraceae - BP104G	No	No	Yes
Rikenellaceae - BP177	No	No	Yes
Oscillospiraceae - BP179	No	No	Yes
Erysipelotrichaceae - KW9 / KW190	No	No	Yes
Oscillospiraceae - KW10 / KW191	No	No	Yes
Oscillospiraceae - KW11 / KW192	Yes - weak	Yes - weak	Yes
Oscillospiraceae - KW12 / KW193	Yes	Yes	Yes
Oscillospiraceae - KW13 / KW194	No	No – very slight	Yes
Oscillospiraceae - KW14 / KW195	No – very slight	No – very slight	Yes
Oscillospiraceae - KW15 / KW196	No – very slight	No – very slight	Yes
Erysipelotrichaceae - KW16 / KW197	Yes	Yes	Yes
Erysipelotrichaceae - KW17 / KW198	No	No	Yes

These results demonstrate the reliable growth of fish gut bacterial isolates on YCFASWU medium, in comparison to unreliable success on a simplified and replicable YCFA-Carbohydrate medium. Interestingly, these results also reveal that if an isolate can grow on YCFA-Mannitol, this isolate will most likely also be able to grow on YCFA-Laminarin. Laminarin is a more complex carbohydrate than the simple mannitol, with laminarin chains often glycosidically linked to a D-mannitol molecule (Painter, 1983). These findings suggest that either these bacteria can breakdown and utilise the energy from this brown algae storage molecule, or they are not able to derive energy from any stage of this process. Further, if fish gut bacteria cannot break down the most common storage molecule from brown algae, laminarin, then these bacteria will not possess the transporting apparatus necessary to make use of energy rich products from this break down process. This is demonstrated by the failure of non-laminarin digesting bacteria to utilise mannitol, which are small and energy rich molecules that require no further digestion for consumption. These findings have interesting implications for our understanding of commensality between various bacterial species within the gut microbiota. As these isolates are present across only the sections IV and V of the *K. sydneyanus* gut, it seems that it is not common for bacteria to beneficially interact in these sections during the digestion of the most common storage carbohydrate in the host fish diet. This may reflect complete digestion and utilisation of all products by bacteria that have the pathways necessary for laminarin digestion, providing the greatest possible energy payoff. This leaves bacteria without this digestion apparatus to survive by deriving energy from separate and different parts of the *K. sydneyanus* diet. In this way, these findings present an example of niche specialisation within the *K. sydneyanus* gut.

Anaerobic bacterial culture methods

Throughout all *in vitro* experimental work strict anaerobic conditions were maintained using the Hungate technique, along with specialized anaerobic equipment.

Hungate technique

This technique, which was first introduced by Hungate in a 1969 paper, enables aseptic and anaerobic transfer of ingredients between containers for generation of sterile anaerobic bacterial culture media (Hungate, 1969). Gas is passed via a pressure reducing valve through a hot reduced copper wire column, removing all trace oxygen present in the source gas (Moore, 1966). The gas passes out of this column through a glass Y tube. The gas then passes through rubber tubes and out of 16-18 gauge bent gassing needles through 2 mL sterile cotton-plugged Luer-Lok syringe barrels. A reducing agent (e.g. cysteine-HCl) is also added to the media to ensure all oxygen is removed.

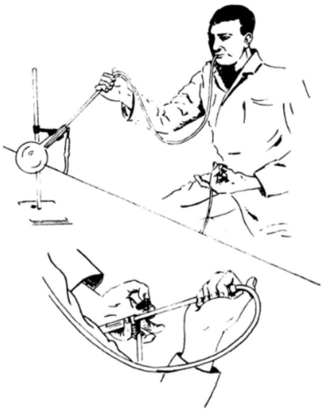


Figure 4: Hungate methods for aseptic and anaerobic transfer of culture media or media ingredients from flasks to culture tubes (Bryant, 1972).

When making usual bacterial culture media using the Hungate method, first autoclavable ingredients (water, cysteine-HCl, yeast extract, tryptone (bacto) and minerals, trace elements, and agar if making plates) are added to large Hungate vial (Bryant, 1972). The mixture is then autoclaved under N_2 gas for 15 min at 121°*C*. The rest of the ingredients can then be added anaerobically under a N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere (Figure 4) (Bryant, 1972). Finally, the media is poured into plates or aliquoted into stoppered Hungate tubes.

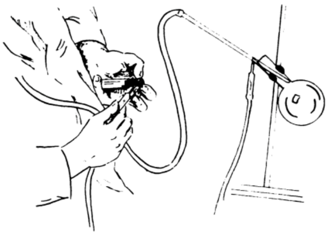


Figure 5: Hungate methods for aseptic removal of the gassing needle and replacement of the stopper into the culture tube after anaerobic transfer of culture media or media ingredients (Bryant, 1972).

Throughout this process the gassing needles remain inserted into all tubes in use and are moved between tubes aseptically (Figure 5) (Bryant, 1972). Agar plates once prepared, before bacterial addition, are stored at 3-5 °*C* in anaerobic jars. The jars must be pressure resistant when closed, jars are gassed with nitrogen to 5 psi. When being used for bacterial culture, agar plates are incubated at 20 °*C* in anaerobic jars, gassed with N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) to 5 psi. Bacterial incubation is maintained at the ambient seawater temperature of 17 to 23 ° *C*, as this is the temperature range of the fish gut (Mountfort, et al., 2002).

Anaerobic chamber

The anaerobic chamber, used for streaking and inoculation of bacterial isolates onto new media, is a flexible glove box (Coy Laboratory Products, Grass Lake, Mich.) kept at room temperature and filled with an atmosphere of N_2, CO_2, and H_2 (87.5%, 5%, 7.5% v/v/v) (H_2 levels kept at 3% inside the chamber), as detailed in Pardesi, et al., (2022). Gas supplied by BOC. The anaerobic conditions in the chamber were monitored with a CAM-12 anaerobic monitor (Coy Laboratory Products, Grass Lake, Mich.).

Measuring bacterial growth rate

The many complications to measuring the growth rate of these fastidious anaerobes have been discussed previously. The following section details methodologies: **Method 1** and **Method 2**, along with trials of these methods in chronological order. Analysis at each stage of method development eventually led to the final anaerobic growth rate measurement method, detailed in **Chapter 3**.

Method 1: 12-well plate agar and liquid media method

A 12-well plate culture method was developed within this PhD project, building on previous work by Dr Svetlana Boycheva, Dr Bikiran Pardesi and Prof. Antony Roberton at the University of Auckland. This method allows an initial period of plate-based growth (Figure 6).

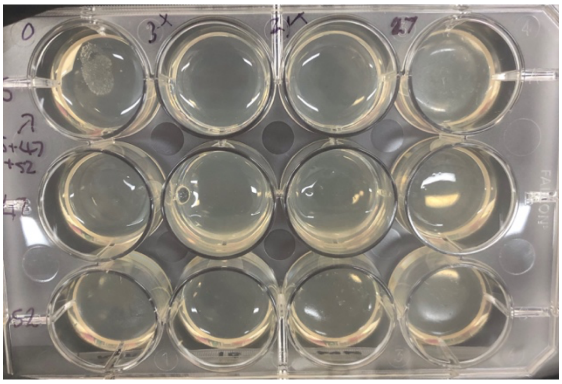


Figure 6: 12-well plate containing 1.5 mL YCFASWU solid agar media per well with isolates BP5G, BP47G and BP52G growing in each of the three descending rows respectively. Top left well has all three bacteria inoculated: BP5G, BP47G and BP52G. Growth displayed by cloudy layer after 27 hours incubation.

The culture can then be suspended into a liquid media layer above the agar (Figure 7). The aim of this method is to overcome issues in the accuracy of liquid culture experimental results. Previous data shows that sub-par growth seen in a liquid media differs from better growth seen on solid agar. It is possible that agar plates show more successful growth as they more closely resemble the intestinal environment, with a solid surface for bacteria to adhere to or rest upon.

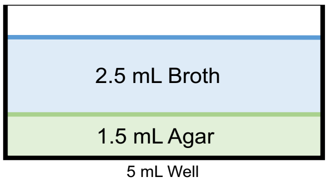


Figure 7: Cross section of one well in the 12-well plate.

Liquid media is preferable to solid agar media in this case as growth rate measurements can be generated spectrophotometrically. To overcome the liquid growth issues, liquid media is added on top of an established solid media culture. Pipetting up and down suspends bacteria cells in the liquid media. This liquid media can then be used in growth rate experiments, including addition of seaweed extract nucleotide and vitamin fractions in the search of a growth factor. This method, being conducted in 12-well plates, enables simultaneous culture of up to seven isolates plus a control in three different media, all under the same environmental conditions.

To generate the 12-well plates all 5 mL wells were first filled with 1.5 mL of YCFASWU agar. Isolates were then anaerobically spread onto the agar from plate culture. Three wells were used for each isolate, also three wells with no isolate were included as negative controls. Plates were incubated in an anaerobic jar for five days at 20°C. After this incubation period growth could be seen in all wells upon visual inspection. Liquid media (2.5 mL) was then pipetted on top of the agar layer in each well (Figure 7).

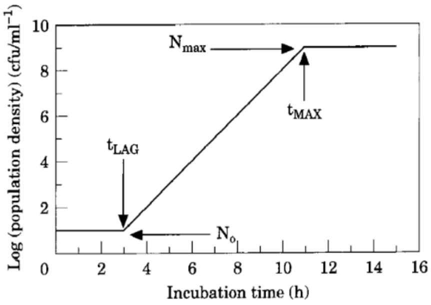


Figure 8: Graphic representation of the three-phase linear bacterial growth model (Buchanan, et al., 1997).

As can be seen from Figure 8 growth rate measurements only apply to the exponential phase. The exponential growth phase of the *K. sydneyanus* gut bacterial isolates of interest in **Method 1** are not yet known. Therefore, absorbance was measured at hours: 0, 3, 21, 27, 47, 63, and 84. These time points are similar to those that Prof. Anthony Roberton used in his growth rate experiments. From the results of **Method 1 Trial 1**, detailed later in this chapter, the exponential growth period of these isolates was established as between 3 and 27 hours.

Data Analysis

Absorbance was measured by re-suspending the culture, pipetting up and down a 200 µL volume ten times. Then taking a 200 µL sample from each well into a 96-well plate.

Absorbance at 600 nm was then measured spectrophotometrically and used as an estimate of bacteria cell density. Samples were taken from the wells at timepoints within the 0 to 27 hour exponential growth phase. The absorbance measurements from each isolate at each timepoint are averaged and plotted graphically against time. The average rate of growth across isolates can be represented by k coefficient of the exponential trendline of these graphs. These values can be translated into an estimate of average doubling time (t) in hours in each media type using k in the formula: $t = \ln(2)/k$.

Method 1 Trial 1: Growth rate measurement in multi-well plate

The aim of this trial was to test for a significant increase on growth rate of fish gut bacteria caused by the addition of seaweed extract to culture media.

Isolate selection

Seven bacterial strains were used, these were chosen to give a good variety of the species previously isolated in the lab by the Clements lab, and of those recently isolated within this PhD. The selection contained the Families Erysipelotrichaceae (isolates KW197 and KW198), Ruminococcaceae (isolates KW193 and KW194), Lachnospiraceae (isolates BP5G and BP104G), and Rikenellaceae (isolate BP38G). Growth was estimated by measuring absorption at 600 nm.

Isolates were also chosen based on their history of growth in laboratory conditions. Preference was given to those isolates with an observed history of reliably strong growth. A good variety in isolate media preference was also selected. Previous plate-based experiments showed that some isolates can grow well on YCFA-X (YCFA only) and YCFA-Mannitol media. Others only grow when seaweed extract is present (YCFASWU media). Isolates with a range of growth preferences were chosen to enable comparison between these groups and to ensure that the study covered a broad range of phenotype (Table 3). Throughout experimentation BP5G and BP38 were the only isolates to grow on YCFA-X.

Isolate Growth Success

	BP5G	BP38	KW193	KW197	BP104G	KW198	KW194
YCFASWU	YES	YES	YES	YES	YES	YES	YES
YCFA-Mannitol	YES	YES	YES	YES	NO	NO	NO
YCFA-X	YES	YES	NO	NO	NO	NO	NO

Table 3: Isolate growth success in three media types; YCFASWU (YCFA media with addition of seaweed extract), YCFA-Mannitol (YCFA media with addition of mannitol) and YCFA-X (YCFA media only).

For each isolate, one well was filled with media containing extract of *U. pinnatifida* (YCFASWU), one with media containing a brown seaweed-derived carbohydrate (YCFA-Mannitol) and one with no additional carbohydrate sources added (YCFA-X) (Figure 9). The culture was suspended into the liquid media by flush pipetting a 900 µL volume, 20 times in each well.

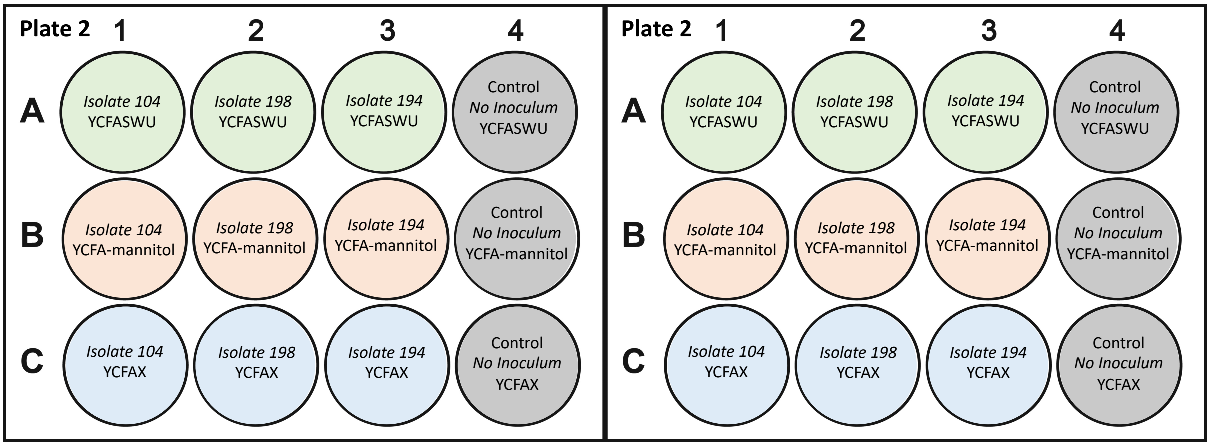


Plate 2	1	2	3	4
A	*Isolate 104* YCFASWU	*Isolate 198* YCFASWU	*Isolate 194* YCFASWU	Control *No Inoculum* YCFASWU
B	*Isolate 104* YCFA-mannitol	*Isolate 198* YCFA-mannitol	*Isolate 194* YCFA-mannitol	Control *No Inoculum* YCFA-mannitol
C	*Isolate 104* YCFAX	*Isolate 198* YCFAX	*Isolate 194* YCFAX	Control *No Inoculum* YCFAX

Plate 2	1	2	3	4
A	*Isolate 104* YCFASWU	*Isolate 198* YCFASWU	*Isolate 194* YCFASWU	Control *No Inoculum* YCFASWU
B	*Isolate 104* YCFA-mannitol	*Isolate 198* YCFA-mannitol	*Isolate 194* YCFA-mannitol	Control *No Inoculum* YCFA-mannitol
C	*Isolate 104* YCFAX	*Isolate 198* YCFAX	*Isolate 194* YCFAX	Control *No Inoculum* YCFAX

Figure 9: Plate layout for experiment, displaying isolate and liquid media used in each well. YCFASWU agar used in every well.

Method 1 Trial 1: Growth rate effects of U. pinnatifida extract and mannitol for seven isolates

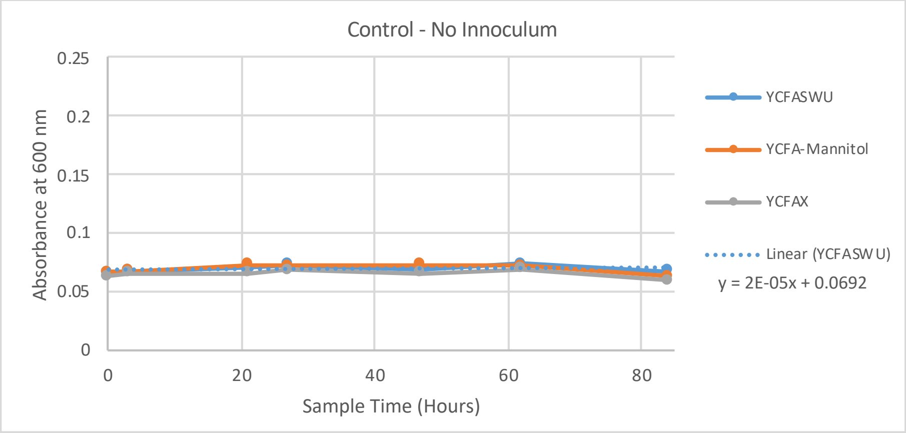
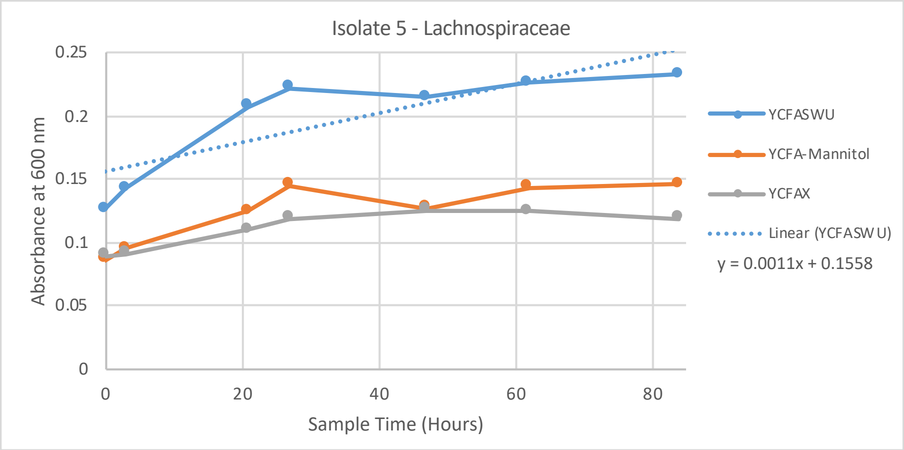


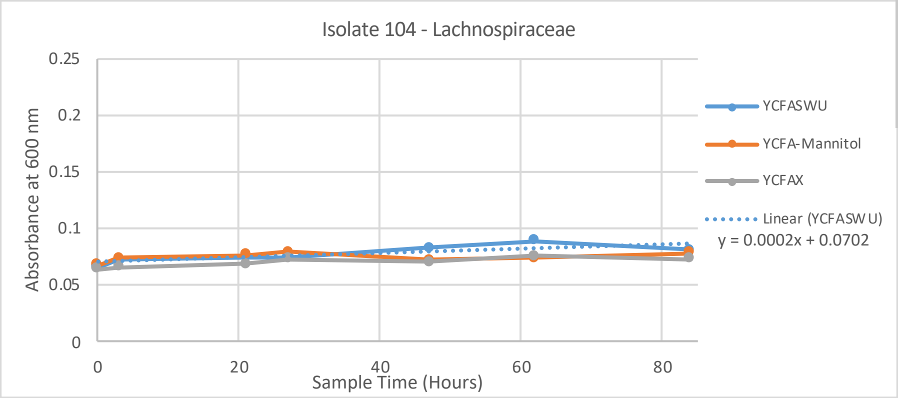
Figure 10: Growth in control wells with no bacterial isolate. Growth measured by absorbance at 600 nm at seven timepoints over 84 hours for YCFASWU, YCFA-Mannitol, and YCFA-X media. Linear trendline applied to YCFASWU growth (dotted line).



Isolate 5	Growth
YCFASWU	Yes
YCFA-Mannitol	Yes
YSFAX	Yes

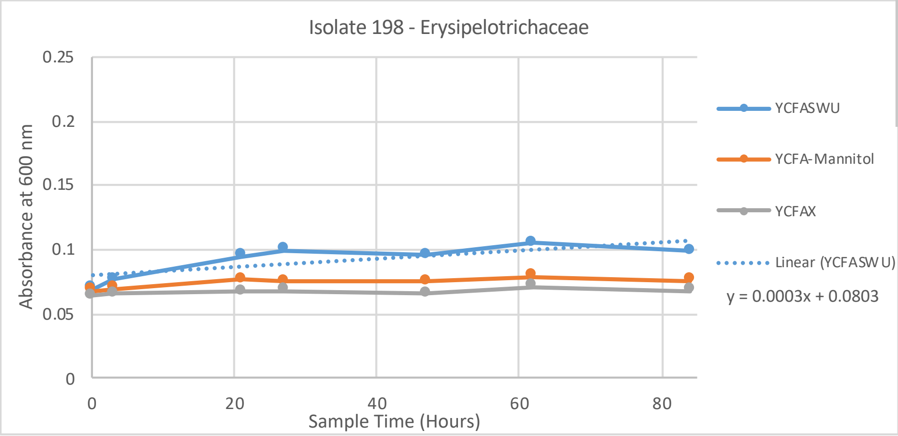
Figure 11: Growth of *Lachnospiraceae* isolate BP5G over 84 hours. Growth measured by absorbance at 600 nm at seven timepoints over 84 hours for YCFASWU, YCFA-Mannitol, and YCFA-X media. Linear trendline applied to YCFASWU growth (dotted line). **Table:**

Known growth success of isolate BP5G on the three media types, as identified in previous work within the present study.



Isolate 104	Growth
YCFASWU	Yes
YCFA-Mannitol	No
YSFAX	No

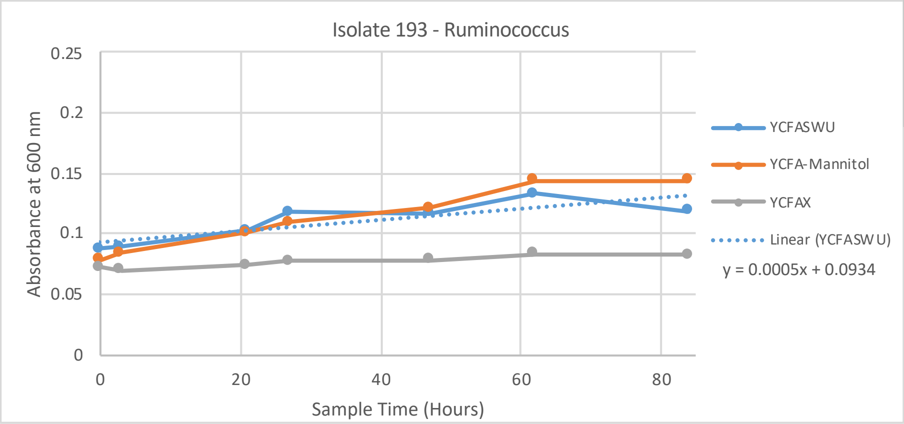
Figure 12: Growth of *Lachnospiraceae* isolate BP104G over 84 hours. Growth measured by absorbance at 600 nm at seven timepoints over 84 hours for YCFASWU, YCFA-Mannitol, and YCFA-X media. Linear trendline applied to YCFASWU growth (dotted line). **Table:** Known growth success of isolate BP104G on the three media types, as identified in previous work within the present study.

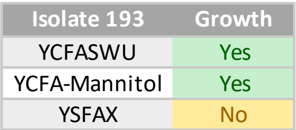


Isolate 198	Growth
YCFASWU	Yes
YCFA-Mannitol	No
YSFAX	No

Figure 13: Growth of *Erysipelotrichaceae* isolate KW198 over 84 hours. Growth measured by absorbance at 600 nm at seven timepoints over 84 hours for YCFASWU, YCFA-Mannitol, and YCFA-X media. Linear trendline applied to YCFASWU growth (dotted line).

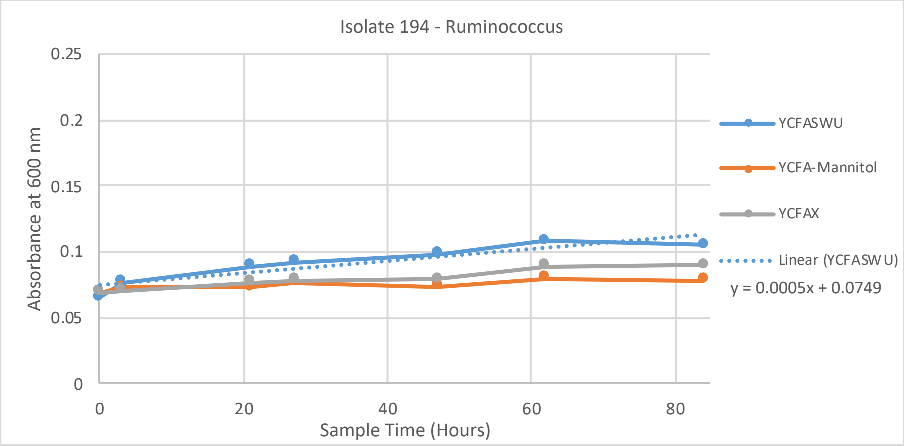
Table: Known growth success of isolate KW198 on the three media types, as identified in previous work within the present study.

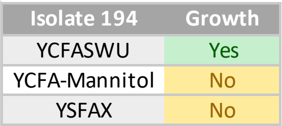




Isolate 193	Growth
YCFASWU	Yes
YCFA-Mannitol	Yes
YSFAX	No

Figure 14: Growth of *Oscillospiraceae* isolate KW193 over 84 hours. Growth measured by absorbance at 600 nm at seven timepoints over 84 hours for YCFASWU, YCFA-Mannitol, and YCFA-X media. Linear trendline applied to YCFASWU growth (dotted line). **Table:** Known growth success of isolate KW193 on the three media types, as identified in previous work within the present study.





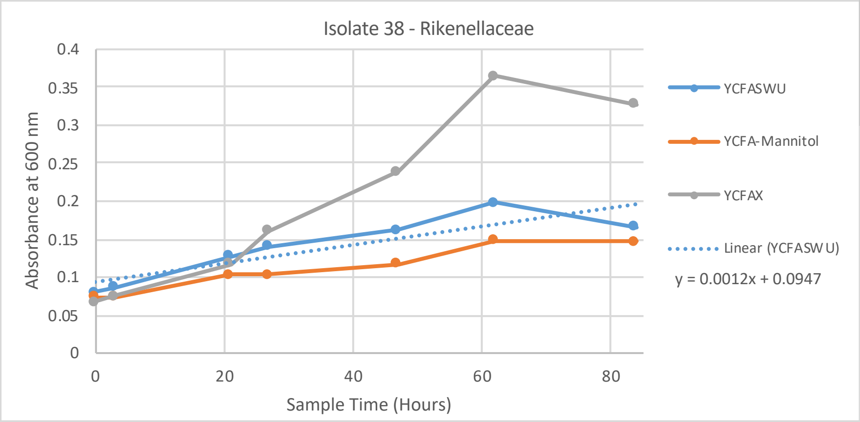
Isolate 194	Growth
YCFASWU	Yes
YCFA-Mannitol	No
YSFAX	No

Figure 15: Growth of *Oscillospiraceae* isolate KW194 over 84 hours. Growth measured by absorbance at 600 nm at seven timepoints over 84 hours for YCFASWU, YCFA-Mannitol, and YCFA-X media. Linear trendline applied to YCFASWU growth (dotted line). **Table:**

Known growth success of isolate KW194 on the three media types, as identified in previous work within the present study.

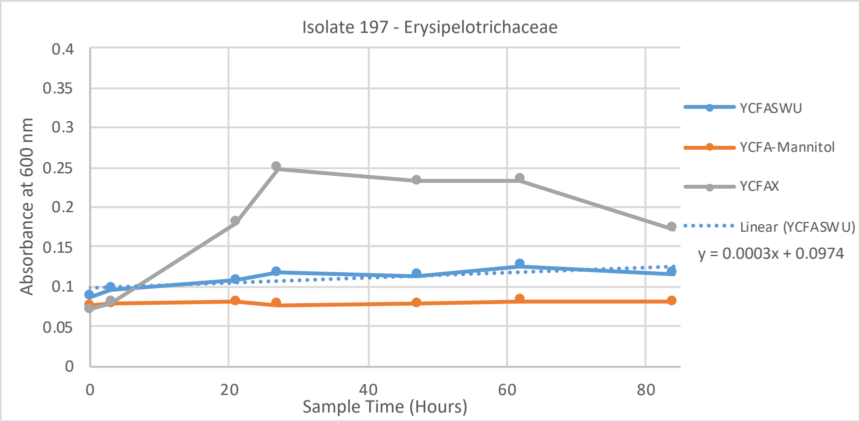
From these results the first of the isolates that begin to finish their exponential growth phase and enter stationary phase at the 27-hour timepoint. The low growth seen in isolate BP104G reflects observed weak growth at the agar stage. After this experiment the exponential growth phase of these bacteria (3 - 27 hours) became clear and this allowed accurate exponential trendline to be produced.

Excluded isolates due to unusual growth



Isolate 38	Growth
YCFASWU	Yes
YCFA-Mannitol	Yes
YSFAX	Yes

Figure 16: Growth of *Rikenellaceae* isolate BP38 over 84 hours. Growth measured by absorbance at 600 nm at seven timepoints over 84 hours for YCFASWU, YCFA-Mannitol, and YCFA-X media. Linear trendline applied to YCFASWU growth (dotted line). **Table:** Known growth success of isolate BP38 on the three media types, as identified in previous work within the present study.



Isolate 197	Growth
YCFASWU	Yes
YCFA-Mannitol	Yes
YSFAX	No

Figure 17: Growth of *Erysipelotrichaceae* isolate KW197 over 84 hours. Growth measured by absorbance at 600 nm at seven timepoints over 84 hours for YCFASWU, YCFA-Mannitol, and YCFA-X media. Linear trendline applied to YCFASWU growth (dotted line). **Table:** Known growth success of isolate KW197 on the three media types, as identified in previous work within the present study.

Isolates BP38 and KW197 showed greatly increased growth in YCFA-X media (Figures 16 and 17). The scales on the absorbance y-axis have been doubled compared to previous graphs so that the results can be visible. The unusual growth seen in isolate 38 was to be expected. This isolate was previously shown to grow on YCFA-X in plate agar experiments. However isolate 197 had not previously shown any growth in YCFA-X, but at some point after the four-hour time point, growth of this isolate in this medium began to exponentially rise. The growth rate of both isolates in YCFA-X exceeded that in YCFASWU.

It is possible that there was an error in measurement some YCFA-X wells. This would also explain the difference in growth pattern between the bacteria in the YCFA-X and YCFASWU wells of the same isolate. These unusual results have been excluded from analysis.

Mean exponential growth phase for five isolates in three different media

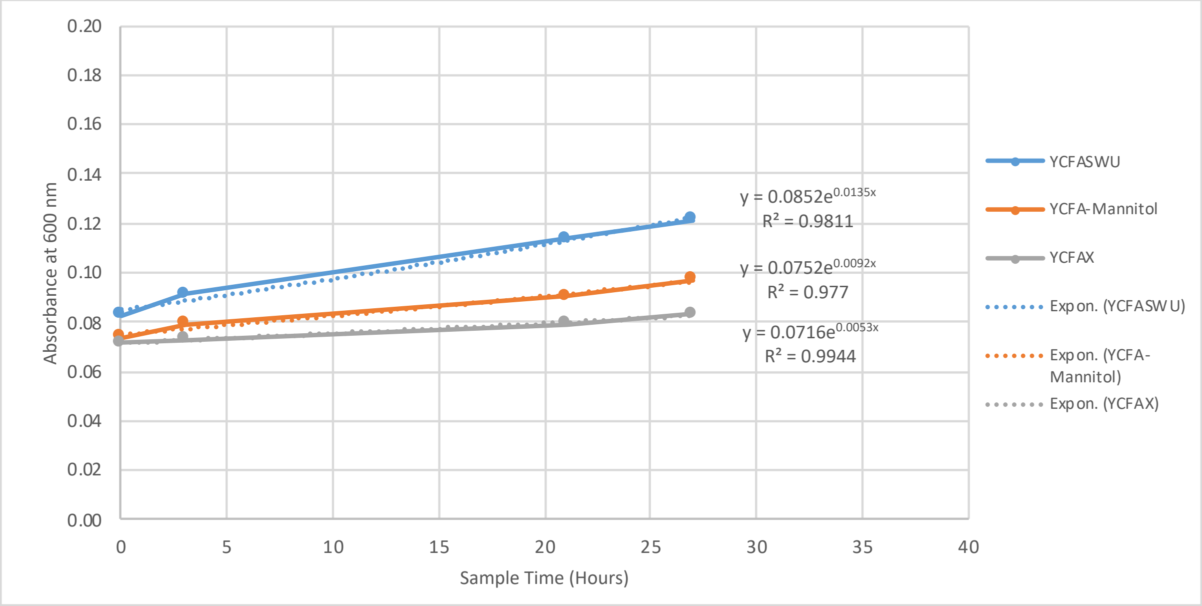


Figure 18: Mean growth measured in absorbance at 600 nm at four timepoints over 27 hours (hour 0, 3, 21 and 27) for five isolates (BP5G, KW193, BP104G, KW198, KW194). All wells used YCFASWU as the solid media layer. Measurements taken in three different liquid media: YCFASWU, YCFA-Mannitol and YCFA-X. Exponential phase only, with exponential trendlines.

Results show a clear increase in growth rate in YCFASWU compared to YCFA-mannitol and YCFA-X. No standard deviation has been given as the difference in initial inoculum and overall growth phenotype between species renders comparison of absorbance readings between isolates unhelpful. This fuelled further development of this method to standardise the inoculum, enabling the generation of directly comparable results using the final methodology.

The average rate of growth of the five isolates (BP5G, KW193, BP104G, KW198, and KW194) in each of the conditions can be represented by k coefficient of each exponential trendline as follows:

YCFASWU = 0.0135
YCFA-Mannitol = 0.0092
YCFA-X = 0.0053

These values can be translated into an estimate of average doubling time (t) in hours in each media type as follows:

$$t = \ln(2)/k$$

YCFASWU: $t = 51.3$ hours
YCFA-Mannitol: $t = 75.3$ hours
YCFA-X: $t = 130.8$ hours

These doubling times (should they prove accurate) are very long for bacteria. Improving culture conditions to shorten these growth times should be a priority if large-scale fermentation is to take place.

Discussion: Method 1 Trial 1

The results of this **Method 1** trial demonstrate that, when compared to normal YCFA-X media, addition of a brown algae-derived carbon source (mannitol) increases fish hindgut bacteria growth rate by ~70%. However, there are growth factor(s) present in *U. pinnatifida* that cause a much more impressive ~150% growth rate increase.

Discovery of these growth factor(s) will greatly increase production potential of proposed fermentation chambers, for an increased yield and decreased incubation time, reducing associated costs. The current average doubling time in YCFASWU is predicted at 51.3 hours. Decreasing this time by optimizing the media will greatly increase fermentation results and ease of laboratory work.

There is also the possibility that isolating the growth factor(s) and using them in increased amounts in media may allow isolation of a greater range of fish hindgut bacteria species than is currently possible, as more fastidious species will be able to grow in these conditions.

This trial showed that **Method 1** has good consistency between conditions and enables high throughput of results. As isolates vary in growth rate on agar the size of the 'inoculum' also varies, along with subsequent growth rate in liquid media. This makes it impossible to compare absorbance readings between species. Therefore, focus of this trial was on observing the overall trends revealed in this study. The issue of directly comparable results was addressed in development of the final methodology.

Method 1 Trial 1: Further testing

Method 1 Trial 1 Further testing 1: Requirement for initial solid media growth period

Isolates cultured in wells containing YCFA-X agar and a YCFA-X, YCFA-Mannitol or YCFASWU liquid media were compared over a 27-hour exponential growth period (Figure 19). The aim of this test was to reduce confounding variables impacting the 12-well plate method by using a defined and replicable media in the agar layer, removing any possible leakage of undefined ingredients into the liquid media layer. The average growth rates were measured for five isolates: BP5G, KW193, BP104G, KW198, KW194, of Families

Erysipelotrichaceae (isolates KW197 and KW198), Ruminococcaceae (isolates KW193 and KW194), Lachnospiraceae (isolates BP5G and BP104G), and Rikenellaceae (isolate BP38).

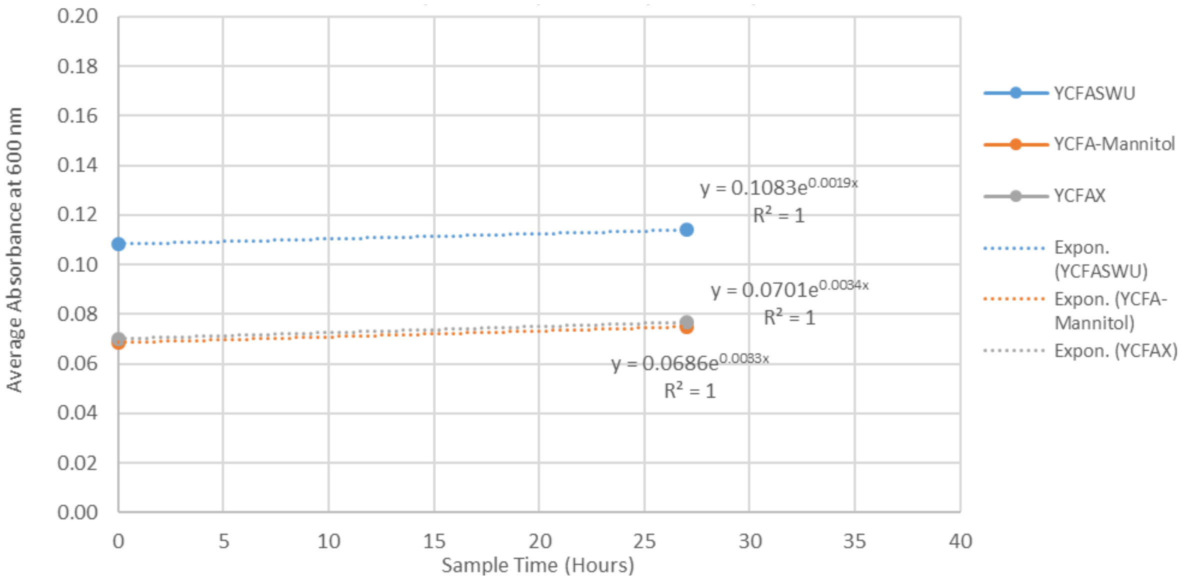


Figure 19: Mean growth measured in absorbance at 600 nm at four timepoints over 27 hours (hour 0 and 27) for five isolates (BP5G, KW193, BP104G, KW198, KW194). All wells used YCFA-X as the solid media layer. Measurements were taken in three different liquid agar media: YCFASWU, YCFA-Mannitol and YCFA-X. Exponential phase only, with exponential trendlines.

Gradient of exponential trendline (k coefficient) for YCFASWU media is lower than that of YCFA-X and YCFA-mannitol. All k coefficients much reduced in comparison to 12-well with YCFASWU agar (as seen in results of **Method 1 Trial 1**). For YCFA-X agar k coefficient is 0.0019, for YCFASWU agar k coefficient is 0.014. Therefore, use of the YCFASWU agar and 5-day incubation step causes a 10 times increase in bacterial growth rate. The low growth rates seen for all conditions in these test results suggest that growth was unsuccessful in all cases in this trial experiment that replaced the YCFASWU agar layer with a YCFA-X agar layer.

The conclusion of these results is that the YCFASWU agar layer used in **Method 1 Trial 1** is likely necessary for survival of these bacteria in the media. The bacteria likely do not just require an agar layer to rest on but require a stable phase of growth on the YCFASWU agar that they are used to, before the liquid media is added. These bacteria may also take up nutrients from this layer when resting on it in culture after the liquid media layer has been added.

Therefore, the initial solid YCFASWU media growth period was found to be a requirement of this novel growth rate measurement method. Subsequent trials found that three hours of incubation on solid media gave the isolates time to stabilise on the YCFASWU agar layer. Then the liquid media was pipetted on, and bacteria resuspended for the zero-hour time point to be taken for growth rate measurement. These method adjustments are applied in **Method 1 Trial 2**.

Method 1 Trial 1 Further testing 2: Another attempt at growth of isolates in a liquid media after culture in 12-well plates

An attempt was made to use this experiment as a method of transitioning bacteria from exclusively surviving on solid agar media to successful growth in a liquid culture. During **Method 1** these bacteria are periodically re-suspended into a liquid media. It was possible that these bacteria, having slowly become accustomed to liquid growth in this transitionary culture methodology, may now grow successfully in a liquid.

It was possible that the positive growth results seen in **Method 1** was the result of the presence of an agar surface upon which the bacteria could rest. This may have provided benefits similar to conditions present in the natural gut environment of these bacteria. However, it was also possible that the five-day YCFASWU plate culture step purely served to reduce the shock of a liquid addition, allowing growth in a liquid media. The purpose of this further work was to find out what is causing the growth success seen in **Method 1**.

A 300 µL sample was taken from the YCFASWU wells of each of the five isolates used in **Method 1 Trial 1** (BP5G, KW193, BP104G, KW198, KW194) into 3 ml YCFASWU in 5 mL Hungate tubes, growth estimated by absorbance at 600 nm across 70 hours (Figure 20).

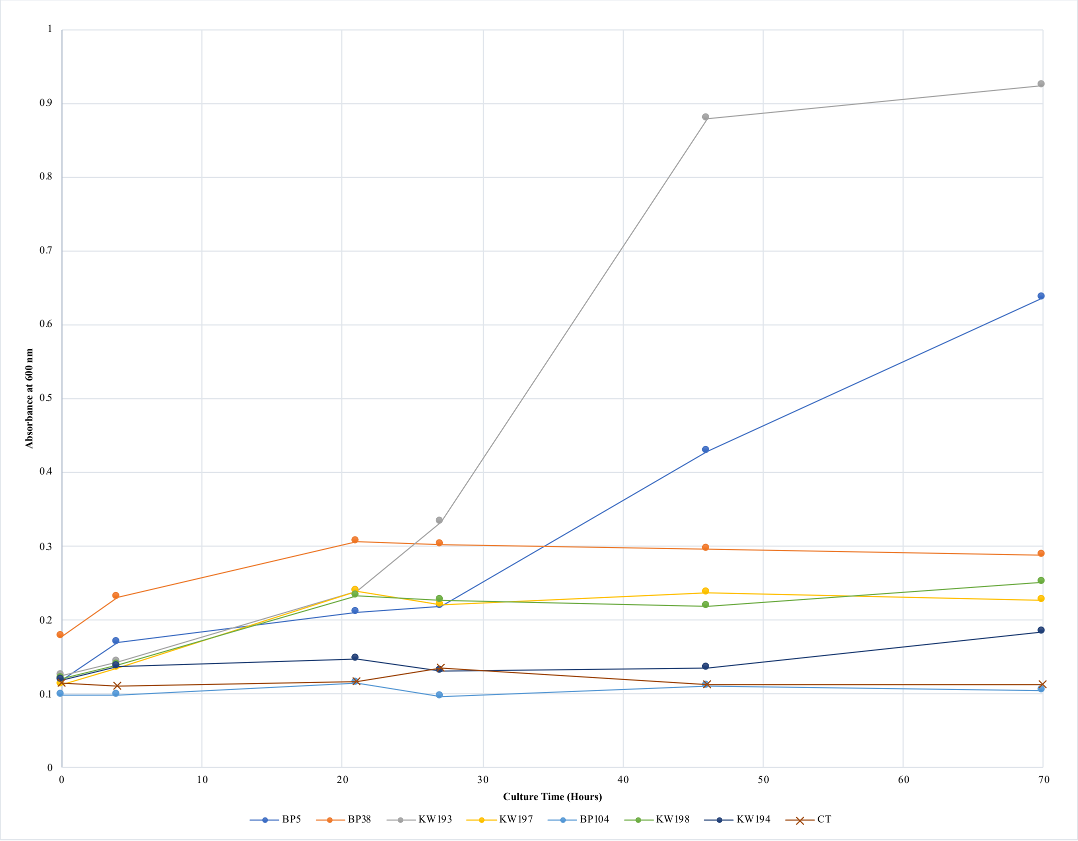


Figure 20: Growth measured by absorbance (600 nm) for isolates BP5G, KW193, BP104G, KW198 and KW194 grown in 3 mL YCFASWU, in 5 mL Hungate tubes. Six measurements over 70 hours.

Successful growth was seen only for two isolates: KW193 and BP5G. These results show that a solid agar media remains a requirement for successful growth rate measurement of most *K. sydneyanus* gut bacterial isolates *in vitro*. Therefore, liquid culture growth rates were not possible. All work from this point onwards built on the solid media growth rate measurement method detailed in **Method 1,** to generate comparable and reliable growth rate results for these fish gut bacteria.

Method 1 Trial 2: Growth rate effects of four carbohydrate sources on *Erysipelotrichaceae* isolate BP52G over 72 hours

The growth rate of *Erysipelotrichaceae* isolate BP52G on YCFA media with four different carbohydrate nutrient additions was measured at three timepoints over 72 hours (24, 48 and 72 hours). The carbohydrate nutrient additions were laminarin (YCFA-Laminarin), mannitol (YCFA-Mannitol) and alginate (YCFA-Alginate). The aqueous extract of the brown seaweed *U. pinnatifida* was the fourth carbohydrate nutrient addition (YCFASWU), this extract contains a large amount of carbohydrate from the brown algae storage molecules.

After the first 48 hours YCFASWU reached similar absorbance to previous results in the first 24 hours, at which point stationary phase began and the bacteria did not grow any further (Figure 21). YCFA-Laminarin, YCFA-Mannitol and YCFA-Alginate had all grown more than YCFASWU by the 48-hour mark (Figure 21). Bacteria continued to grow on media with Mannitol and Laminarin up to 72 hours.

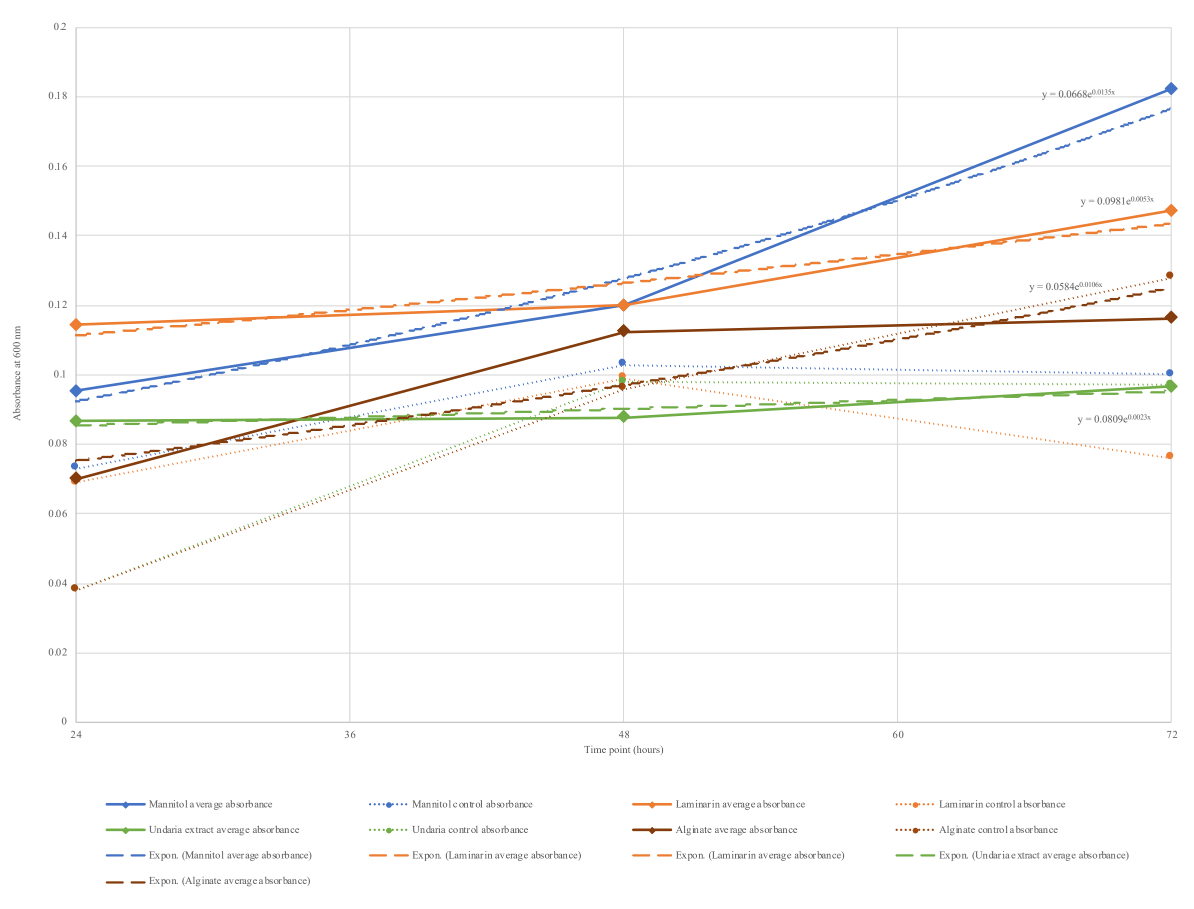


Figure 21: Mean growth measured in absorbance at 600 nm at three timepoints over 72 hours (hour 0, 48, and 72) for *Erysipelotrichaceae* isolate BP52G. Measurements taken on YCFASWU and YCFA media with carbohydrate additions of: mannitol, laminarin, alginate. With linear trendlines.

These results show the differing *Erysipelotrichaceae* isolate BP52G growth rates produced by various carbohydrates. The YCFA-Alginate and YCFASWU media wells reach stationary phase before the end of the 72 hours. Whereas the YCFA-Mannitol and YCFA-Laminarin media wells are still in exponential growth phase. Therefore, these different nutrients are utilised at different rates by this isolate, even if only one carbohydrate source is present for digestion.

The results of this **Method 1 Trial 2** (Table 4) confirmed that the exponential growth phase of the initial 27 hours is the best reflection of bacterial growth rate and therefore optimal media concentrations. The later timepoints missed the initial exponential growth phase for the *Erysipelotrichaceae* isolate BP52G. Therefore, these growth rate measurements taken after the 27-hour mark are not reflective of the optimal growth and nutrient uptake of these organisms.

Table 4: Mean doubling time (t) in hours for five fish gut bacterial isolates (BP5G, KW193, BP104G, KW198, KW194) measured using **Method 1** in **Trial 1** and **Trial 2**. Growth measured on five different media: YCFA-X, YCFASWU, YCFA-Mannitol, YCFA-Laminarin, YCFA-Alginate.

	Mean doubling time (*t*) in hours for five fish gut bacterial isolates (BP5G, KW193, BP104G, KW198, KW194)	
Media	**Method 1 Trial 1**	**Method 1 Trial 2**
YCFA-X	130.8	-
YCFASWU	51.3	301.4
YCFA-Mannitol	75.3	115.5
YCFA-Laminarin	-	266.6
YCFA-Alginate	-	65.4

Discussion: Method 1 Trial 2

Alginate, laminarin and mannitol all cause greater positive effects on growth of isolate BP52G over 72 hours than the aqueous extract of *U. pinnatifida* (Table 4). This would suggest that *U. pinnatifida* extract is not a necessary media ingredient for this isolate. Previous work of this study showed that isolate BP52G of the *Erysipelotrichaceae* family (Pardesi., et al, 2022), does grow well on laminarin and mannitol. Therefore, some fish gut bacteria do not require aqueous *U. pinnatifida* for *in vitro* growth. Instead, this additional nutrient source can be replaced with a carbohydrate source that is present in *U. pinnatifida*, such as mannitol, laminarin, or alginate.

However, this is likely not true for all fish gut bacteria species as some were found in previous work within the present study to have poor growth on mannitol and laminarin. For these isolates, along with potentially many other species in the *K. sydneyanus* gut, many other nutrients present in *U. pinnatifida* aqueous extract are also necessary for successful isolate growth *in vitro*.

These results of **Method 1 Trial 2** demonstrate that the novel methodology developed within the present study allow novel growth factors to be identified for fastidious anaerobic *K. sydneyanus* gut bacterial isolates. These results can be used to develop a defined and replicable media so that these fish gut bacteria may be cultured and analysed *in vitro*.

Method 2: Creating an inoculum of known bacterial concentration, so that growth rate measurements are more accurate.

The previous development of **Method 1** went some way to measuring the growth rate of *K. sydneyanus* gut bacteria on a solid agar surface. **Method 1** overcame issues that prevented these fastidious bacteria from growing well in a liquid media by culturing in a layer of liquid media above a solid agar surface. However, measuring growth in a liquid media pipetted onto an agar layer creates many confounding experimental factors, complicating the interpretation of results. Various trials of **Method 1** also still produced unsatisfactory growth results for many isolates in many conditions, compared to the reliable growth success seen on agar plates.

Method 2 aims to solve these issues by culturing bacteria on solid media in multi-well plates, then removing the bacteria with a water-gel wash for growth measurement by absorbance at 600 nm. This work is informed by the plate washing and absorbance measurement performed in Casciato, et al., (1974).

These methods also solve the issue of zero-hour inoculum size, which in **Method 1** is not standardised, resulting in growth rate results that cannot be reliably directly compared. In **Method 1** bacteria is spread onto agar inside a well, incubated for a period of three days to generate a good size inoculum, then liquid media is pipetted on top. In **Method 2** this step has been improved and a standardised inoculum of a predetermined absorbance is used for every isolate in every condition. This methodology improvement allows all growth rate results to be directly compared as every well contained the same size zero-hour inoculum. This improvement enables the building of a comprehensive picture of bacterial species nutritional preferences using **Method 2**.

Five isolates currently in culture in the laboratory of Clements, et al. are fully genome sequenced and available in public databases. These isolates will be used in all future growth rate experiments as this allows identification of the bacteria used in a public database. The genome sequenced isolates are of the Families: BP5G (Lachnospiraceae), BP47G (*Oscillospiraceae*, Family Ruminococcaceae), BP38 (Rikenellaceae), BP52G (Erysipelotrichaceae) and BP104G (Lachnospiraceae).

Method 2: Initial Testing

The first stage of method development was to test if our sequenced fish gut bacterial isolates survive the transfer and concentration alteration in water-gel. These isolates need to grow well after being suspended in water-gel for up to half an hour, as this situation will occur in **Method 2** when the bacterial cell concentration is measured spectrophotometrically, then adjusted to a consistent known concentration. This allows generation of an inoculum of known bacterial concentration, which can then be used in experiments to test the growth rate of these isolates. Growth rates can be compared between isolates cultured on media containing various nutrient additions to discover species-specific optimal growth conditions.

0.1% water gel was made using FreAlagin R gelatin (type A: Porcine Skin, G2625, Lot 60H0477, SIGMA). This lyophilised powder is powdered gelatin from porcine skin, recommended for use as a stabilizer or blocking reagent. Gelatin is a heterogeneous mixture of water-soluble proteins of high average molecular masses present in collagen. For 40 mL of 0.1% water-gel, 0.04 g of gelatin was added to 40 ml sterile H_2O in a falcon, vortexed for 30 seconds until mixed, warmed in a water bath at $60^\circ C$ for 15 minutes. The solution was then transferred in 5 mL aliquots to 10 mL Hungate tubes, and gassed with anaerobic gas mix (N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v)). These tubes were moved into an anaerobic chamber overnight, where the bungs on the Hungate tubes were loosened, allowing anaerobic gas flow into the tubes to remove all oxygen in the water gel.

The McFarland standards were the chosen method of converting absorbance readings to bacterial concentration in **Method 2**. Using an equation developed based on these standards (detailed from Stage 4 Step 7), the absorbance at 600 nm can be converted to an estimate of bacteria concentration in colony forming unit number per mL (Casciato, et al., 1975; McFarland, 1907).

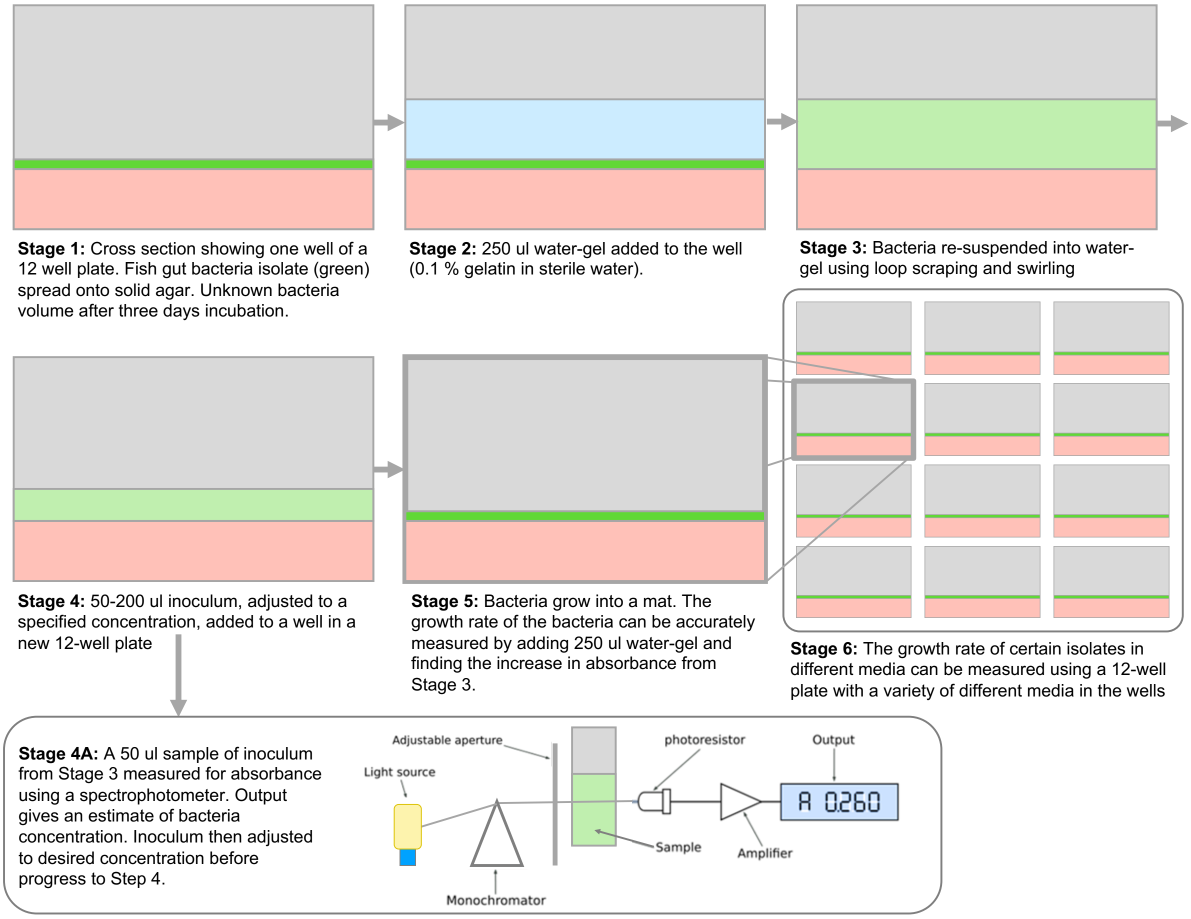
To test survivability in water-gel, all isolates to be used in experiment further work on **Method 2** (*Lachnospiraceae* BP5G, *Oscillospiraceae* BP47G and *Erysipelotrichaceae* BP52G) were spread in separate wells of a 12-well plate containing 1.5 mL of YCFASWU in each well. After 27 hours of incubation in an anaerobic jar at 20 ° *C*, the bacteria were harvested by washing each well with 2.5 mL of water-gel. The concentration of bacteria in this water-gel was measured spectrophotometrically at 600 nm. This bacterial suspension was then adjusted to approximately match the absorbance of McFarland standard 1, which has an absorbance of 0.257 at 600 nm, corresponding to an approximate cell density of 3 x 10^8 CFU/mL. This was achieved by centrifugation at 2000 g for 10 min, removal of the supernatant, then re-suspension of the pellet in the appropriate volume of water-gel. A 0.1 mL sample of this inoculum was added to corresponding wells of a new 12-well plate containing 1.5 mL of YCFASWU in each well. After four days of incubation in an anaerobic jar at 20 ° *C*, the bacteria were harvested by washing each well with 2.5 mL of water-gel. Bacterial concentration was measured spectrophotometrically at 600 nm wavelength. Results, detailed in Table 5, show that all isolates tested survived this process. With an average final absorbance of greater than McFarland Standard 1, which has an absorbance of 0.257 at 600 nm, corresponding to an approximate cell density of 3 x 10^8 CFU/mL for all isolates. Therefore, this methodology can be used to generate an inoculum for this growth rate measurement method. Four replicates were used for both isolate BP47G and BP52G, Table 5 presents the average absorbance across these replicates, with the corresponding standard deviation. Isolate BP5G had only one replicate, so no standard deviation is given.

Table 5: Mean growth success of isolates BP5G, BP47G and BP52G measured by absorption at 600 nm after four days culture on YCFASWU media

Isolate	**Average absorbance at 600 nm (± standard deviation)**
BP5G	0.316
BP47G	0.367 (± 0.033)
BP52G	0.304 (± 0.016)

Method 2: Trial 1

The process of **Trial 1** of **Method 2** process is detailed by a flow chart in Figure 22. In this method the inoculum is standardised between all isolates in all conditions, allowing direct comparison of all growth rate results. This is an improvement to the uncontrolled inoculum generated in **Method 1** by direct sterile spreading onto agar in a well of a 12-well plate, followed by incubation for three days. In this second method the initial culture is also generated by spreading the isolates onto agar in a well of a 12-well plate. However, this culture is then adjusted to a desired concentration before inoculation into the wells of a second 12-well plate.



Stage 1: Cross section showing one well of a 12 well plate. Fish gut bacteria isolate (green) spread onto solid agar. Unknown bacteria volume after three days incubation.

Stage 2: 250 ul water-gel added to the well (0.1 % gelatin in sterile water).

Stage 3: Bacteria re-suspended into water-gel using loop scraping and swirling

Stage 4: 50-200 ul inoculum, adjusted to a specified concentration, added to a well in a new 12-well plate

Stage 5: Bacteria grow into a mat. The growth rate of the bacteria can be accurately measured by adding 250 ul water-gel and finding the increase in absorbance from Stage 3.

Stage 6: The growth rate of certain isolates in different media can be measured using a 12-well plate with a variety of different media in the wells

Stage 4A: A 50 ul sample of inoculum from Stage 3 measured for absorbance using a spectrophotometer. Output gives an estimate of bacteria concentration. Inoculum then adjusted to desired concentration before progress to Step 4.

Figure 22: Method 2 Trial 1 All stages detailed. Fish gut bacteria is first spread onto solid agar at **Stage 1.** A 0.1% water gel is added to the well at **Stage 2**. The bacterial culture is re-suspended in the water-gel in **Stage 3**. At Stage 4, a sample of the suspension is removed and measured for absorbance at 600 mn in a spectrophotometer in **Stage 4A**, which produces an estimate of bacteria concentration. The concentration of bacteria is adjusted to be the same in each well, then pipetted onto a new 12-well plate in **Stage 5**. Growth rate of these wells can be measured by addition of water-gel and spectrophotometric measurement in **Stage 6**, method as detailed in **Stage 3 and 4**.

Protocol: Method 2 Trial 1

Stage 1

Create two 12-well plates with 1.5 mL YCFASWU agar in each well. Incubate at 20°C in anaerobic jars overnight, so the agar media becomes completely anaerobic.

Stage 2

Spread a chosen isolate into all wells of one 12-well YCFASWU plate with a sterile loop. Gas jar with gas mix and incubate in an anaerobic jar at 20 ° *C* for 3 days. For this trial one 12-well plate was used for *Lachnospiraceae* isolate BP5G, and one was used for *Oscillospiraceae* isolate BP47G.

Stage 3

Make two 12-well plates with 1.5 mL of chosen agar media in each well. These plates will be used to measure effect of certain media ingredients on bacteria growth rate.

Stage 3: Making agar plates for growth rate comparison

Two 12-well plates with four different solid media types: YCFASWU, YCFA-Vitamin, YCFA-Nucleotide and the YCFA-X control were created for **Method 2 Trial 1** (Figure 23). These plates were used to test the effectiveness of **Method 2** in identifying growth factors for *K. sydneyanus* gut bacteria among the vitamin and nucleotide extracts from the aqueous extract of *U. pinnatifida*. The methods used to obtain these nutrient fractions were developed within the present study and are detailed in **Chapter 3 Section B**. One of these plates (Figure 23) was used to test *Lachnospiraceae* isolate BP5G, one to test *Oscillospiraceae* isolate BP47G. The total media required to fill each well of these 12-well plates with 1.5 mL of media was 18 mL. The media was kept in a water bath at 60 ˚ *C* throughout the plate making process to prevent it from solidifying.

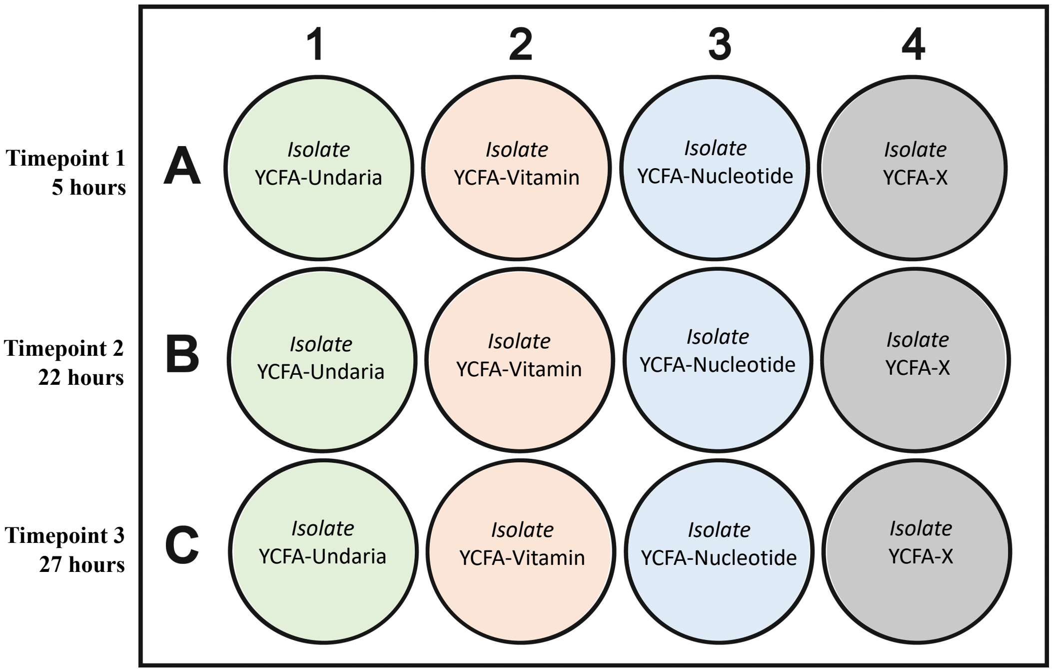


Figure 23: Plate layout for a 12-well plate used for growth rate comparison of one *K. sydneyanus* gut bacterial isolate on four different solid media types: YCFASWU, YCFA-Vitamin, YCFA-Nucleotide and the YCFA-X control. Three timepoints were used in **Method 2 Trial 1** within the exponential phase: either 5, 22 and 27 hours, or 5, 22 and 48 hours.

To generate the six YCFA-Vitamin wells, 9 mL of YCFA media was pipetted into one 2 mL dried vitamin aliquot in a 10 mL Hungate tube (generated using the protocols outlined in **Chapter 3 Section B)**. Then, 1.5 mL of this YCFA-Vitamin media was injected into each of six wells of two 12-well plates in a sterile hood.

To generate the six YCFA-Nucleotide wells, six aliquots of 300 µL Nucleotide extract in H_2O (generated using the protocols outlined in **Chapter 3 Section B)** were added to 9 mL of

YCFA media in a 10 mL Hungate tube. Then, 1.5 mL of this YCFA-Nucleotide media was injected into each of six wells of two 12-well plates in a sterile hood.

To generate the six YCFASWU wells, 378 µL *U. pinnatifida* extract was pipetted into 9 mL of YCFA media. Then, 1.5 mL of this YCFASWU media was injected into each of six wells of two 12-well plates in a sterile hood.

To generate the YCFA-X control wells, 9 mL of remaining YCFA media was removed from the batch stored in the 60 ˚ *C* water bath throughout the plate making process. Then, 1.5 mL of this YCFA-X media was injected into each of six wells of two 12-well plates in a sterile hood.

Stage 4

1. Pipette 300 µL water-gel (0.1% gelatin) into all wells of one of the 12-well plates of culture generated in **Stage 2** of this **Method 2**
2. Resuspend bacteria into the water-gel using a disposable sterile loop
3. Transfer 50 µL of the suspension into a 96-well plate for absorbance testing at 600 nm

   This result can also be used as a zero hr growth rate time point during analysis,
4. Transfer the remaining 250 µL of the suspension into 1.5 mL tubes in an anaerobic hood
5. Centrifuge these 1.5 mL tubes at 2000 g for 10 minutes
6. Remove and discard supernatant
7. Calculate amount of water-gel needed resuspend the pellet at McFarland Standard 3 (9 x 10^8 CFU/mL)

Stage 4 Step 7: Expanded Explanation

McFarland standards were used to calculate the approximate cell density of the two chosen isolates BP5G and BP47G with each media variation. To convert absorbance readings at 600 nm to an approximate bacterial cell concentration in CFU/mL, an exponential growth curve of the McFarland standard relationship between these two measurements was created (Figure 24)

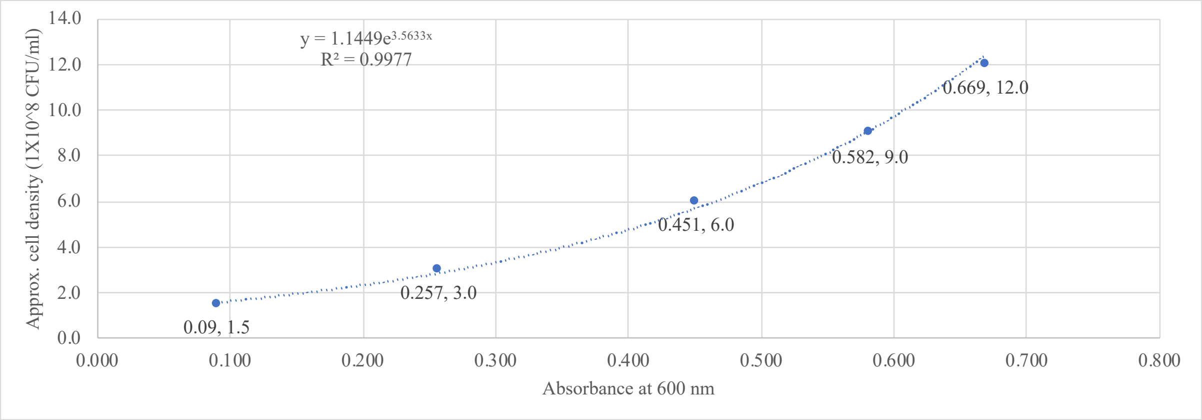


Figure 24: McFarland Standards, Absorbance at 600 nm against approximate cell density (1 x 10^8 CFU/ml). Exponential trendline with exponential equation and R value.

To convert the absorbance readings generated in Method 2 Trial 1 to approximate bacterial cell concentrations in CFU/mL use the McFarland Standard exponential curve Figure 24, the exponential equation of the exponential trendline for this graph was used (Equation 1).

$$y = 1.1449e^{3.5633x}$$

Equation 1

The absorbance of each isolate at each timepoint was used as the χ-value in Equation 1, the resulting γ-value of this equation is the approximate cell density at 1 x 10^8 CFU/mL.

8. Resuspend pellet in correct amount of water-gel in a 1.5 mL tube by gently pipetting up and down
9. Add this McFarland Standard 3 inoculum into all wells of the second 12-well plate, generated in **Method 2 Stage 3**
10. Incubate in an anaerobic jar at 20°C for 3 hours
11. Pipette 250 µL of water-gel into the 5-hour wells
12. Resuspend bacteria into the water-gel using a disposable sterile loop
13. Transfer 250 µL of suspension into 96 well plate for absorbance measurement at 600 nm
14. Convert this value to CFU/mL using the McFarland Standards
15. Repeat this process for the 22 and 27 (or 48) hour time point wells

Stage 5

Equation 2 from Hall, et al., (2013) was used to convert the first order growth rate constant (α) to doubling time (hours) for easy comparison of the growth factor properties of different media ingredients.

$$\text{Doubling time (hours)} = \frac{ln_2}{\kappa\ coefficient}$$

Equation 2

> *Growth rates are estimates and interpreting them requires some judgement, many confounding factors can affect absorbance readings and therefore growth rate calculations (Hall, et al., 2013). Hall, et al., (2013) recommends that if the standard error is >3% of the growth, or if the correlation coefficient is <0.995, then the results can be considered unreliable.*

The doubling time (hours) is calculated by finding the κ coefficient associated with exponential bacterial growth. The κ coefficient can be found by plotting mean absorbance values at 600 nm against time (hours) and applying an exponential trendline to the graph. The equation associated with this exponential trendline contains the κ coefficient as the exponential index. This is used in Equation 2 to calculate the doubling time.

Method 2 Trial 1: Results

The growth effects of vitamin and nucleotide extracts of the seaweed *Undaria pinnatifida* were tested on fish gut bacterial isolate BP5G (*Lachnospiraceae*) and isolate BP52G (*Erysipelotrichaceae*). The novel 12-well plate **Method 2** was used to measure the growth rate changes caused by various media additions. A shorter doubling time demonstrates quicker bacterial replication and a faster growth rate, this occurs when essential nutrients for growth have been introduced into the media.

Isolate BP5G (Lachnospiraceae)

Extracts Tested: Undaria, Vitamin, Mannitol

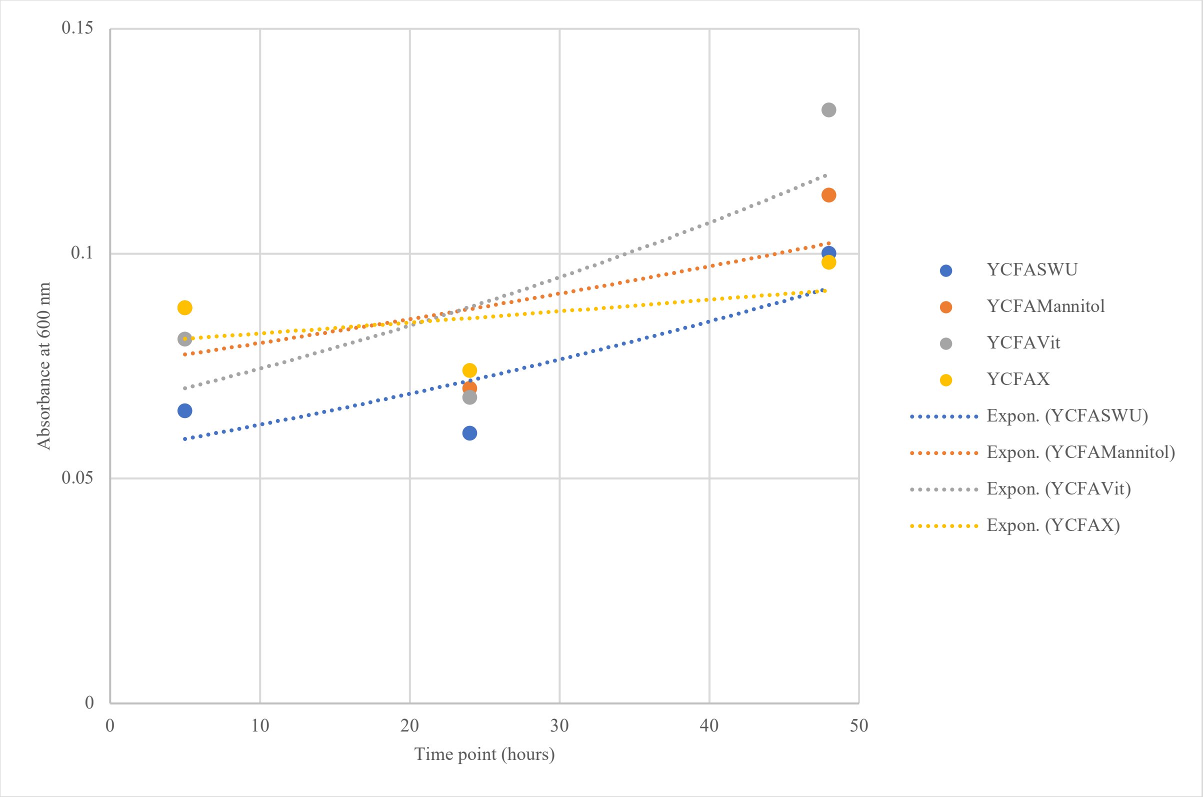


Figure 25: Growth of *Lachnospiraceae* isolate BP5G over 48 hours. Growth measured by absorbance at 600 nm at three timepoints (5, 22 and 48) over 48 hours with linear trendlines for YCFASWU (YCFASWU), YCFA-Mannitol, YCFA-Vitamin and YCFA-X media.

For *Lachnospiraceae* isolate BP5G **Method 2** was altered slightly. A longer growth period was used, this adjustment was made because the absorbance reading at 22 hours were surprisingly low (Figure 25). A delayed third timepoint at 48 hours was used to provide the bacteria with a long period of uninterrupted anaerobic growth in stable conditions. This was done to compensate for suspected sampling process effects on growth success. This lengthened culture period was not repeated in future experiments because prior evidence for a usual 27-hour exponential phase of growth was strong from the results of **Method 1 Trial 1**.

Instead, the results of this experiment were marked as poor growth caused by possible failure to maintain strictly anaerobic conditions during sampling for growth rates. This being the first time **Method 2** was performed, the use of this method on all other isolates (detailed in this chapter) did not encounter this problem.

The results of this experiment showed that for isolate BP5G, the Undaria and vitamin extracts produced a growth rate comparable to previous results of average fish gut bacteria growth rate in YCFASWU (51 hours doubling time). The vitamin extract from *U. pinnatifida* produces a growth rate similar to YCFASWU, both being ~30 hours doubling time. This result suggests that there are vitamin(s) present in Undaria which cause this isolate to multiply at optimum rates *in vitro*.

There was a large (~5x) increase in growth rate seen between YCFA-X and the addition of Undaria extract and the vitamin extract. This shows that addition of either of these extracts causes far superior growth in comparison to the YCFA media alone. YCFA media contains all growth factors for these bacteria known by this lab. This is further confirmation that there are nutrient(s) unknown to our lab that are required by fish hindgut bacteria for optimum growth *in vitro*. As the increase in growth rate caused by the addition of mannitol was much reduced at only around 2x, this compound is not the only bacterial growth factor that is present in brown seaweed extract.

Isolate BP52G (Erysipelotrichaceae)

Extracts Tested: Undaria, nucleotide and vitamin

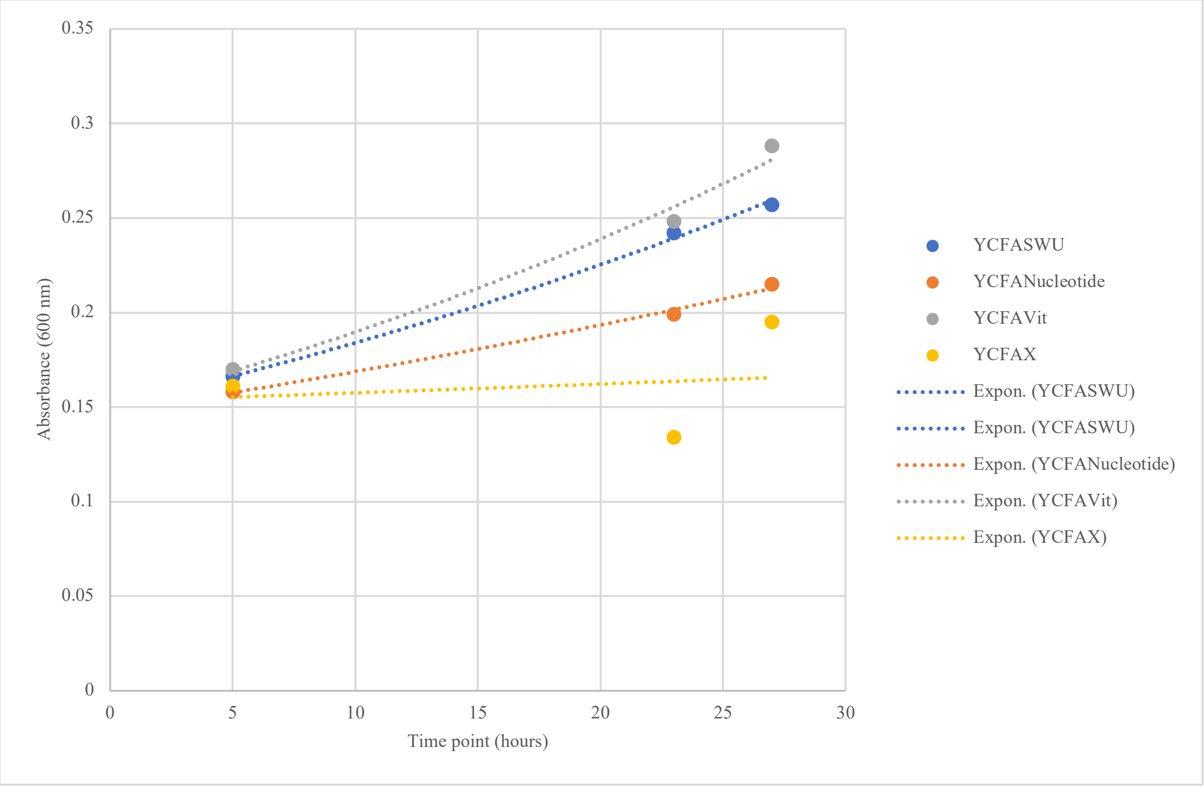


Figure 26: Growth of *Erysipelotrichaceae* isolate BP52G over 27 hours. Growth measured by absorbance at 600 nm at three timepoints (5, 22 and 27) over 48 hours with linear trendlines for YCFASWU, YCFA-Mannitol, YCFA-Vitamin and YCFA-X media.

For *Erysipelotrichaceae* isolate BP52G the Undaria, nucleotide and vitamin extracts all produce a growth rate comparable to previous results of average fish gut bacteria growth rate in YCFASWU (51 hours doubling time) (Figure 26). Also, the vitamin extract of *U. pinnatifida* produces a growth rate similar to YCFASWU, both being ~30 hours doubling time. These results suggest that there are vitamin(s) present in *U. pinnatifida* that cause this isolate to multiply at faster rates *in vitro*.

There was a large (>5x) increase in growth rate seen between the control YCFA-X wells and the addition of each of the three extracts. This result shows all of these nutrient additions cause far superior growth in comparison to the YCFA media that has no added carbohydrates. YCFA media contains many of the growth factors for these bacteria known by this lab. Therefore, this result is encouraging in the use of this media to search for growth factors required by fish hindgut bacteria for optimum growth *in vitro*. Both the nucleotide and vitamin extracts cause >5x increase in growth rate, so both compound types must contribute towards increased growth rate of isolate BP52G *in vitro*.

Comparison of all Method 2 Trial 1 results

Both isolates BP5G and BP52G had shorter doubling times when the vitamin extract of *U. pinnatifida* was added when compared to either the nucleotide extract or to mannitol (Table 5). These results suggest that there are vitamin(s) present in *U. pinnatifida* aqueous extract that are essential for optimal *in vitro* growth of both fish gut bacterial isolates. These results also suggest that this vitamin is not one of those already currently used in the normal YCFA-X media, which contains a vitamin solution. The growth rate produced by the vitamin extract of *U. pinnatifida* is faster than the total *U. pinnatifida* aqueous extract in both cases (Table 5). This may be due to the vitamin extraction process concentrating the vitamins slightly compared to aqueous extract. These questions are tested in further work within the present study (**Chapter 5**).

Table 5: Doubling time (in hours) of two fish gut bacterial isolates in YCFA base medium or YCFA supplemented with: the total aqueous extract of *U. pinnatifida*; *U. pinnatifida* vitamin extract; *U. pinnatifida* nucleotide extract; or mannitol. Isolate 5 (*Lachnospiraceae*) was not tested for growth with *U. pinnatifida* nucleotide extract and isolate 52 (*Erysipelotrichaceae*) was not tested for growth with mannitol. Compared to mean doubling time of five isolates (BP5G, KW193, BP104G, KW198, KW194), results from previous work within the present study.

	Doubling time (t) in hours:		
Extracts added to YCFA base medium	Isolate BP5G (*Lachnospiraceae*)	Isolate BP52G (*Erysipelotrichaceae*)	Mean for five isolates (BP5G, KW193, BP104G, KW198, KW194)
Total U. pinnatifida aqueous extract	66	34	51.3
Vitamin extract from U. pinnatifida aqueous extract	57	30	-
Nucleotide extract from U. pinnatifida aqueous extract	-	51	-
Mannitol (common carbohydrate in U. pinnatifida)	108	-	75.3
None (normal YCFA-X media)	239	239	130.8

Method Development Conclusion

Results from the trials of **Method 1** and **Method 2** in this chapter were used to develop the final growth rate measurement method published in Wornell, et al., 2022 (**Appendix 1**). Various improvements were made to produce this final methodology, improving the reliability of results. The use of a 48-well plate in the final method increased the throughput by allowing more growth conditions to be tested per plate. Also, the increase in well number allowed four rather than three timepoints to be taken for each growth rate curve, improving the reliability of growth rate curves. The trial and error of these early methodologies developed the precise experimental procedures required for each stage, this enabled the achievement of reliable growth success of all isolates in the final method, detailed in **Chapter 3**.

Chapter 4 Section C: Methodology Specifics for Chapter 5 Results

Earlier Sections A and B of this Chapter detailed the general methodological approach used throughout the experimental work of this PhD. This Section C details specific methodology and rationale that provides context for the results set out in Section C.

Four timepoints of 3, 6, 19 and 22 hours within the 27-hour exponential growth period were used to calculate the growth rates. Each of the three rounds of testing was separately plotted then averaged. Growth rates were calculated from the gradient of a linear regression line associated with the absorbance readings over time, graphs presented in **Chapter 5**, equations used detailed in **Chapter 3**. Growth rates are presented as an increase in colony forming units (CFUs) per hour, in tables throughout **Chapter 5**. Significance was found using one way or two-way ANOVAs and post hoc Tukey's tests in IBM SPSS Statistics for Macintosh, version 28.0.1 (IBM Corp., Armonk, N.Y., USA). Significant increases in growth rate in comparison to YCFA with gut fluid due to a nutrient addition demonstrate an alternative media recipe.

Twelve nutrient additions to YCFA media were trialled. Four polysaccharides were purchased from industry, with potential to generate a new defined and simplified YCFA medium. Aqueous extracts from four different algae were trialled, three of which were brown algae (*U. pinnatifida, E. radiata* and *C. torulosa)*, with one red alga (*C. ustulatus*). Both *E. radiata* and *C. ustulatus* regularly make up a large component of the Northeastern caught *K. sydneyanus* diet. Vitamin extracts from *U. pinnatifida,* along with an increased concentration of commercially available vitamins, were used to find the importance of *U. pinnatifida* water soluble vitamin content on successful bacterial growth.

Three individual isolates of anaerobic bacteria known to be present in the fish gut were used in this study: Oscillospiraceae (BP47G), Lachnospiraceae (BP5G), Rikenellaceae (BP38), and a mix of all three. The phylogenetic diversity of these isolates provides a good representation of the variety of families present *in vivo* in the *K. sydneyanus* gut (Stevenson, et al., 2022). Individual isolates were included as use of only a mix of all bacteria from a gut sample in culture dependent work can cause the community composition and function to shift, skewing digestive abilities of bacteria away from those seen *in vivo* (Shiina, et al., 2006; Preston-Mafham, et al., 2002; Ray, et al., 2012; Clements, 1997). The use of three different *K. sydneyanus* gut bacteria from the same section of the gut revealed divergence in nutrient preference between isolates. The use of a mixture of all three bacteria together revealed growth advantages provided by the presence of multiple isolates, and suggested community digestion behaviour.

The *Oscillospiraceae* (BP47G) and Lachnospiraceae (BP5G) isolates are from the same phylum, Bacillota, whereas the Rikenellaceae (BP38) is from the Bacteroidota phylum. All three of these isolates are from different families. The *Oscillospiraceae* (BP47G) is from the Ruminococcaceae Family, Order Clostridiales; the Lachnospiraceae (BP5G) is classified at Family level, also in the Order Clostridiales; the Rikenellaceae (BP38) is also classed at Family level, in the Order Bacteriodales. The Oscillospiraceae (BP47G) and Lachnospiraceae (BP5G) isolates are more closely related to each other than to the Rikenellaceae isolate as they are both in the Clostridiales order.

Chapter 5: Results Part 2 – Growth Rates

Introduction

This Chapter includes the results of the novel growth rate measuring method developed in this PhD and published as Wornell, et al., (2022) to trial various nutrient additions to the standard Yeast extract-casein hydrolysate-volatile fatty acids (YCFA) media. The aim of these experiments was to discover novel growth factors for bacterial isolates from the hindgut of the herbivorous marine fish *Kyphosus sydneyanus*. Isolates BP47G (*Oscillospiraceae,* Family Ruminococcaceae), BP5G (family Lachnospiraceae) and BP38 (family Rikenellaceae) were chosen for these experiments due to the full genome sequences for these isolates being available in public databases, along with the good performance of these isolates in preliminary studies.

The growth rates of these individual isolates and mixtures of multiple isolates were measured in the multi-well method with various nutrient additions to YCFA media. These nutrient additions were trialled in batches. **Section A** presents the growth rates of each isolate on YCFA media with the complex nutrient addition of *K. sydneyanus* gut fluid. This addition of host gut fluid represents a standard rate of *in vitro* growth against which the alternative nutrient additions can be compared. **Section B** presents four different polysaccharides present in brown algae: mannitol, laminarin, alginic acid and glucose. **Section C** presents the growth rate effects of the aqueous extract from four seaweed species: *Undaria pinnatifida, Ecklonia radiata, Cystophora torulosa* and *Caulacanthus ustulatus*. **Section D** presents the growth rate effects of a normal amount of vitamin solution in YCFA, versus ten times the normal vitamin solution, versus the vitamin extract from the aqueous extract of *U. pinnatifida*. In each section the growth rates of chosen isolates with the novel nutrient additions are compared with the growth rates with either *K. sydneyanus* gut fluid or *U. pinnatifida* aqueous extract. These are complex and undefined nutrient additions that most closely resemble the nutrient make-up of the environment these bacteria were isolated from the *K. sydneyanus* hindgut. These two media are the media recipes most used to culture *K. sydneyanus* gut bacterial isolates *in vitro* in the laboratory of Clements, et al. YCFA-X control media was also included in all cases.

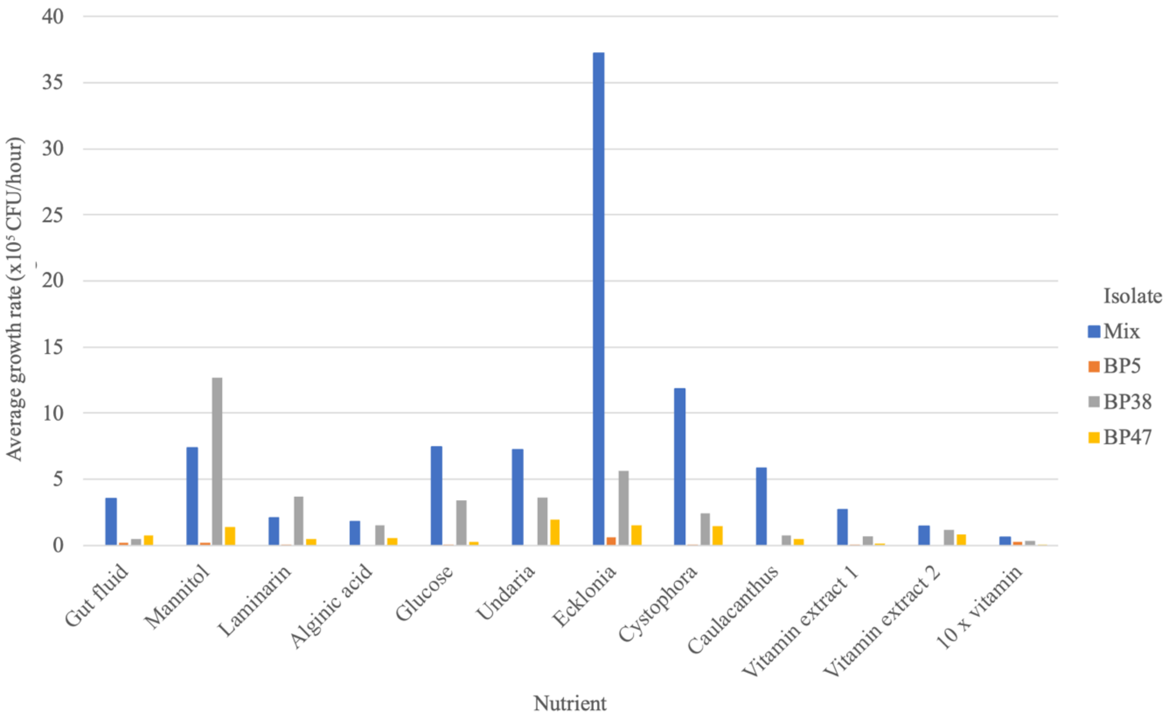


Figure 34: Comparison of mean growth rates (CFU/hour) across all three isolates: *Oscillospiraceae* (BP47G), *Lachnospiraceae* (BP5G), *Rikenellaceae* (BP38) and the Mix of all three. Growth measured on YCFA media with all twelve different YCFA media variations.

The best performing inoculum overall was the mixture of all three species. This shows that the *Rikenellaceae* isolate (BP38), *Oscillospiraceae* isolate (BP47G) and *Lachnospiraceae* isolate (BP5G) can be successfully cultured together. This result also suggests that mutually beneficial digestive mechanisms are shared between these three isolates. This is a major finding of this PhD, the implications of which will be explored in the Discussion Chapter (**Chapter 6**).

Summary of prior growth factor trial results

The preliminary results in Table 8 show that the out of YCFA-*Undaria*, YCFA-Mannitol and YCFA-X media, the nutrient addition with the biggest positive effect of growth is the aqueous extract of *U. pinnatifida*. This is unsurprising as this complex and nutrient rich addition will most resemble the nutrient make-up of the *K. sydneyanus* gut environment from which these bacteria were isolated, as phaeophyceae (brown algae) are the main food source for these herbivorous marine fish, observed at 93.9% of total diet (Rimmer & Wiebe, 1986).

Table 8: Average growth rates in doubling time (*t*) in hours for five isolates (BP5G, KW193, BP104G, KW198, KW194), on three different media: YCFA-*Undaria*, YCFA-Mannitol and YCFA-X.

Medium	**Average doubling time (*t*) in hours for five isolates (BP5G, KW193, BP104G, KW198, KW194)**
YCFA-Undaria	51.3
YCFA-Mannitol	75.3
YCFA-X	130.8

Further previous experimental trials, detailed in **Chapter 4**, found that the vitamin and nucleotide extracts of *U. pinnatifida* produced a faster rate of growth than total *U. pinnatifida* extract for some *K. sydneyanus* gut bacteria (Table 9). These results suggest the vitamin and nucleotide extracts contain growth factor(s) of equal importance to these bacteria as those present in the total aqueous extract of *U. pinnatifida*. These results also suggest that the vitamin growth factor(s) are not one of those already present in the YCFA base recipe, which contains a vitamin solution. This may be due to the vitamin extraction process concentrating the vitamins slightly compared to aqueous extract. These questions are tested in further work within this **Chapter 5**.

Table 9: Doubling time in hours of two fish gut bacterial isolates in normal YCFA media or YCFA supplemented with: the total aqueous extract of *U. pinnatifida*; *U. pinnatifida* vitamin extract; *U. pinnatifida* nucleotide extract; or mannitol. Isolate 5 (*Lachnospiraceae*) was not tested for growth with *U. pinnatifida* nucleotide extract and isolate 52 (*Erysipelotrichaceae*) was not tested for growth with mannitol. Compared to mean doubling time of five isolates (BP5G, KW193, BP104G, KW198, KW194), results from previous work within this PhD.

	Doubling time (t) in hours:		
Extracts added to normal YCFA media	**Isolate BP5G (*Lachnospiraceae*)**	**Isolate BP52G (*Erysipelotrichaceae*)**	**Mean for five isolates (BP5G, KW193, BP104G, KW198, KW194)**
Total U. pinnatifida aqueous extract	66	34	51.3
Vitamin extract from U. pinnatifida aqueous extract	57	30	-
Nucleotide extract from U. pinnatifida aqueous extract	-	51	-
Mannitol (common carbohydrate in U. pinnatifida)	108	-	75.3
None (normal YCFA-X media)	239	239	130.8

The preliminary results detailed in Tables 8 and 9 can be used as a reference against which the results of **Chapter 5** can be compared. These preliminary results show that YCFA-*Undaria* was previously the best performing medium for the culture of *K. sydneyanus* gut bacteria *in vitro*, with an average doubling time across five isolates of 51.3 hours. In **Chapter 5**, various alternative nutrient additions are trialled using the novel growth rate measurement method, developed in this PhD and published as Wornell, et al., (2022). If these nutrient additions have the effect of producing growth rates comparable to the 51.3 hours produced by YCFA-*Undaria*, these nutrient additions must contain growth factors of equal importance to those in the nutritionally complex and undefined *U. pinnatifida* aqueous extract. Therefore, trialled nutrient extracts that produce an average doubling time of less than or equal to that produced by YCFA-*Undaria* are of interest in the search for novel growth factors for the *in vitro* culture of *K. sydneyanus* gut bacteria.

Chapter 5 Section A: Growth rates of *K. sydneyanus* gut bacterial isolates on YCFA media with a nutrient addition of *K. syndneyanus* gut fluid

First, the growth rates of the three *K. sydneyanus* gut bacterial isolates of interest: *Oscillospiraceae* BP47G, *Lachnospiraceae* BP5G and *Rikenellaceae* BP38 were measured on YCFA media with the nutrient addition of *K. sydneyanus* gut fluid. This gut fluid should contain all nutrients required for these bacteria as it is taken from the natural environment of these organisms. Many anaerobic gut bacteria are still cultured in rich complex media of undefined chemical composition (Tramonatano, et al., 2018). A complex and undefined nutrient addition of host gut fluid is often used to isolate these bacteria *in vitro* (Lagier, et al., 2015). Use of media containing gut fluid reduces experimental reproducibility as the nutrient profile varies between individual host organisms, including in fish (Egerton, et al., 2018). Therefore, the use of rich media with gut fluid limits the accuracy of metabolic and physiological investigation of microbes in culture (Tramonatano, et al., 2018).

In this chapter, the results of growth rates of the chosen fish gut bacterial isolates in the presence of various alternative defined and replicable nutrient additions are presented. The results of this growth rates on YCFA-Gut fluid media will be compared to all other alternative nutrient additions that will be investigated for growth factor effects later in this chapter. This comparison will reveal if there are significant growth factors for these bacteria present in these various nutrient additions.

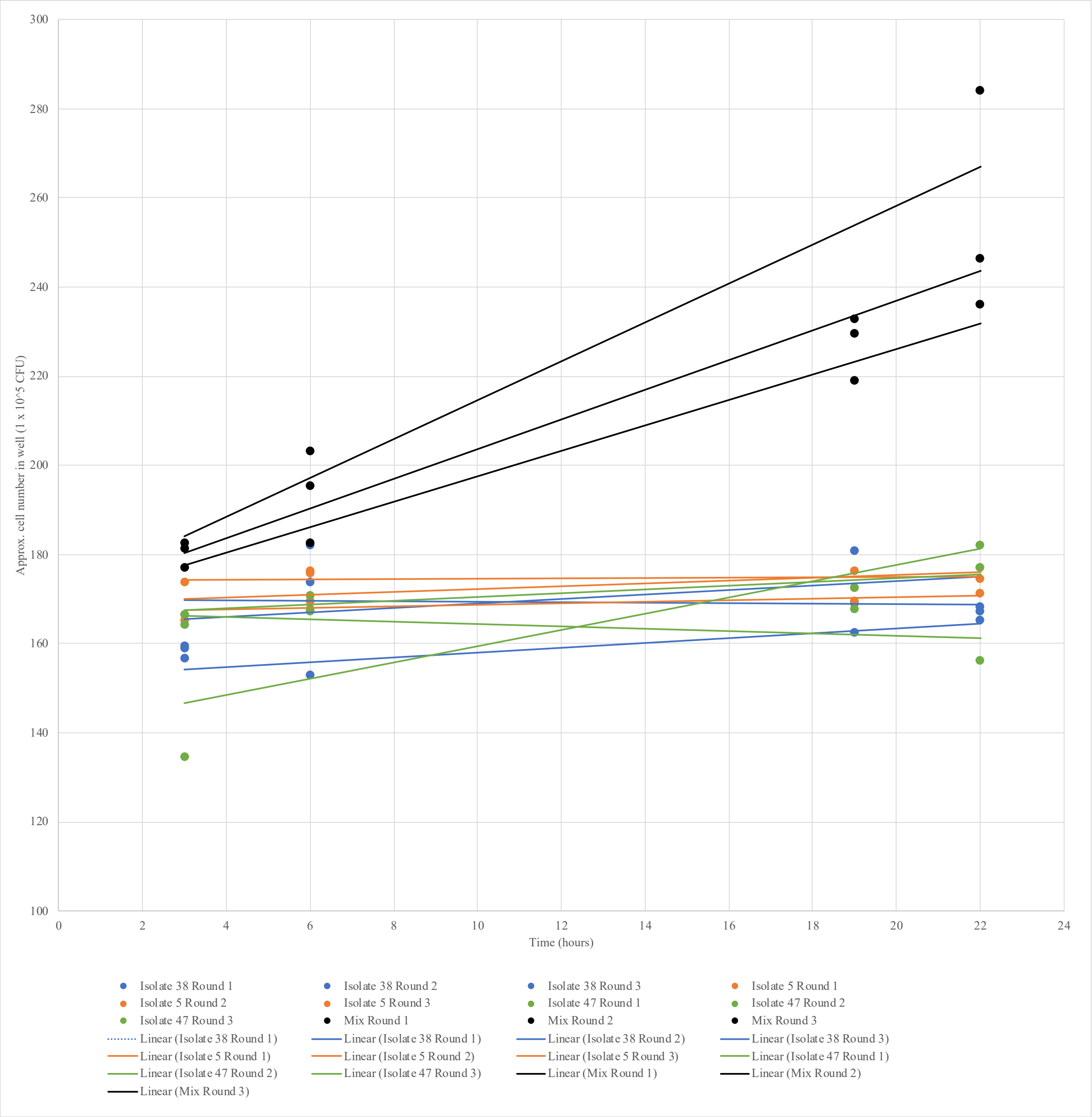


Figure 35: Growth of *Oscillospiraceae* BP47G, *Lachnospiraceae* BP5G and *Rikenellaceae* BP38, and a mix of all three over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on YCFA-Gut fluid media.

The results presented in Figure 35 show that the mix of all three isolates presented the fastest rate of growth with the nutrient addition of *K. sydneyanus* gut fluid to standard YCFA medium. The individual isolates all produced a similar rate of growth to each other, which was lower than that achieved by the mix.

The growth rates, an increase in estimated colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 35. These results are presented in Table 10, along with average growth rates across all three rounds and associated standard deviations. These growth rates will be included in all further tables in this chapter, allowing comparison between trial nutrient additions and *K. sydneyanus* gut fluid.

Table 10: Growth rates for *Oscillospiraceae* BP47G, *Lachnospiraceae* BP5G and *Rikenellaceae* BP38, and a mix of all three over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each bacterium on YCFA-Gut fluid media. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA-Gut fluid	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Rikenellaceae Isolate BP38	1	0.542	0.349	0.303
	2	0.505		
	3	0.000		
Lachnospiraceae Isolate BP5G	1	0.036	0.178	0.145
	2	0.325		
	3	0.173		
Oscillospiraceae Isolate BP47G	1	0.000	0.747	0.955
	2	0.418		
	3	1.823		
Mix	1	3.324	3.513	0.771
	2	4.360		
	3	2.854		

The results in Table 10 show a positive average growth rate for all isolates on the YCFA-GF media. A growth rate of zero is given for *Oscillospiraceae* isolate BP47G Round 1 despite the CFU number in these wells decreasing over time, this is done as there cannot be a negative rate of growth, the growth in this case was zero.

The average growth rates across all three rounds for the *K. sydneyanus* gut fluid nutrient addition, calculated in Table 10, are presented in Figure 36 as a bar chart with standard deviation. Direct comparison of these results in Figure 36 shows that the mix of three isolates had a significantly faster average growth rate on YCFA-Gut fluid media than each of the individual isolates.

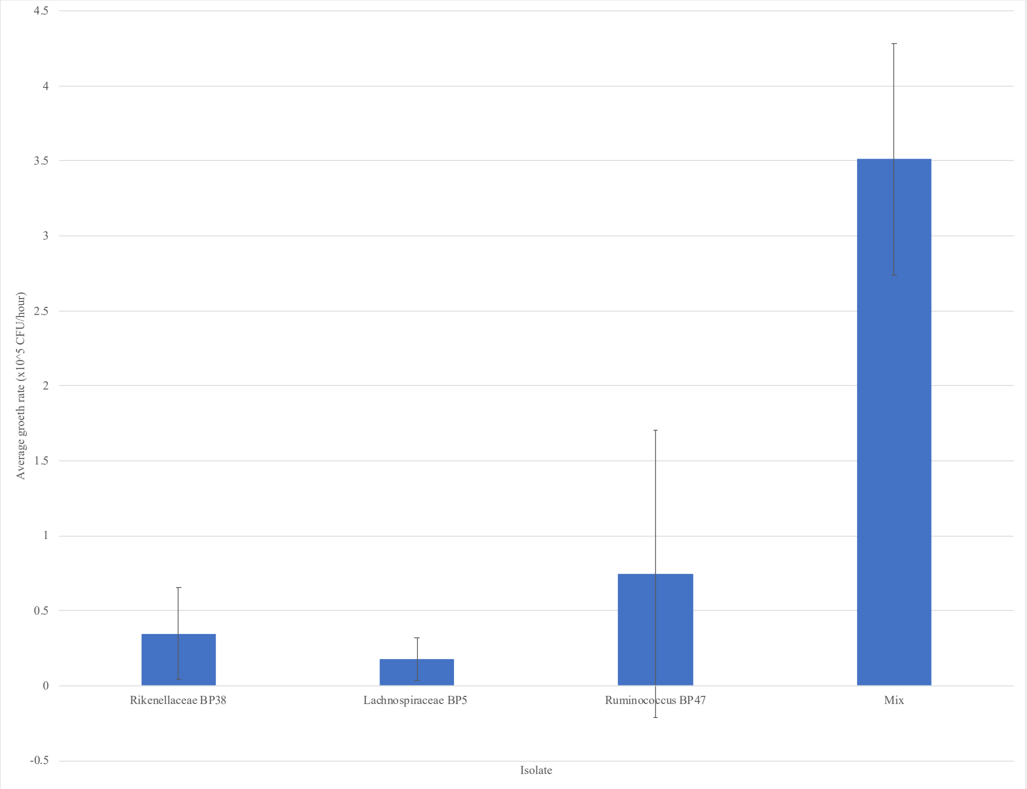


Figure 36: Average growth rates of *Oscillospiraceae* BP47G, *Lachnospiraceae* BP5G and *Rikenellaceae* BP38, and a mix of all three on YCFA-Gut fluid media. Average growth rates

measured by increase in number of colony forming units (CFUs) over time (hours). With standard deviation.

The three individual isolates, *Oscillospiraceae* BP47G, *Lachnospiraceae* BP5G and *Rikenellaceae* BP38, were shown in Figure 36 to not vary significantly in average growth rate on YCFA-Gut fluid media. However, the average growth rate of a mix of all three isolates produced a large increase in average growth rate. The significance of these results will be further investigated using statistical tests. There is a large amount of variation seen across the three rounds of these results, with large standard deviation for all isolates. Particularly for *Oscillospiraceae* isolate BP47G (Table 10) which had one round display no growth. The large amount of variation seen in bacteria growth between replicates reflects the fastidious nature of these organisms. These strict anaerobes are difficult to work with, with unavoidable confounding variables between replicates, such as amount of time between pipetting each replicate, having an outsized effect on growth rate. When these bacteria already struggle to survive *in vitro*, the mission to improve culture conditions is hindered by this poor rate of growth.

The further work within **Sections B, C** and **D** of this chapter trial various alternative defined nutrient additions in a search for growth factors for these *K. sydneyanus* gut bacteria. Some of the polysaccharide, seaweed and vitamin nutrient additions produce a positive impact on isolate growth rate. The growth rates on many of these alternative media are more consistent than the growth rates seen in this **Section A**. Some of these nutrient additions result in a faster rate of growth than that seen in this **Section A** on YCFA-Gut fluid media. These results are presented in the following sections and discussed later in this chapter.

Statistical significance of growth rate results

The mean growth rates of three fish gut bacterial isolates and a mix of all three together were measured on YCFA medium with the nutrient addition of *K. sydneyanus* gut fluid. A one way ANOVA and post hoc Tukey's test on these results was conducted in IBM SPSS Statistics for Macintosh, version 28.0.1 (IBM Corp., Armonk, N.Y., USA). This software was used for all subsequent statistical testing in this chapter.

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were significant differences in growth rates between some variables.

The one way ANOVA was performed to compare the effect of isolate on growth rate in CFU per hour YCFA medium during culture on YCFA medium with *K. sydneyanus* gut fluid.

The one way ANOVA revealed a statistically significant difference in mean growth rate between at least two isolates tested (F(3, 8) = [18.099], p = <0.001). Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between a mix of all three isolates and all three individual isolates: isolate BP5G (p = <0.001, 95% C.I. = [1.6715, 4.998]), isolate BP38 (p = 0.001, 95% C.I. = [1.501, 4.827]) and isolate BP47G (p = 0.003, 95% C.I. = [1.103, 4.429]).

There was no statistically significant difference in growth rate between isolates BP5G and BP38 (p = 0.987), isolates BP5G and BP47G (p = 0.702), or isolates BP38 and BP47G (p = 0.867).

This analysis shows that the mix of all three isolates had a significantly increased mean growth rate compared to all three individual isolates. The mean growth rates of isolates BP5G, BP38 and BP47G were not significantly different from each other at the 0.05 level.

Chapter 5 Section B: Variable polysaccharide utilisation by bacteria isolated from the same *Kyphosus sydneyanus* hindgut section

The microbial community of the fish gut is strictly anaerobic and therefore difficult to manipulate *in vitro*. This has caused a lack of functional studies on these bacteria. This study is the first to demonstrate the functional polysaccharide preference of three bacteria derived from the fish gut. Novel methodology developed within this PhD was used in this chapter to measure the growth rate of hindgut bacteria in YCFA media with four different polysaccharide nutrient additions. Three bacterial isolates from the hindgut of the herbivorous marine fish *Kyphosus sydneyanus* were used in these experiments: *Oscillospiraceae* BP47G, *Lachnospiraceae* BP5G and *Rikenellaceae* BP38. A mixture of all three of these isolates was also used, in the first study to investigate *in vitro* mutualistic relationships between fish gut bacteria species.

The polysaccharide nutrient additions of mannitol, laminarin, alginic acid and glucose were chosen due to their presence in brown algae, a major dietary component of the host fish *K. sydneyanus*. For these reasons, these polysaccharides were used as nutrient additions to YCFA media in these experiments to measure nutrient growth rate effects.

Rikenellaceae isolate BP38

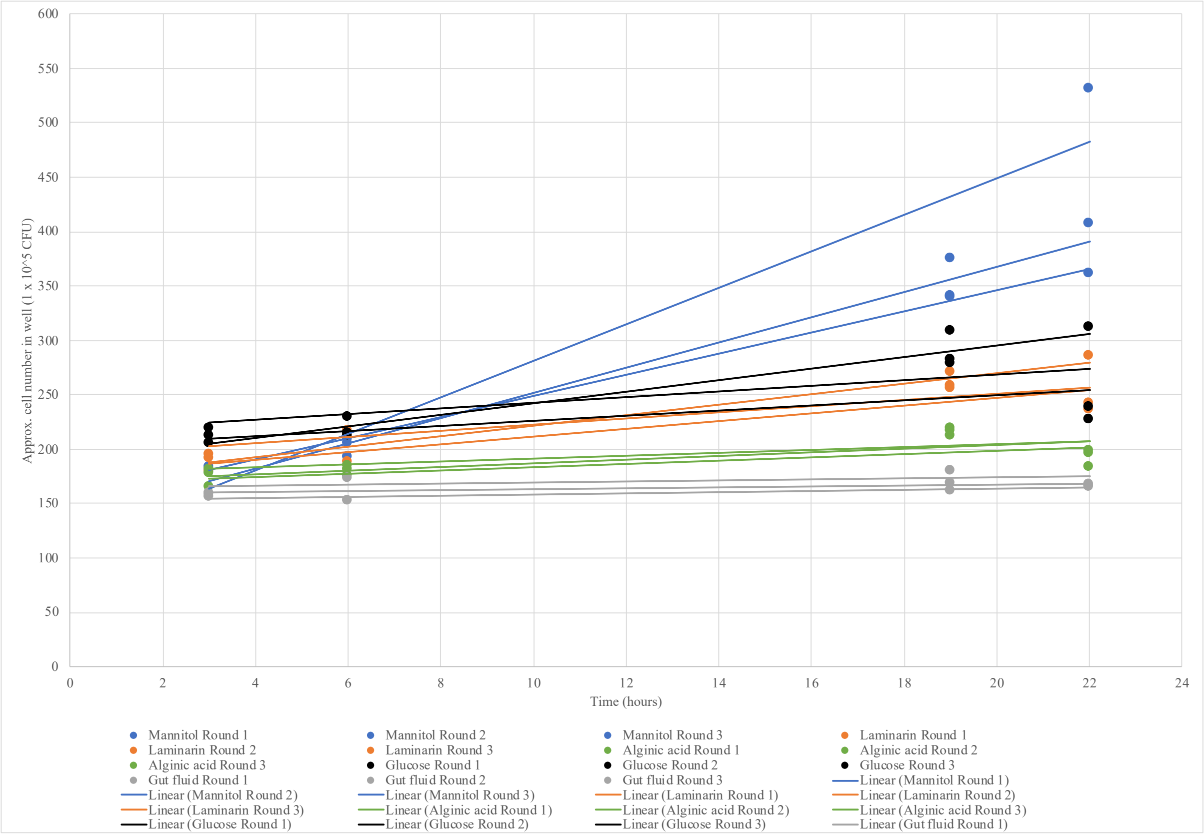


Figure 37: Growth of *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid.

The results presented in Figure 37 show that *Rikenellaceae* isolate BP38 presented the fastest rate of growth with the nutrient addition of mannitol to standard YCFA medium. The glucose and laminarin produced a similar rate of growth to each other, which was slower than that produced by mannitol. The nutrient addition of alginic acid produced the slowest rate of growth out of all polysaccharides tested in this experiment.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 37. These results are presented in Table 11, along with average growth rates across all three rounds and associated standard deviations.

Table 11: Growth rates for *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Mannitol	1	9.703	12.697	3.661
	2	16.778		
	3	11.609		
Laminarin	1	2.818	3.726	1.028
	2	3.519		
	3	4.842		
Alginic acid	1	1.548	1.507	0.177
	2	1.660		
	3	1.313		
Glucose	1	2.644	3.429	1.630
	2	5.303		
	3	2.340		
Gut fluid	1	0.542	0.349	0.303
	2	0.505		
	3	0.000		

The average growth rates across all three rounds for each media nutrient addition, calculated in Table 11 show a positive average growth rate for *Rikenellaceae* isolate BP38 on all media. The results in Table 11 are presented in Figure 38 as a bar chart with standard deviation.

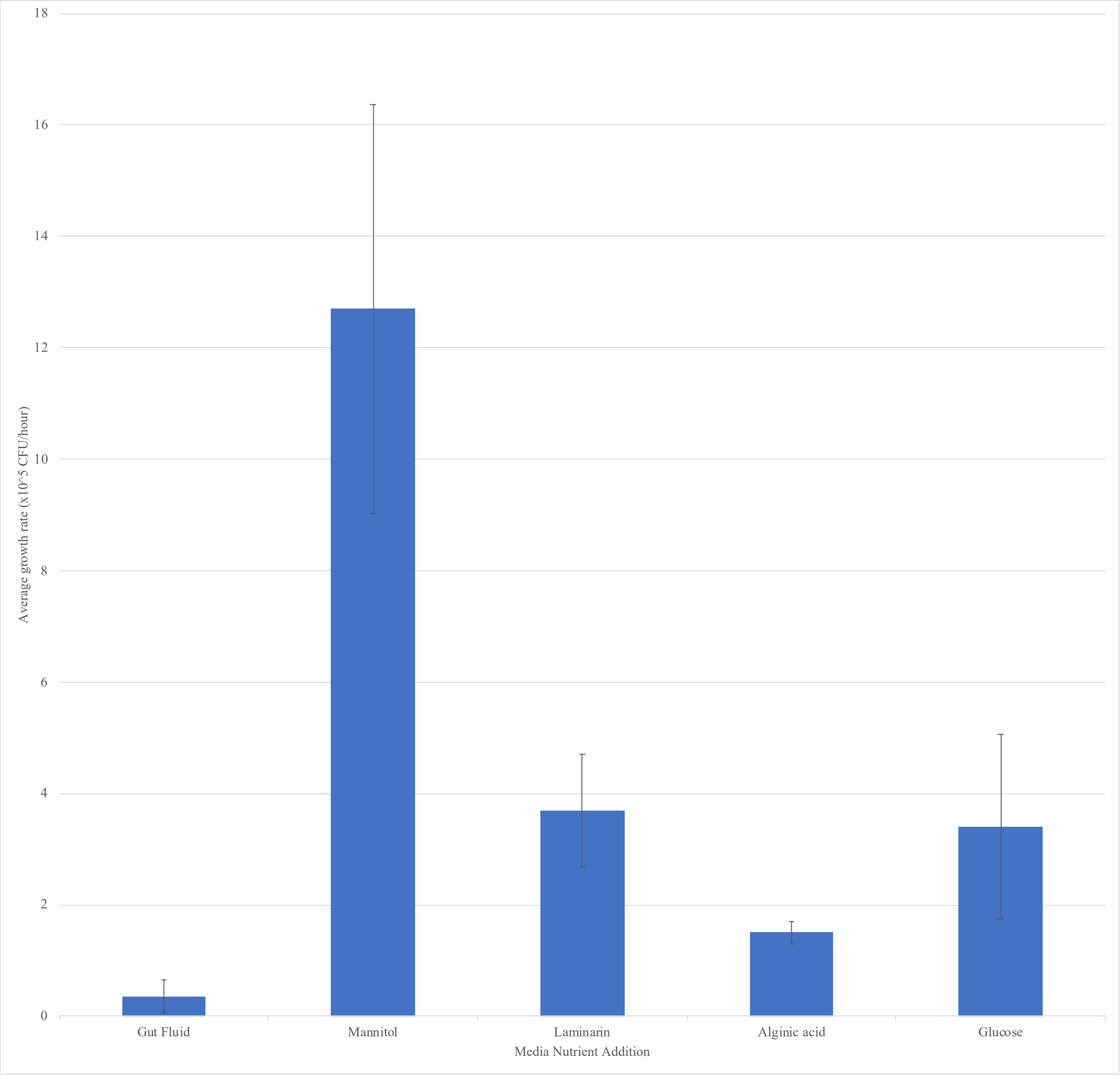


Figure 38: Average growth rate of *Rikenellaceae* isolate BP38, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. With standard deviation.

The average growth rate of *Rikenellaceae* isolate BP38 was shown in Figure 38 to increase with the nutrient addition of mannitol compared to all other nutrient additions. All nutrient additions produced a faster average growth rate in *Rikenellaceae* isolate BP38 than gut fluid

(Figure 38). The significance of these differences will be further investigated using statistical tests. These results suggest that for *Rikenellaceae* isolate BP38, all of these polysaccharides can be used in YCFA as alternative nutrient additions to gut fluid.

Statistical significance of growth rate results

The mean growth rates of three fish gut bacterial isolates and a mix of all three together were measured on YCFA medium with the nutrient additions of: mannitol, laminarin, alginic acid, glucose and *K. sydneyanus* gut fluid. A one way ANOVA and post hoc Tukey's test on these results was conducted in IBM SPSS Statistics for Macintosh, version 28.0.1 (IBM Corp., Armonk, N.Y., USA).

The one way ANOVA was followed by a post hoc Tukey test to compare the effects of nutrient additions to YCFA medium on the growth rate of isolate BP38 in CFU per hour. These tests found that there were significant differences in growth rates between some variables.

The one way ANOVA revealed a statistically significant difference in mean growth rate between at least two nutrients tested (F(4, 10) = [20.510], p = <0.001). Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between mannitol and all four other nutrient additions: laminarin (p = 0.001, 95% C.I. = [3.987, 14.013]), alginic acid (p = <0.001, 95% C.I. = [6.187, 16.213]), glucose (p = <0.001, 95% C.I. = [4.287, 14.313]) and gut fluid (p = <0.001, 95% C.I. = [7.338, 17.364]).

There was no statistically significant difference in growth rate between any of the other nutrient additions to YCFA medium: laminarin and alginic acid (p = 0.616), laminarin and glucose (p = 1.000), laminarin and gut fluid (p = 0.254), alginic acid and glucose (p = 0.726), alginic acid and gut fluid (p = 0.938), glucose and laminarin (p = 1.000), glucose and alginic acid (p = 0.726) or glucose and gut fluid (p = 0.330).

The results of the one way ANOVA and post hoc Tukey test show that only the YCFA medium with mannitol added caused a significantly increased mean rate of growth for isolate BP38. With a significant difference value of ≤0.001 compared to all four other nutrient additions. The mean growth rates of YCFA with laminarin, alginic acid, glucose and *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level.

Summary

All four polysaccharide additions to YCFA medium produced a growth rate of *Rikenellaceae* isolate BP38 that was equal to or exceeding that of gut fluid. Therefore, alternative defined media have been discovered for this isolate. Mannitol produced a significantly faster growth rate then gut fluid (p = <0.001). Laminarin, alginic acid and glucose produced growth rates that were faster than gut fluid on average, but not significantly different at the 0.05 level. Therefore, for *Rikenellaceae* isolate BP38 the optimal defined medium tested is YCFA with mannitol, with an average growth rate of 12.697 (±3.661) x 10^5 CFU/hour however all tested polysaccharides may be used in an alternative YCFA media for this isolate.

Lachnospiraceae isolate BP5G

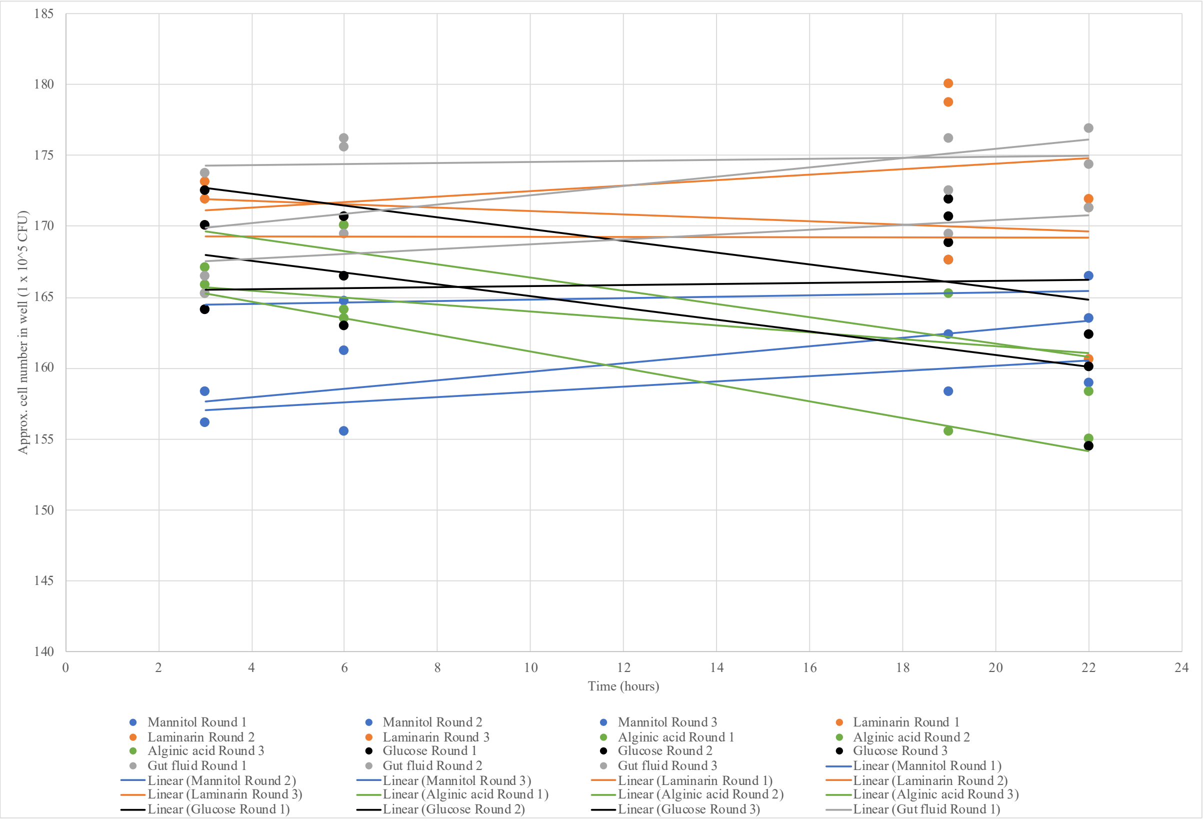


Figure 39: Growth of *Lachnospiraceae* isolate BP5G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid.

The results presented in Figure 39 show that *Lachnospiraceae* isolate BP5G presented a similar rate of growth with all five of these polysaccharide nutrient additions to standard YCFA medium.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 39.

These results are presented in Table 12, along with average growth rates across all three rounds and associated standard deviations.

Table 12: Growth rates for *Lachnospiraceae* isolate BP5G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Mannitol	1	0.301	0.180	0.125
	2	0.187		
	3	0.052		
Laminarin	1	0.000	0.064	0.110
	2	0.191		
	3	0.000		
Alginic acid	1	0.000	0.000	0.000
	2	0.000		
	3	0.000		
Glucose	1	0.041	0.014	0.024
	2	0.000		
	3	0.000		
Gut fluid	1	0.036	0.178	0.145
	2	0.325		
	3	0.173		

The results in Table 12 show a positive average growth rate for *Lachnospiraceae* isolate BP5G on all media except YCFA media with alginic acid, which showed an average growth rate of zero. The average growth rates across all three rounds for each media nutrient addition, calculated in Table 12, are presented in Figure 40 as a bar chart with standard deviation.

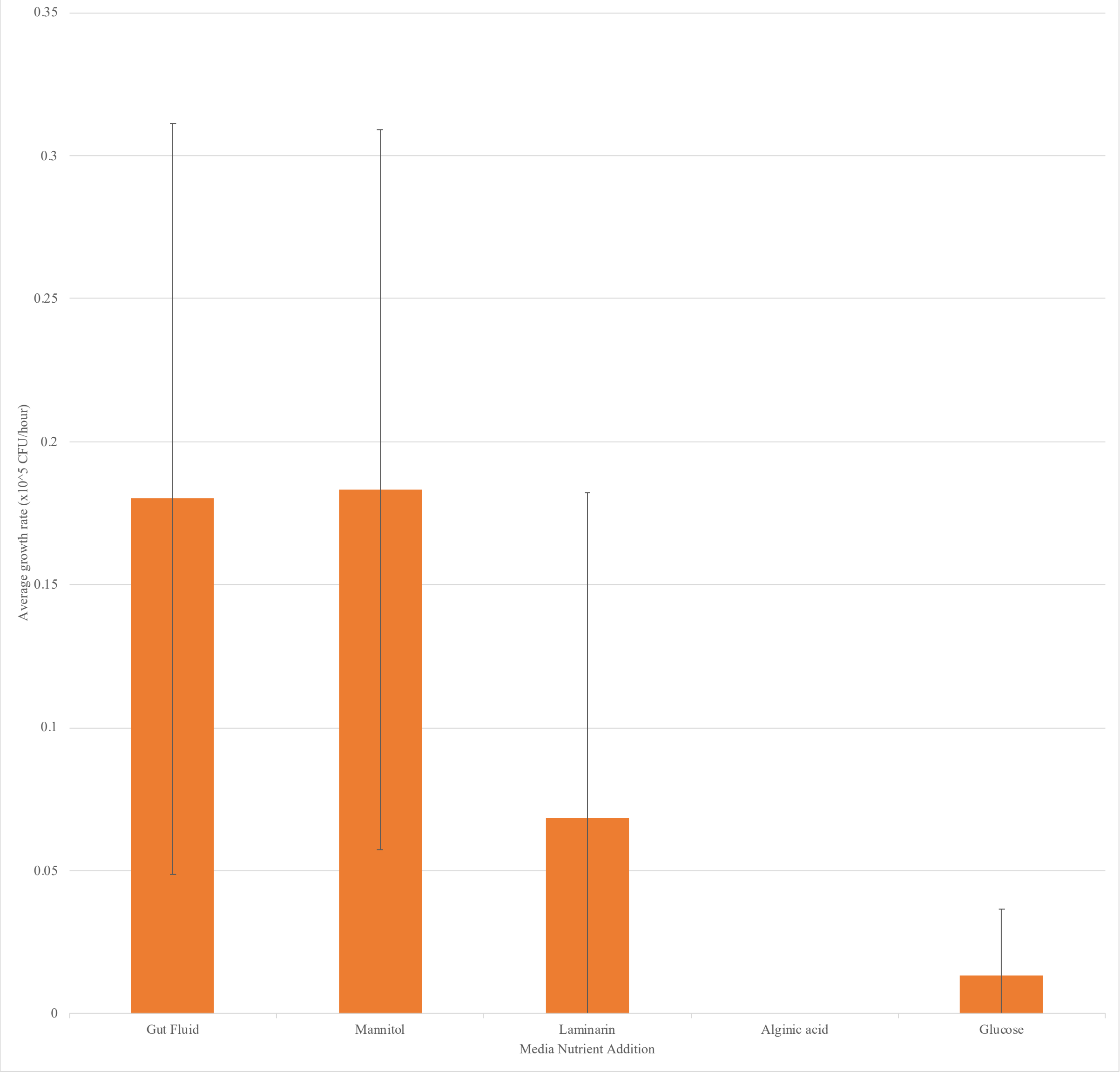


Figure 40: Average growth rate of *Lachnospiraceae* isolate BP5G, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. With standard deviation.

The results displayed in Figure 40 show that the growth rates of *Lachnospiraceae* isolate BP5G on YCFA medium with all four nutrient additions had a large amount of variation between replicates. The four polysaccharide nutrient additions produced an equal or slower average growth rate to that produced with gut fluid. The significance of these differences will be further investigated using statistical tests. The results in Figure 40 suggest that none of these alternative defined media recipes are viable alternatives to YCFA with gut fluid for *Lachnospiraceae* isolate BP5G.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were no significant differences in growth rates between any of these variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of isolate BP5G in CFU per hour.

The one way ANOVA revealed that there was not a statistically significant difference at the 0.05 level in mean growth rate between any of the nutrients tested (F(4, 10) = [2.516], p = 0.108).

The results of the one way ANOVA and post hoc Tukey test show that none of the nutrients added to YCFA medium caused a significantly different mean rate of growth for isolate BP38. The mean growth rates of YCFA with mannitol, laminarin, alginic acid, glucose and *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level.

Summary

The four polysaccharide additions to YCFA medium produced a growth rate of *Lachnospiraceae* isolate BP5G that was not statistically different to that of gut fluid at the 0.05 level. Mannitol produced the fastest average growth rate, which at 0.180 (± 0.125) x 10^5

CFU/hour was not statistically different at the 0.05 level to that of gut fluid 0.178 (± 0.145) x 10^5 CFU/hour. Therefore, YCFA with mannitol is an alternative defined media for this isolate. Laminarin, alginic acid and glucose produced growth rates that were not statistically different to that of gut fluid on average. Therefore, for *Lachnospiraceae* isolate BP5G the optimal defined medium tested is YCFA with mannitol, with an average growth rate of 0.180 (± 0.125) x 10^5 CFU/hour however all tested polysaccharides may be used in an alternative YCFA media for this isolate.

Oscillospiraceae isolate BP47G

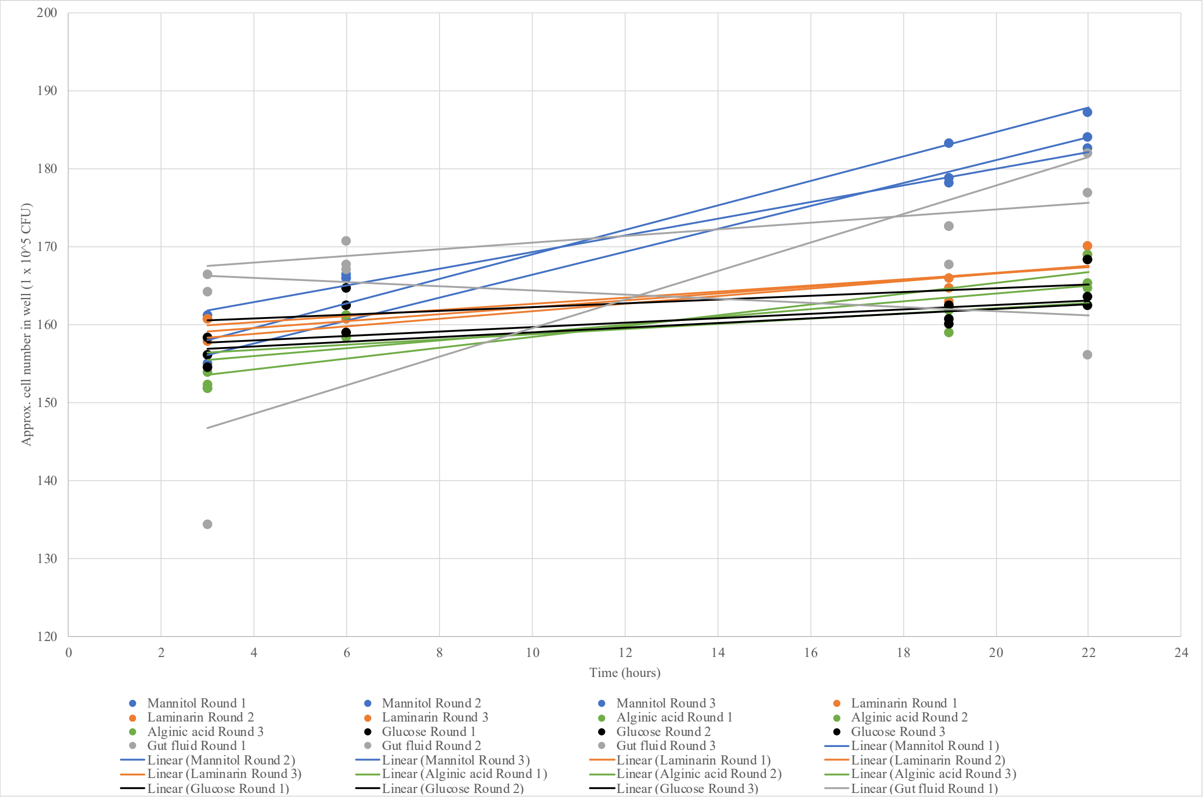


Figure 41: Growth of *Oscillospiraceae* isolate BP47G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid.

The results presented in Figure 41 show that *Oscillospiraceae* isolate BP47G presented the fastest rate of growth with the nutrient addition of mannitol to standard YCFA medium. The glucose, alginic acid and laminarin additions produced a similar rate of growth to each other, which was slower than that produced by mannitol. The nutrient addition of *K. sydneyanus* gut fluid produced a median rate of growth compared to other additions tested in this experiment.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 41. These results are presented in Table 13, along with average growth rates across all three rounds and associated standard deviations.

Table 13: Growth rates for *Oscillospiraceae* isolate BP47G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Mannitol	1	1.470	1.368	0.265
	2	1.068		
	3	1.567		
Laminarin	1	0.394	0.440	0.044
	2	0.443		
	3	0.482		
Alginic acid	1	0.331	0.508	0.184
	2	0.698		
	3	0.494		
Glucose	1	0.283	0.277	0.033
	2	0.241		
	3	0.307		
Gut fluid	1	0.000	0.747	0.955

2	0.418		
3	1.823		

The results in Table 13 show a positive average growth rate for *Oscillospiraceae* isolate BP47G on all media. The average growth rates across all three rounds for each media nutrient addition, calculated in Table 13, are presented in Figure 42 as a bar chart with standard deviation.

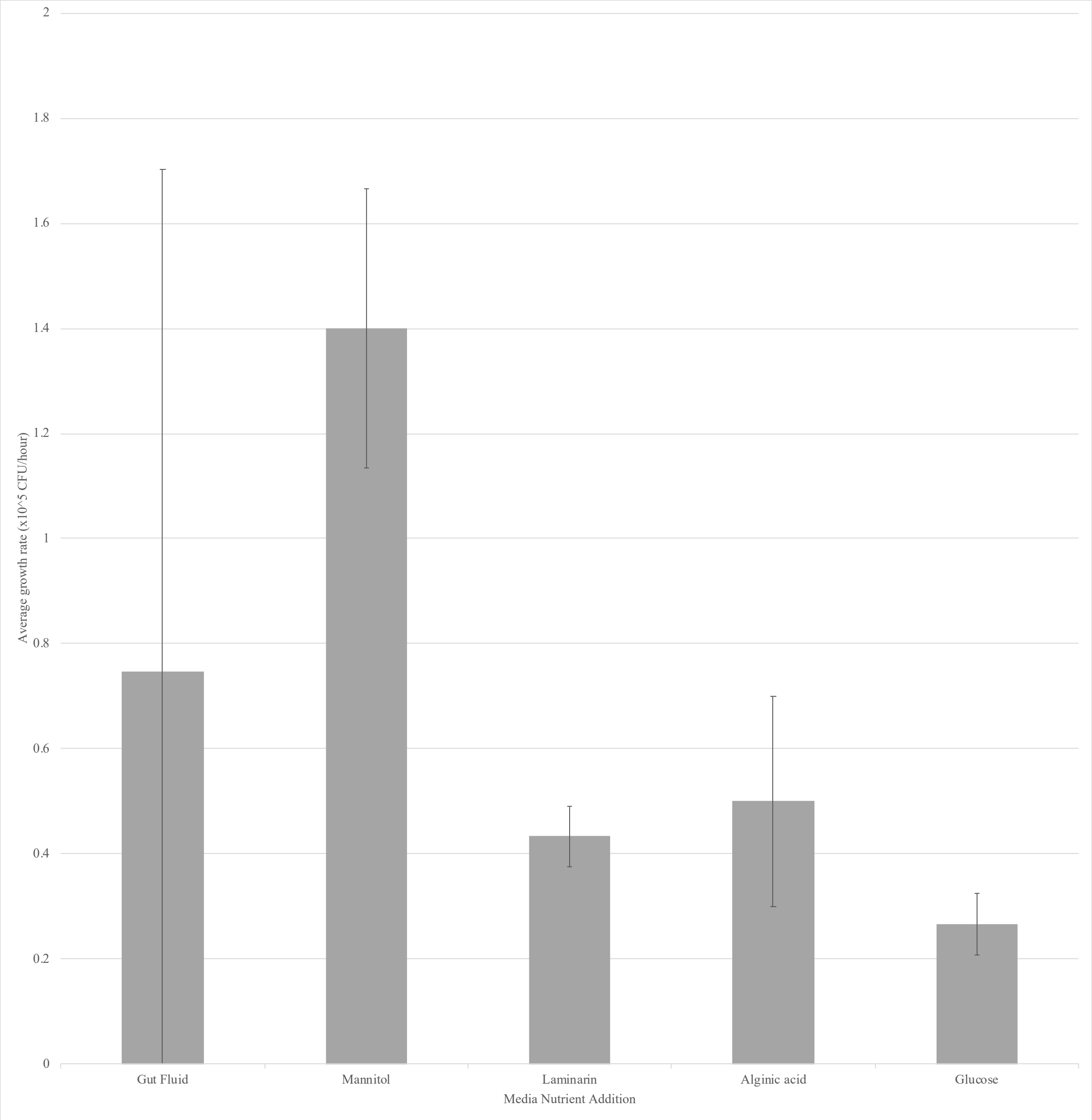


Figure 42: Average growth rate of *Oscillospiraceae* isolate BP47G, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. With standard deviation.

The average growth rate of *Oscillospiraceae* isolate BP47G was shown in Figure 42 to increase with the nutrient addition of mannitol compared to all other nutrient additions. All other nutrient additions produced a slower average growth rate in *Oscillospiraceae* isolate BP47G than gut fluid (Figure 42). The significance of these differences will be further investigated using statistical tests. These results suggest that for *Oscillospiraceae* isolate BP47G, only mannitol can be used as an alternative to the gut fluid nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were no significant differences in growth rates between any of these variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of isolate BP47G in CFU per hour.

The one way ANOVA revealed that there was not a statistically significant difference at the 0.05 level in mean growth rate between any of the nutrients tested (F(4, 10) = [2.913], p = 0.078).

Summary

The four polysaccharide additions to YCFA medium produced a growth rate of *Oscillospiraceae* isolate BP47G that was not statistically different to that of gut fluid. The mannitol addition produced the fastest average growth rate, which at 1.386 (± 0.265) x 10^5 CFU/hour was faster that of gut fluid at 0.747 (± 0.955) x 10^5 CFU/hour. Therefore, YCFA with mannitol is the best option for an alternative defined media for this isolate. Laminarin, alginic acid and glucose produced growth rates that were not statistically different to that of gut fluid on average. Therefore, for *Oscillospiraceae* isolate BP47G the optimal defined medium tested is YCFA with mannitol, with an average growth rate of 1.386 (± 0.265) x 10^5 CFU/hour, however all tested polysaccharides may be used in an alternative YCFA media for this isolate.

Mixture of all three isolates (BP47G, BP5G and BP38)

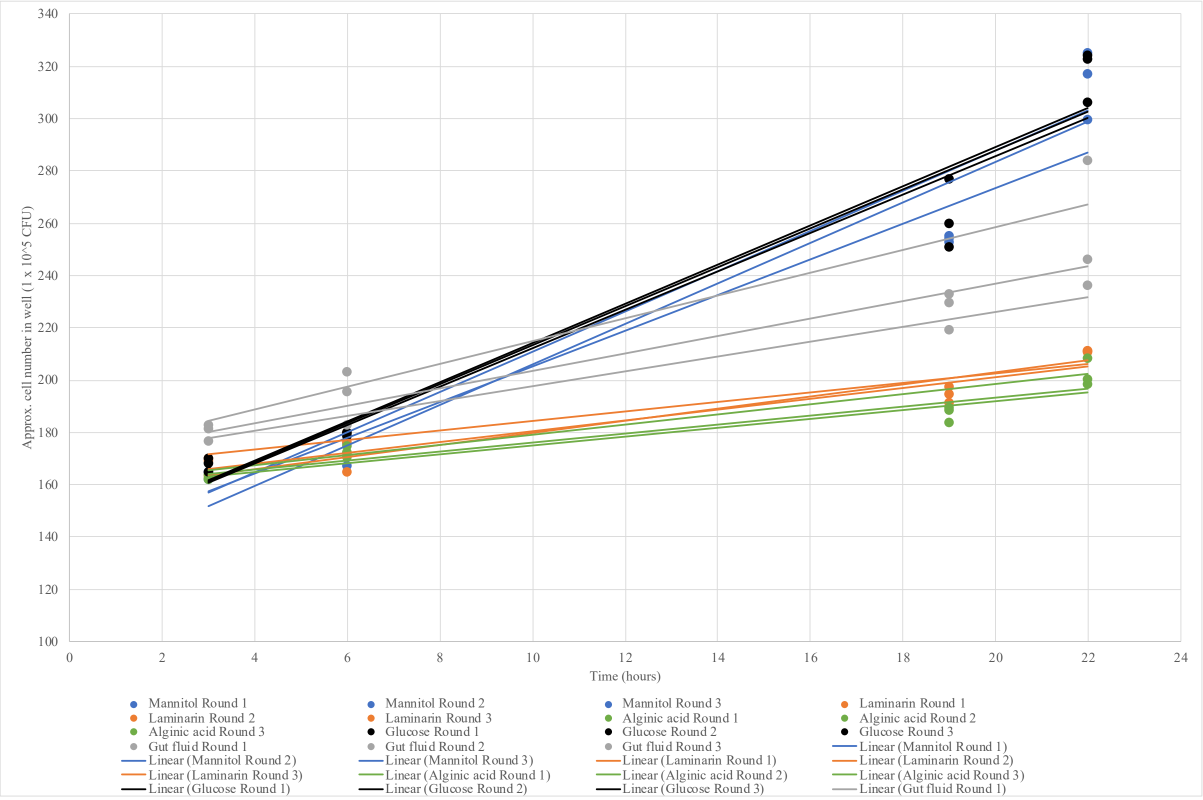


Figure 43: Growth of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid.

The results presented in Figure 43 show that a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 presented the fastest rate of growth with the nutrient additions of mannitol or glucose to standard YCFA medium. The *K. sydneyanus* gut fluid addition produced a median rate of growth. The alginic and laminarin additions produced a similar rate of growth to each other, which was slower than that produced by the other three additions tested in this experiment.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 43. These results are presented in Table 14, along with average growth rates across all three rounds and associated standard deviations.

Table 14: Growth rates for a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Mannitol	1	7.737	7.414	0.518
	2	7.688		
	3	6.817		
Laminarin	1	2.328	2.075	0.258
	2	1.813		
	3	2.083		
Alginic acid	1	1.687	1.775	0.127
	2	1.920		
	3	1.717		
Glucose	1	7.337	7.419	0.072
	2	7.470		
	3	7.451		
Gut fluid	1	3.324	3.513	0.771
	2	4.360		
	3	2.854		

The results in Table 14 show a positive average growth rate for a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 on all media.

The average growth rates across all three rounds for each media nutrient addition, calculated in Table 14, are presented in Figure 44 as a bar chart with standard deviation.

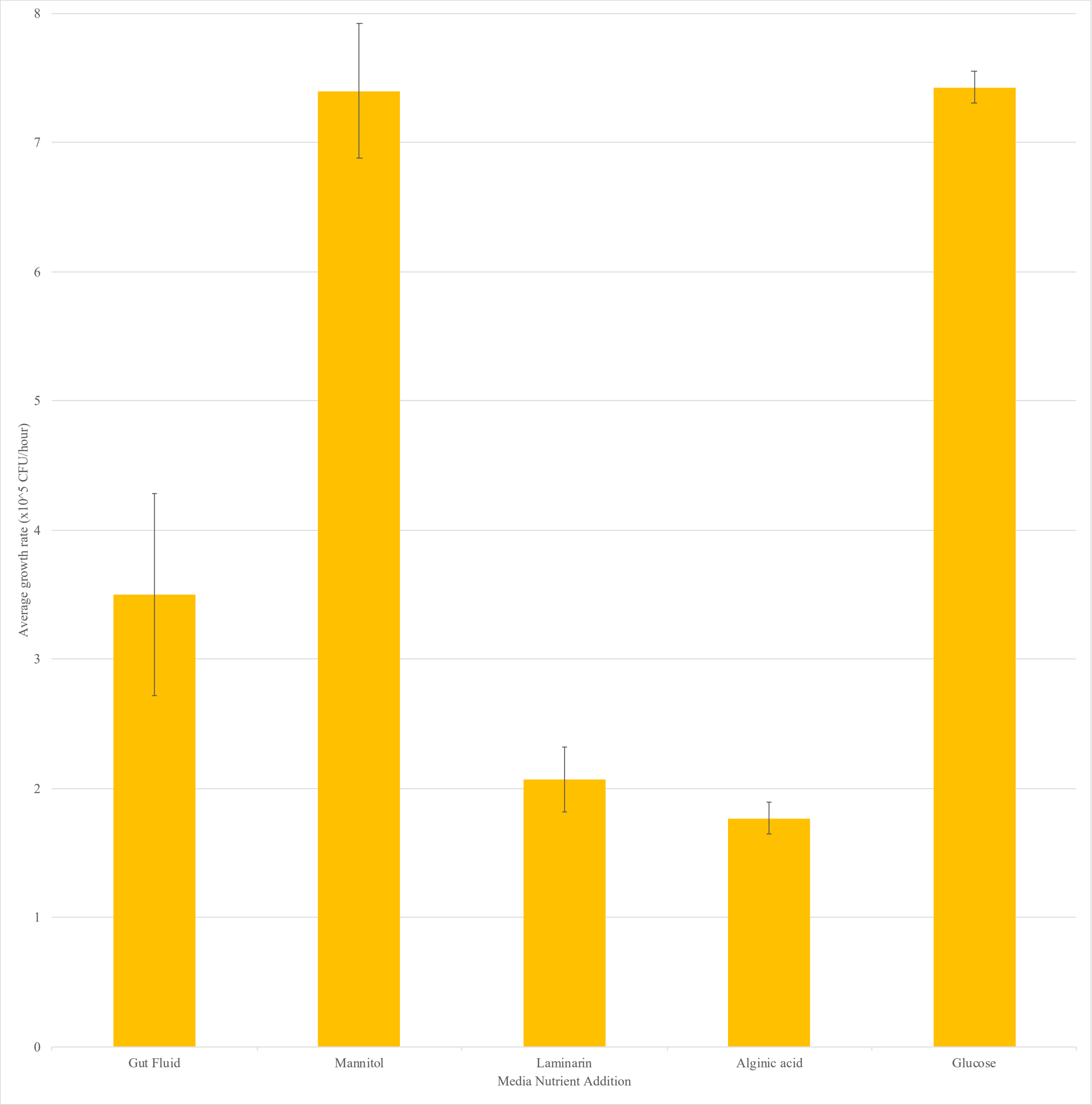


Figure 44: Average growth rate of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5 and *Rikenellaceae* isolate BP38, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. With standard deviation.

The average growth rate of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 was shown in Figure 44 to increase with the nutrient addition of both mannitol and glucose compared to all other nutrient additions. The laminarin and alginic acid nutrient additions both produced a slower average growth rate than gut fluid for a mix of all three isolates (Figure 44). The significance of these differences will be further investigated using statistical tests. These results suggest that for a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 both mannitol and glucose can be used as an alternative to the gut fluid nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were significant differences in growth rates between some variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 in CFU per hour.

The one way ANOVA revealed a statistically significant difference in mean growth rate between at least two nutrients tested (F(4, 10) = [121.933], p = <0.001).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between mannitol and three other nutrient additions: laminarin (p =

<0.001, 95% C.I. = [4.154, 6.513]), alginic acid (p = <0.001, 95% C.I. = [4.454, 6.813]), and gut fluid (p = <0.001, 95% C.I. = [2.687, 5.046]).

Tukey's HSD test for multiple comparisons also found that the mean value of growth rate was significantly different between glucose and three other nutrient additions: laminarin (p = <0.001, 95% C.I. = [4.187, 6.546]), alginic acid (p = <0.001, 95% C.I. = [4.487, 6.846]), and gut fluid (p = <0.001, 95% C.I. = [2.721, 5.080]).

Tukey's HSD test for multiple comparisons also found that the mean value of growth rate was significantly different between gut fluid and all four other nutrient additions: mannitol (p = <0.001, 95% C.I. = [-5.046, -2.687]), laminarin (p = 0.015, 95% C.I. = [0.287, 2.646]), alginic acid (p = 0.004, 95% C.I. = [0.587, 2.946]), and glucose (p = <0.001, 95% C.I. = [-5.080, -2.721]).

There was no statistically significant difference in growth rate between mannitol and glucose (p = 1.000) or laminarin and alginic acid (p = 0.913).

The results of the one way ANOVA and post hoc Tukey test show that YCFA medium with mannitol or glucose added caused the fastest mean rate of growth for isolate BP38 with a significant increase of ≤0.001 for these two polysaccharides compared to all three other nutrient additions. The growth rates for these two additions were not significantly different from each other at the 0.05 level. The mean growth rates of YCFA with laminarin or alginic acid caused the lowest growth rates, which were not significantly different from each other at the 0.05 level. The addition of *K. sydneyanus* gut fluid to YCFA caused a growth rate significantly faster that laminarin or alginic acid, but significantly slower that glucose or mannitol, all at the 0.05 level.

Summary

The growth rates of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 on YCFA medium with all four polysaccharide additions was statistically different to that of gut fluid. Mannitol and glucose produced the fastest average growth rates, which at 7.414 (± 0.518) x 10^5 CFU/hour for mannitol and 7.419 (± 0.072) x 10^5 CFU/hour were not significantly different from each other. These rates were also significantly faster that of gut fluid at 3.513 (± 0.771) x 10^5 CFU/hour. Therefore, YCFA with mannitol or glucose are the best options for an alternative defined media for this isolate. Laminarin and alginic acid produced growth rates that were significantly reduced in comparison to that of gut fluid. Therefore, for the mix of three isolates the optimal defined medium tested is YCFA with or glucose, with an average with the other tested polysaccharides producing significantly inferior growth.

All isolates polysaccharide utilisation

This summary allows comparison of all growth rate results for fish gut bacterial isolates cultured on YCFA media with the addition of one of four polysaccharides or *K. sydneyanus* gut fluid. These results are displayed in Figure 45 and analysed for significance in further statistical tests. This analysis reveals multiple viable defined and simplified alternative to the complex YCFA-gut fluid media for the *in vitro* culture of some fish gut bacterial isolates.

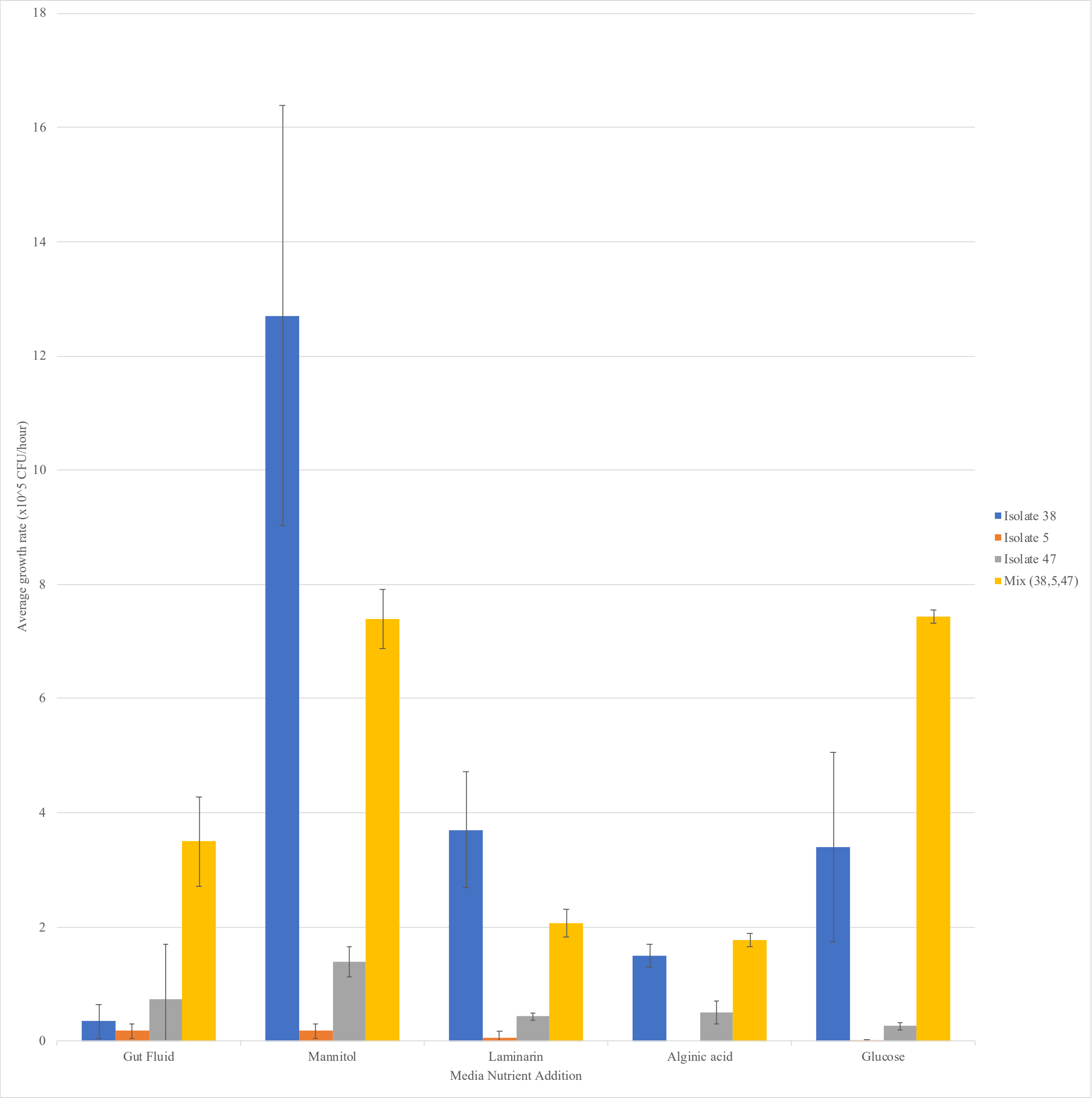


Figure 45: Average growth rates of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G, *Rikenellaceae* isolate BP38 and a mix of all three measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with mannitol, laminarin, acrylic acid, glucose or gut fluid. With standard deviation.

Figure 45 allows the comparison of average growth rates on each variation of YCFA media for all isolates, including *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G,

Rikenellaceae BP38 and a mix of all three. This cross-isolate comparison reveals that the fastest average growth rate was achieved by *Rikenellaceae* BP38 on YCFA with mannitol. The comparison in Figure 45 reveals that *Rikenellaceae* BP38 produced the fastest average growth rate of all the isolates on both YCFA with mannitol and laminarin. Whereas the mix of all three isolates grew produced the fastest average growth rate of all the isolates on glucose and gut fluid. Isolate BP38 and a mix of all three isolates performed better than isolate BP47G and isolate BP5G on all media except YCFA with *K. sydneyanus* gut fluid, where isolate BP47G had a similar growth rate to BP38. Overall, Figure 45 shows that YCFA with mannitol presents the most successful media alternative to YCFA with gut fluid, as all isolates presented successful growth. Also, the highest average growth rate was met for all isolates with this medium, higher or equal to that seen on YCFA with gut fluid. The significance of these differences will be further investigated using statistical tests. These results suggest that for all isolates mannitol can be used as an alternative to the gut fluid nutrient addition to YCFA media.

Statistical significance of growth rate results

A two-way ANOVA was performed to analyse the effect of both isolate and polysaccharide (or gut fluid) nutrient addition on mean value growth rate.

The two-way ANOVA revealed that there was a statistically significant interaction at the 0.05 level between the effects of both isolate and nutrient addition on growth rate (F(12, 41) = [18.874], p = <0.001).

Simple main effects analysis showed that isolate had a significant effect on growth rate (p = <0.001). A post-hoc Tukey’s HSD test for multiple comparisons found that the mean value growth rate was significantly different between: a mix of all three isolates and isolate BP5G (p = <0.001, 95% C.I. [3.387, 5.315]), a mix of all three isolates and isolate BP47G (p = <0.001, 95% C.I. [2.809, 4.738]), isolate BP5G and isolate BP38 (p = <0.001, 95% C.I. [-5.315, -3.387]), isolate BP38 and isolate BP47G (p = <0.001, 95% C.I. [2.696, 4.624]). There was no significant difference in mean value growth rate between a mix of all three isolates and isolate BP38 (p = 0.989) or isolate BP5G and isolate BP47G (p = 0.387).

Simple main effects analysis showed that nutrient addition also had a significant effect on mean growth rate across all isolates (p = <0.001). A post-hoc Tukey's HSD test for multiple comparisons found that the mean value growth rate was significantly different between mannitol and all other nutrient additions: gut fluid (p = <0.001, 95% C.I. = [3.077, 5.374]), laminarin (p = <0.001, 95% C.I. = [2.705, 5.003]), alginic acid (p = <0.001, 95% C.I. = [3.330, 5.628]) and glucose (p = <0.001, 95% C.I. = [1.494, 3.791]). There was also a significant difference between alginic acid and glucose (p = <0.001, 95% C.I. = [-2.985, -0.688]) and glucose and laminarin (p = 0.034, 95% C.I. = [0.063, 2.360]) and gut fluid and glucose (p = 0.003, 95% C.I. = [-2.732, -0.402]). There was no significant difference in mean value growth rate between gut fluid and laminarin (p = 0.885), gut fluid and alginic acid (p = 0.969), laminarin and alginic acid (p = 0.534).

Summary

Post-hoc Tukey's testing of the isolates in this experiment found that across all YCFA media variations the *Rikenellaceae* isolate BP38 and a mix of all three isolates grew significantly faster than both *Oscillospiraceae* isolate BP47G and *Lachnospiraceae* isolate BP5G (p =

<0.001). With the rate of growth within these two groups of isolates being significantly different from each other at the 0.05 level.

Post-hoc Tukey's testing also found that YCFA with mannitol, glucose, laminarin or alginic acid can be used as alternative media to YCFA with gut fluid for the *in vitro* cultivation of these *K. sydneyanus* gut bacterial isolates as the average growth rate across all isolates in these simple and defined media alternatives is equal to or exceeding that of YCFA with gut fluid. The mannitol addition to YCFA media produced a significantly faster rate of growth than all other nutrient additions. Glucose produced a significantly faster growth rate than gut fluid, which was slower than that caused by mannitol. The growth rate across all isolates on YCFA media with laminarin and alginic acid was not significantly different to gut fluid. Therefore, these results show YCFA with mannitol produces the best growth for these bacteria, YCFA with glucose the second best, and YCFA with alginic acid or laminarin can also be used as an alternative to gut fluid. Therefore, these experiments have produced four new alternative defined media recipes that can be used for future experimental work on these *K. sydneyanus* isolates *in vitro*. Two of these media alternatives have the potential to improve the performance of these bacteria in laboratory experiments, therefore easing the difficulty of future laboratory experimentation.

Chapter 5 Section C: Variable seaweed utilisation by bacteria isolated from the same *Kyphosus sydneyanus* hindgut section

For the first time, completely anaerobic *in vitro* culture methods have been used to assess algal preference of three bacteria species isolated from the hindgut of *K. sydneyanus*. These studies reveal the highly specialised nature of individual bacteria species, even when isolated from the same gut section. These studies also reveal the mutualistic relationships between fish gut bacteria species. Use of all three species had a positive effect on growth rate in the presence of all four seaweed species. This suggests greater energy efficiency in substrate digestion is achieved when multiple species are present.

Rikenellaceae isolate BP38

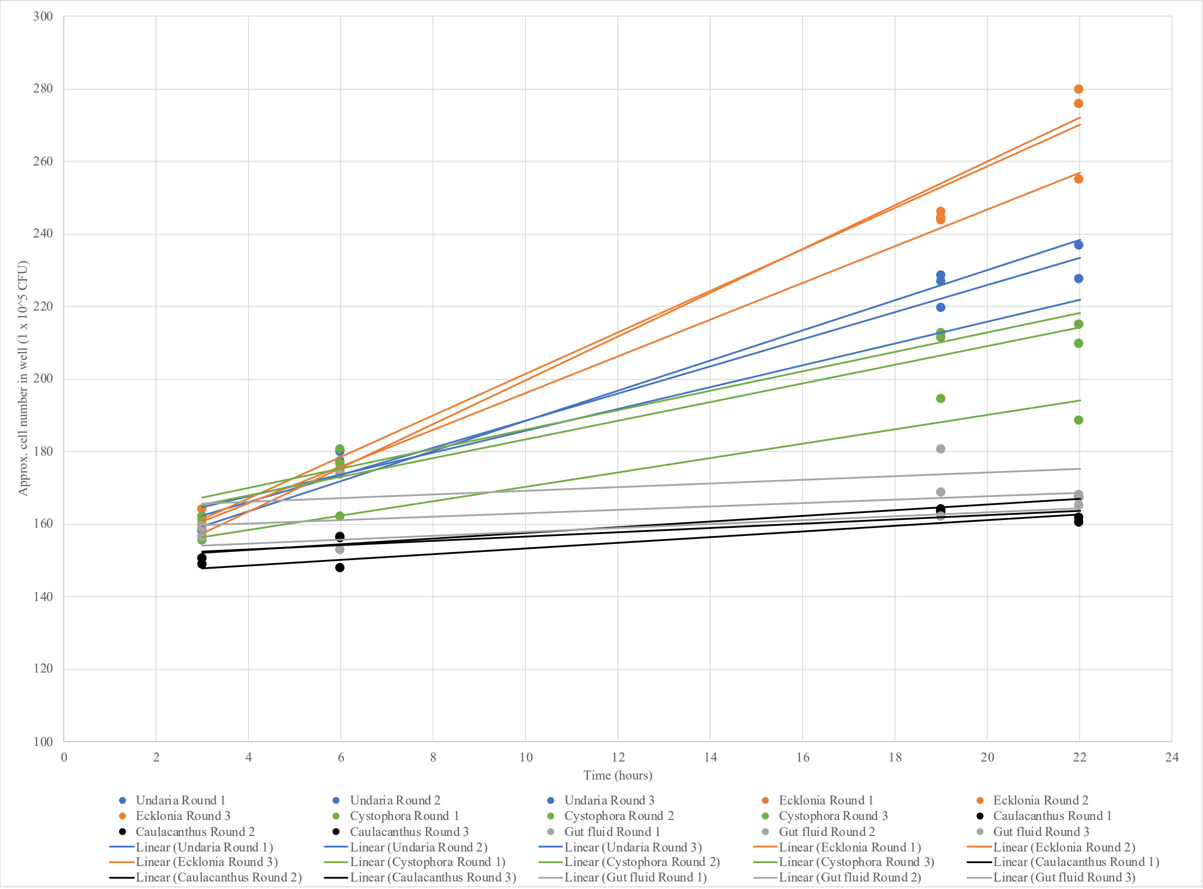


Figure 46: Growth of *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with *Undaria*, *Ecklonia, Caulacanthus, Cystophora* or gut fluid.

The results presented in Figure 46 show that *Rikenellaceae* isolate BP38 presented the fastest rate of growth with the nutrient addition of *Ecklonia* to standard YCFA medium. The *Undaria* nutrient addition produced the second fastest rate of growth. The nutrient addition of *Caulacanthus* produced the third fastest rate of growth. *Cystophora* and *K. sydneyanus* gut fluid produced the slowest rates of growth of all seaweed extracts tested in this experiment.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 46. These results are presented in Table 15, along with average growth rates across all three rounds and associated standard deviations.

Table 15: Growth rates for *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Undaria pinnatifida	1	3.011	3.634	0.577
	2	3.741		
	3	4.150		
Ecklonia radiata	1	5.718	5.600	0.494
	2	5.058		
	3	6.024		
Cystophora torulosa	1	2.589	2.416	0.383
	2	2.681		
	3	1.977		
Caulacanthus ustulatus	1	0.595	0.723	0.111
	2	0.777		
	3	0.796		
Gut fluid	1	0.542	0.349	0.303
	2	0.505		
	3	0.000		

The results in Table 15 show a positive average growth rate for *Rikenellaceae* isolate BP38 on all media.

The average growth rates across all three rounds for each media nutrient addition, calculated in Table 15, are presented in Figure 47 as a bar chart with standard deviation.

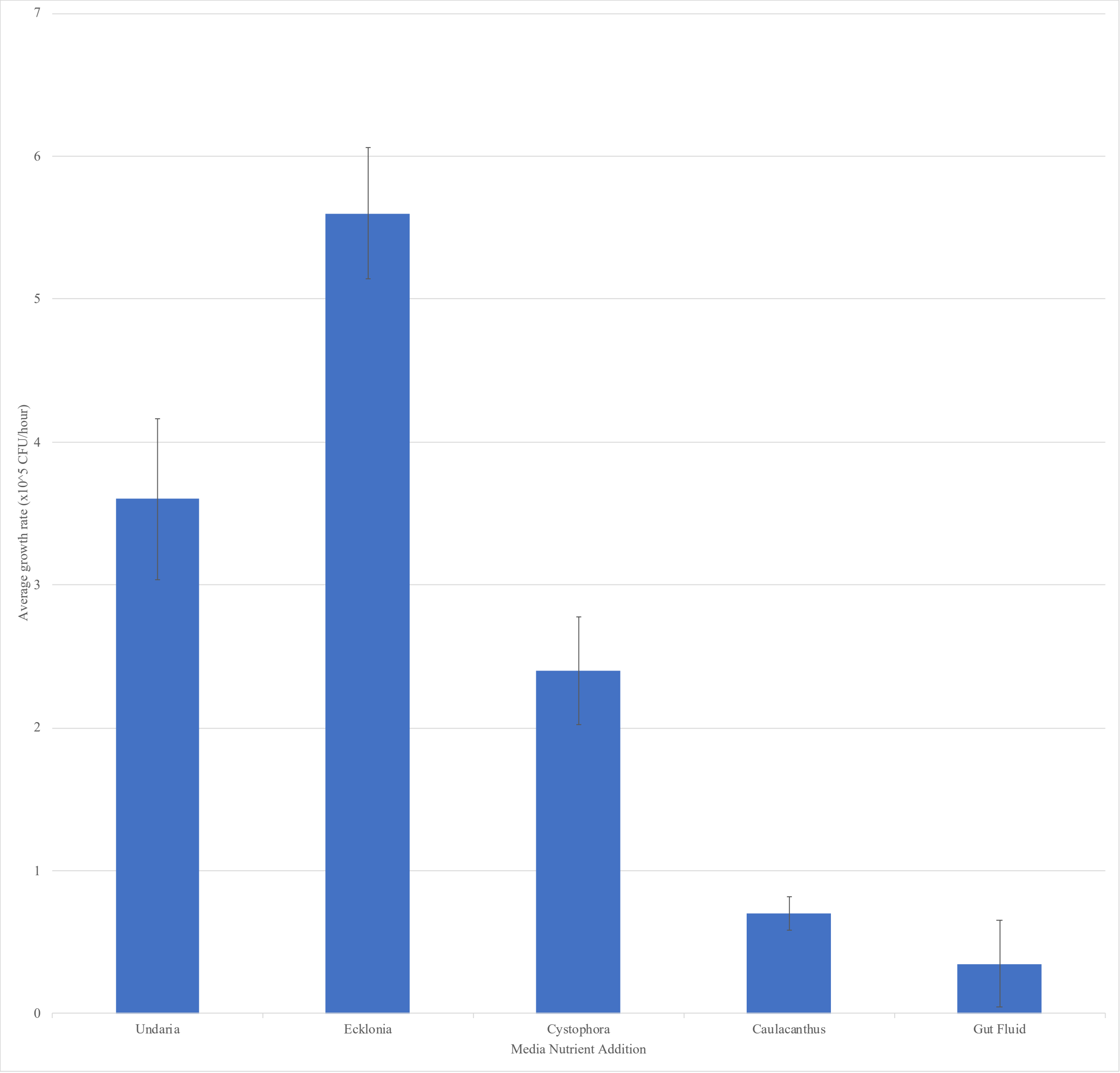


Figure 47: Average growth rate of *Rikenellaceae* isolate BP38, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. With standard deviation.

The average growth rate of *Rikenellaceae* isolate BP38 on YCFA media was shown in Figure 47 to increase compared to gut fluid with the nutrient additions of *Undaria, Ecklonia* and *Cystophora* extract (Figure 47). The average growth rate of *Rikenellaceae* isolate BP38 on YCFA media with *Caulacanthus* was more similar to gut fluid. The significance of these differences will be further investigated using statistical tests. These results suggest that, for *Rikenellaceae* isolate BP38 *Undaria, Ecklonia* and *Cystophora* extract can be used as defined alternatives to gut fluid as a nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were significant differences in growth rates between some variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of *Rikenellaceae* isolate BP38 in CFU per hour.

The one way ANOVA revealed a statistically significant difference in mean growth rate between at least two nutrients tested (F(4, 10) = [84.690], p = <0.001).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly increased between all nutrient additions to YCFA medium except between the additions of gut fluid and *Caulacanthus* extract, which was not statistically significant at the 0.05 level (p = 0.918).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between *Ecklonia* and all four other nutrient additions: *Undaria* (p = 0.001, 95% C.I. = [0.873, 3.059]), *Cystophora* (p = <0.001, 95% C.I. = [2.091, 4.277]), *Caulacanthus* (p = <0.001, 95% C.I. = [3.784, 5.970]), and gut fluid (p = <0.001, 95% C.I. = [4.158, 6.344]).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between *Undaria* and all four other nutrient additions: *Ecklonia* (p = 0.001, 95% C.I. = [-3.059, -0.873]), *Cystophora* (p = 0.028, 95% C.I. = [0.125, 2.311]), *Caulacanthus* (p = <0.001, 95% C.I. = [1.818, 4.004]), and gut fluid (p = <0.001, 95% C.I. = [2.192, 4.004]).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between *Cystophora* and all four other nutrient additions: *Undaria* (p = 0.028, 95% C.I. = [-2.311, -0.125]), *Ecklonia* (p = <0.001, 95% C.I. = [-4.277, -2.091]), *Caulacanthus* (p = 0.003, 95% C.I. = [0.600, 2.786]), and gut fluid (p = <0.001, 95% C.I. = [0.974, 3.160]).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between *Caulacanthus* and three of the four other nutrient additions: *Undaria* (p = <0.001, 95% C.I. = [-4.004, -1.818]), *Ecklonia* (p = <0.001, 95% C.I. = [-5.970, -3.784]) and *Cystophora* (p = 0.003, 95% C.I. = [-2.786, -0.600]).

There was no statistically significant difference in growth rate between *Caulacanthus* and gut fluid ($p = 0.790$).

Summary

The results of the one way ANOVA and post-hoc Tukey test show that YCFA medium with *Ecklonia* extract added caused the fastest mean rate of growth for *Rikenellaceae* isolate BP38 with a significant increase of ≤ 0.001 compared to all four other nutrient additions. YCFA medium with *Undaria* extract caused the second fastest mean rate of growth for isolate BP38, with a significant increase of ≤ 0.030 compared to *Cystophora, Caulacanthus* and *K. sydneyanus* gut fluid. This was a significantly reduced rate of growth compared to that produced by *Ecklonia* ($p < 0.001$). The third fastest rate of growth was achieved by the addition of *Cystophora* extract to YCFA, which this mean growth rate being significantly different to all other rates at ≤ 0.02. The mean growth rates of YCFA with *Caulacanthus* and *K. sydneyanus* gut fluid were the lowest and were not significantly different from each other at the 0.05 level. Therefore, for *Rikenellaceae* isolate BP38 the optimal defined medium tested is YCFA with *Ecklonia* aqueous extract, with an average growth rate of $5.600\ (\pm 0.494) \times 10^5$ CFU/hour, however all tested seaweed extracts may be used in an alternative YCFA media for this isolate.

Lachnospiraceae isolate BP5G

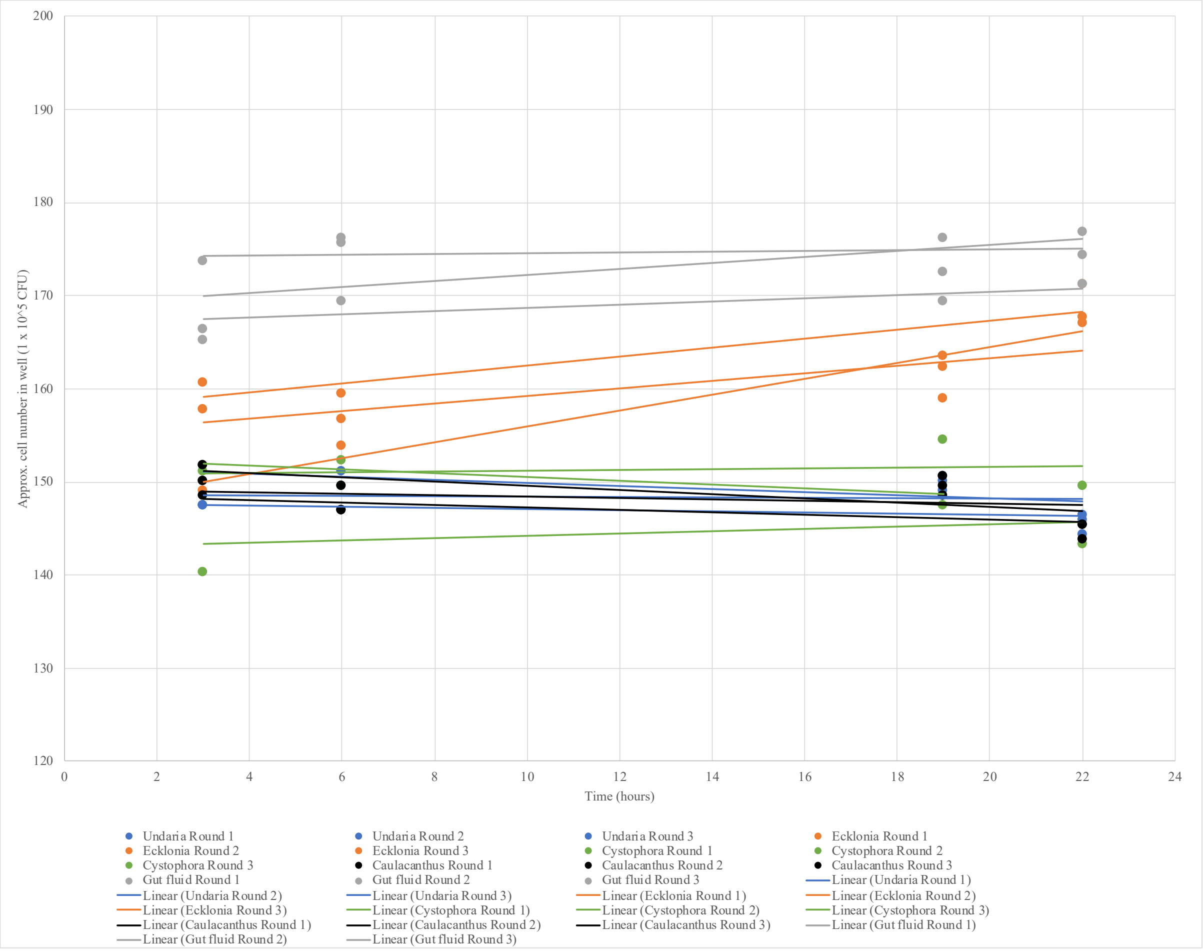


Figure 48: Growth of *Lachnospiraceae* isolate BP5G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid.

The results presented in Figure 48 show that *Lachnospiraceae* isolate BP5G presented the fastest rate of growth with the nutrient addition of *K. sydneyanus* gut fluid to standard YCFA medium. The *Ecklonia* addition produced the fastest growth rate. The other three nutrient additions produced a similar rate of growth to each other, which was slower than that produced by gut fluid or *Ecklonia*.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 48. These results are presented in Table 16, along with average growth rates across all three rounds and associated standard deviations.

Table 16: Growth rates for *Lachnospiraceae* isolate BP5G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Undaria pinnatifida	1	0.000	0.000	0.000
	2	0.000		
	3	0.000		
Ecklonia radiata	1	0.853	0.580	0.239
	2	0.480		
	3	0.408		
Cystophora torulosa	1	0.122	0.055	0.062
	2	0.000		
	3	0.043		
Caulacanthus ustulatus	1	0.000	0.000	0.000
	2	0.000		
	3	0.000		
Gut fluid	1	0.036	0.178	0.145
	2	0.325		
	3	0.173		

The results in Table 16 show a positive average growth rate for *Lachnospiraceae* isolate BP5G on YCFA media with *Ecklonia, Caulacanthus* or Gut fluid nutrient additions. Zero growth was seen with the *Undaria* and *Cystophora* nutrient additions.

The average growth rates across all three rounds for each media nutrient addition, calculated in Table 16, are presented in Figure 49 as a bar chart with standard deviation.

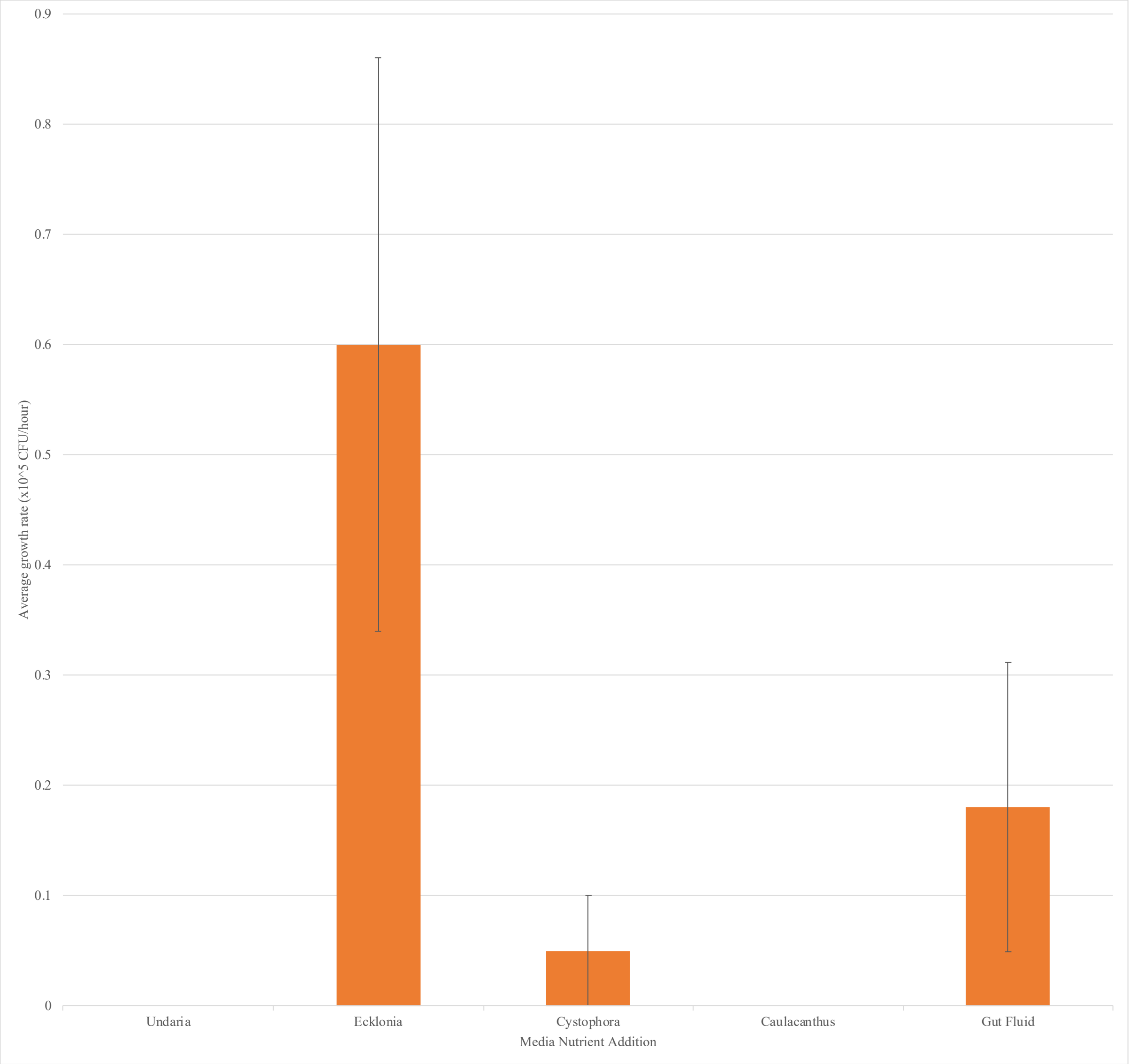


Figure 49: Average growth rate of *Lachnospiraceae* isolate BP5G, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. With standard deviation.

The average growth rate of *Lachnospiraceae* isolate BP5G on YCFA media was shown in Figure 49 to increase compared to gut fluid with the nutrient addition of *Ecklonia* extract

(Figure 49). Growth of *Lachnospiraceae* isolate BP5G was also successful on YCFA media with *Caulacanthus* extract. The significance of these differences will be further investigated using statistical tests. These results suggest that, for *Lachnospiraceae* isolate BP5G, *Ecklonia* extract can be used as a defined alternative to gut fluid as a nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were significant differences in growth rates between some variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of *Rikenellaceae* isolate BP5G in CFU per hour.

The one way ANOVA revealed a statistically significant difference in mean growth rate between at least two nutrients tested (F(4, 10) = [10.788], p = 0.001).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between *Ecklonia* and all four other nutrient additions: *Undaria* (p = 0.002, 95% C.I. = [0.240, 0.960]), *Cystophora* (p = 0.003, 95% C.I. = [0.193, 0.913]), *Caulacanthus* (p = 0.002, 95% C.I. = [0.240, 0.960]), and gut fluid (p = 0.021, 95% C.I. = [0.060, 0.780]).

There was no statistically significant difference in growth rate between any of the other nutrient additions to YCFA medium: *Undaria* and *Cystophora* (p = 0.614), *Undaria* and *Caulacanthus* (p = 1.000), *Undaria* and gut fluid (p = 0.285), *Cystophora* and *Caulacanthus* (p = 0.724), *Cystophora* and gut fluid (p = 0.959), *Caulacanthus* and gut fluid (p = 1.000).

The results of the one way ANOVA and post hoc Tukey test show that YCFA medium with *Ecklonia* extract added caused the fastest mean rate of growth for a mix of isolate BP38 with a significant increase of ≤0.003 compared to all four other nutrient additions. YCFA medium with *Undaria, Cystophora, Caulacanthus* or *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level.

Summary

The results of the one way ANOVA and post-hoc Tukey test show that YCFA medium with *Ecklonia* extract added caused the fastest mean rate of growth for *Lachnospiraceae* isolate BP5G with a significant increase of ≤0.02 compared to all four other nutrient additions. The mean growth rates of YCFA with *Undaria, Cystophora, Caulacanthus* and *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level. However, the average growth of each of the alternative seaweed extracts was less than that of the gut fluid. Therefore, for *Lachnospiraceae* isolate BP5G the optimal defined medium tested is YCFA with *Ecklonia* aqueous extract, with an average growth rate of 0.580 (± 0.239) x 10^5 CFU/hour, with this media being the only viable alternative YCFA media for this isolate.

Oscillospiraceae isolate BP47G

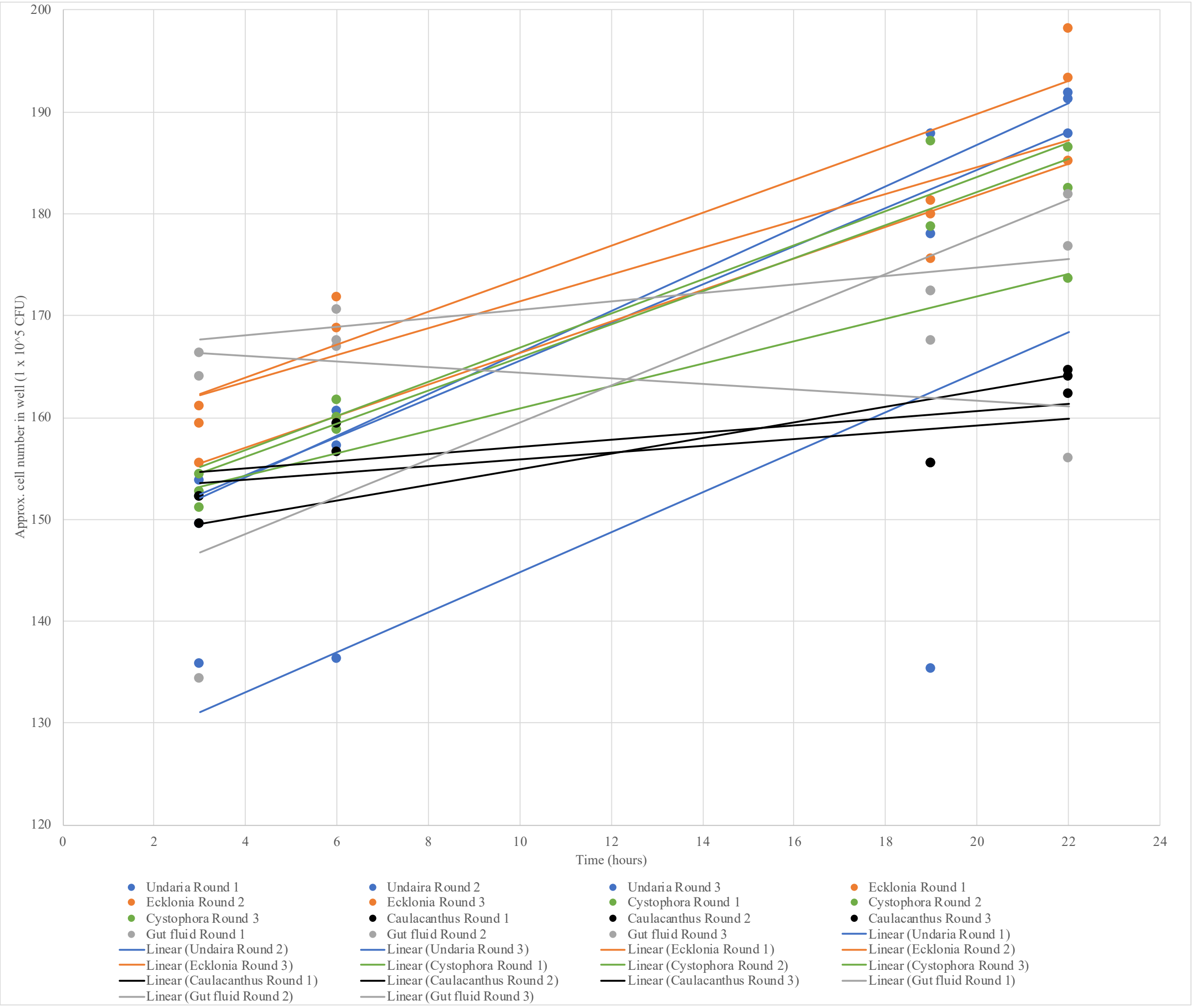


Figure 50: Growth of *Oscillospiraceae* isolate BP47G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid.

The results presented in Figure 50 show that *Oscillospiraceae* isolate BP47G presented the fastest rate of growth with the nutrient additions of *Ecklonia, Undaria* or *Caulacanthus* to standard YCFA medium. The *K. sydneyanus* gut fluid nutrient addition produced a median rate of growth. The nutrient addition of *Caulacanthus* produced the slowest rate of growth out of all seaweed extracts tested in this experiment.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 50. These results are presented in Table 17, along with average growth rates across all three rounds and associated standard deviations.

Table 17: Growth rates for *Oscillospiraceae* isolate BP47G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Undaria pinnatifida	1	2.042	1.960	0.083
	2	1.876		
	3	1.963		
Ecklonia radiata	1	1.620	1.496	0.159
	2	1.317		
	3	1.551		
Cystophora torulosa	1	1.676	1.467	0.319
	2	1.626		
	3	1.100		
Caulacanthus ustulatus	1	0.334	0.482	0.244
	2	0.349		
	3	0.764		
Gut fluid	1	0.000	0.747	0.955
	2	0.418		
	3	1.823		

The results in Table 17 show a positive average growth rate for *Oscillospiraceae* isolate BP47G on all media. Replicate 3 for YCFA with a nutrient addition of *U. pinnatifida* extract was removed from analysis as these results were an outlier. The average growth rates across all three rounds for each media nutrient addition, calculated in Table 17, are presented in Figure 51 as a bar chart with standard deviation.

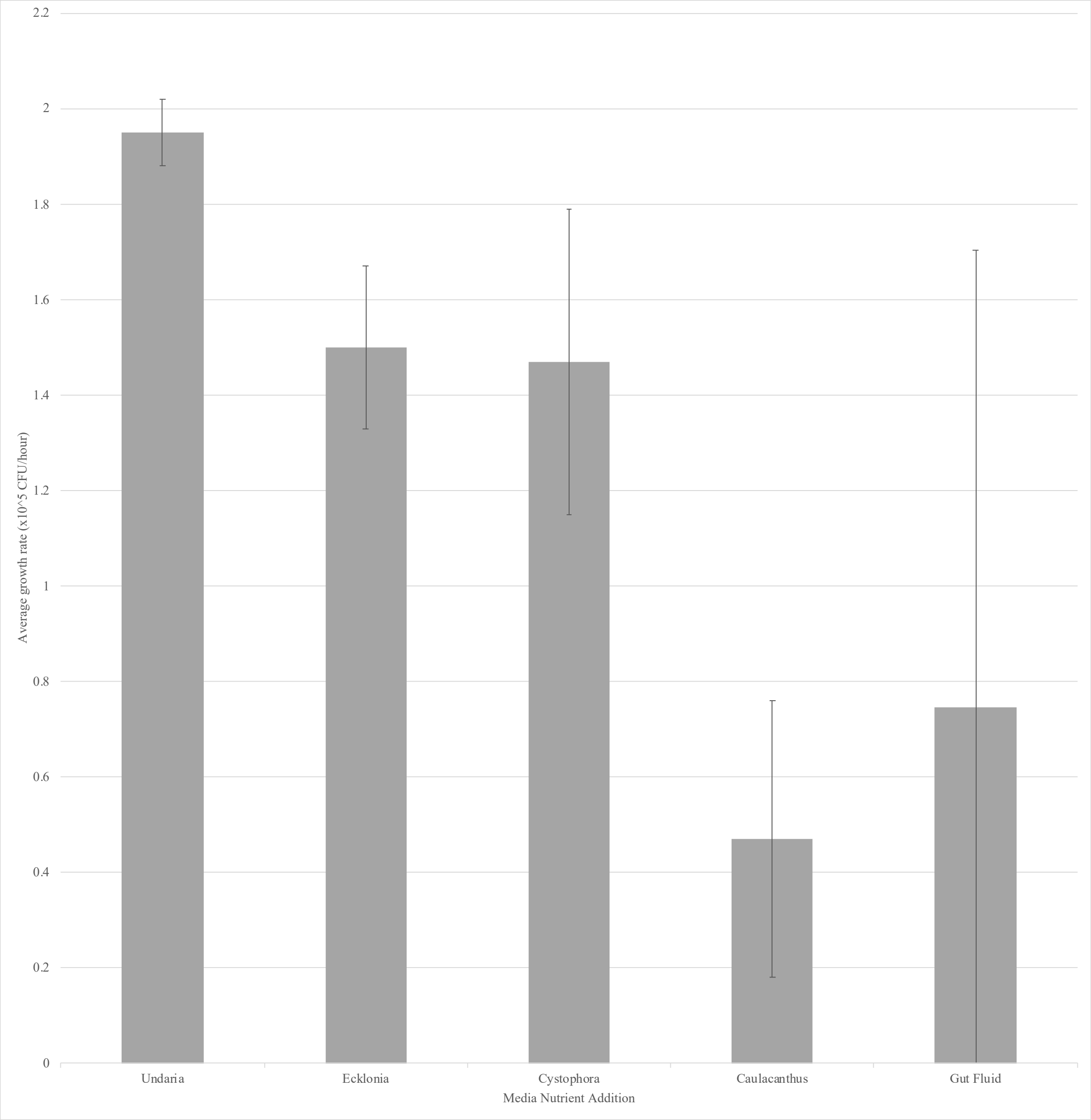


Figure 51: Average growth rate of *Oscillospiraceae* isolate BP47G, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. With standard deviation.

The average growth rate of *Oscillospiraceae* isolate BP47G on YCFA media was shown in Figure 51 to increase compared to gut fluid with the nutrient additions of *Undaria, Ecklonia* and *Cystophora* extract (Figure 51). The average growth rate of *Oscillospiraceae* isolate BP47G on YCFA media with *Caulacanthus* was reduced in comparison to gut fluid. The significance of these differences will be further investigated using statistical tests. These results suggest that, for *Oscillospiraceae* isolate BP47G, *Undaria, Ecklonia* and *Cystophora* extract can be used as defined alternatives to gut fluid as a nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were no significant differences in growth rates between any of the variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of *Oscillospiraceae* isolate BP47G in CFU per hour.

The one way ANOVA revealed a statistically significant difference in mean growth rate between at least two nutrients tested (F(4, 10) = [4.928], p = 0.019). The within group degrees of freedom are reduced from 10 to 9 in this test as there was one outlier within the *Undaria* group of growth rates, which was excluded from analysis.

A Tukey's HSD test for multiple comparisons found that the mean value of growth rate was only statistically significant different at the 0.05 between *Undaria* and *Caulacanthus* (p = 0.021, 95% C.I. = [0.215, 2.742]).

The was no significant difference in growth rate between any of the other nutrients tested: *Undaria* and *Ecklonia* (p = 0.853), *Undaria* and *Cystophora* (p = 0.820), *Undaria* and gut fluid (p = 0.135), *Ecklonia* and *Cystophora* (p = 1.000), *Ecklonia* and *Caulacanthus* (p = 0.163), *Ecklonia* and gut fluid (p = 0.387), *Cystophora* and *Caulacanthus* (p = 0.183), *Cystophora* and gut fluid (p = 0.425), *Caulacanthus* and gut fluid (p = 0.961).

The results of the one way ANOVA and post hoc Tukey test show that none of the nutrients added to YCFA medium caused a significantly different mean rate of growth for isolate BP47G. The mean growth rates of YCFA with *Undaria, Ecklonia, Cystophora, Caulacanthus* or *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level.

Summary

The results of the one way ANOVA and post-hoc Tukey test show that average growth rate of *Oscillospiraceae* isolate BP47G on YCFA medium with all four seaweed extracts did not vary significantly from *K. sydneyanus* gut fluid at the 0.05 level. However, YCFA with *Undaria, Ecklonia* and *Cystophora* all produced faster average growth rates than gut fluid, with *Undaria* being the fastest. The average growth rate of *Caulacanthus* was slower than that of gut fluid. The only significant difference between growth rates was seen between *Undaria* and *Caulacanthus* (p = 0.021). Therefore, for *Oscillospiraceae* isolate BP47G the optimal defined medium tested is YCFA with *Undaria* aqueous extract, with an average growth rate of 1.960 (± 0.083) x 10^5 CFU/hour. However, *Ecklonia*, and *Cystophora* seaweed extracts may also be used in an alternative YCFA media for this isolate.

Mixture of all three isolates (BP47G, BP5G and BP38)

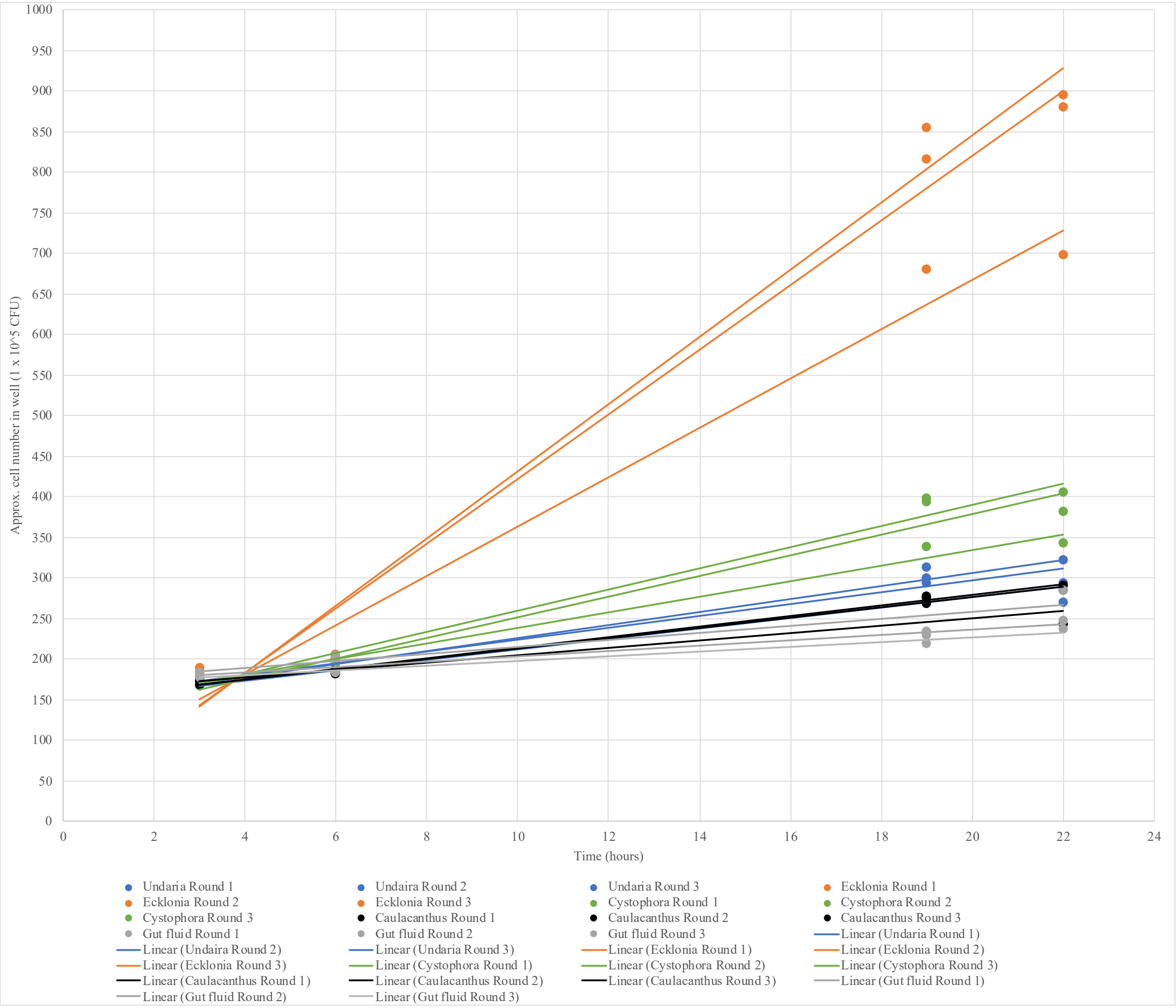


Figure 52: Growth of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid.

The results presented in Figure 52 show that a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 presented the fastest rate of growth with the nutrient addition of *Ecklonia* to standard YCFA medium. The other four nutrient additions in this experiment produce a similar rate of growth, which was slower than the *Ecklonia* extract.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 52. These results are presented in Table 18, along with average growth rates across all three rounds and associated standard deviations.

Table 18: Growth rates of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Undaria pinnatifida	1	6.433	7.266	0.797
	2	8.021		
	3	7.344		
Ecklonia radiata	1	41.355	37.191	5.964
	2	39.859		
	3	30.358		
Cystophora torulosa	1	12.753	11.796	1.922
	2	13.027		
	3	9.569		
Caulacanthus ustulatus	1	6.361	5.796	1.141
	2	6.544		
	3	4.483		
Gut fluid	1	3.324	3.513	0.771
	2	4.360		
	3	2.854		

The results in Table 18 show a positive average growth rate for a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 on all media. The average growth rates across all three rounds for each media nutrient addition, calculated in Table 18, are presented in Figure 53 as a bar chart with standard deviation.

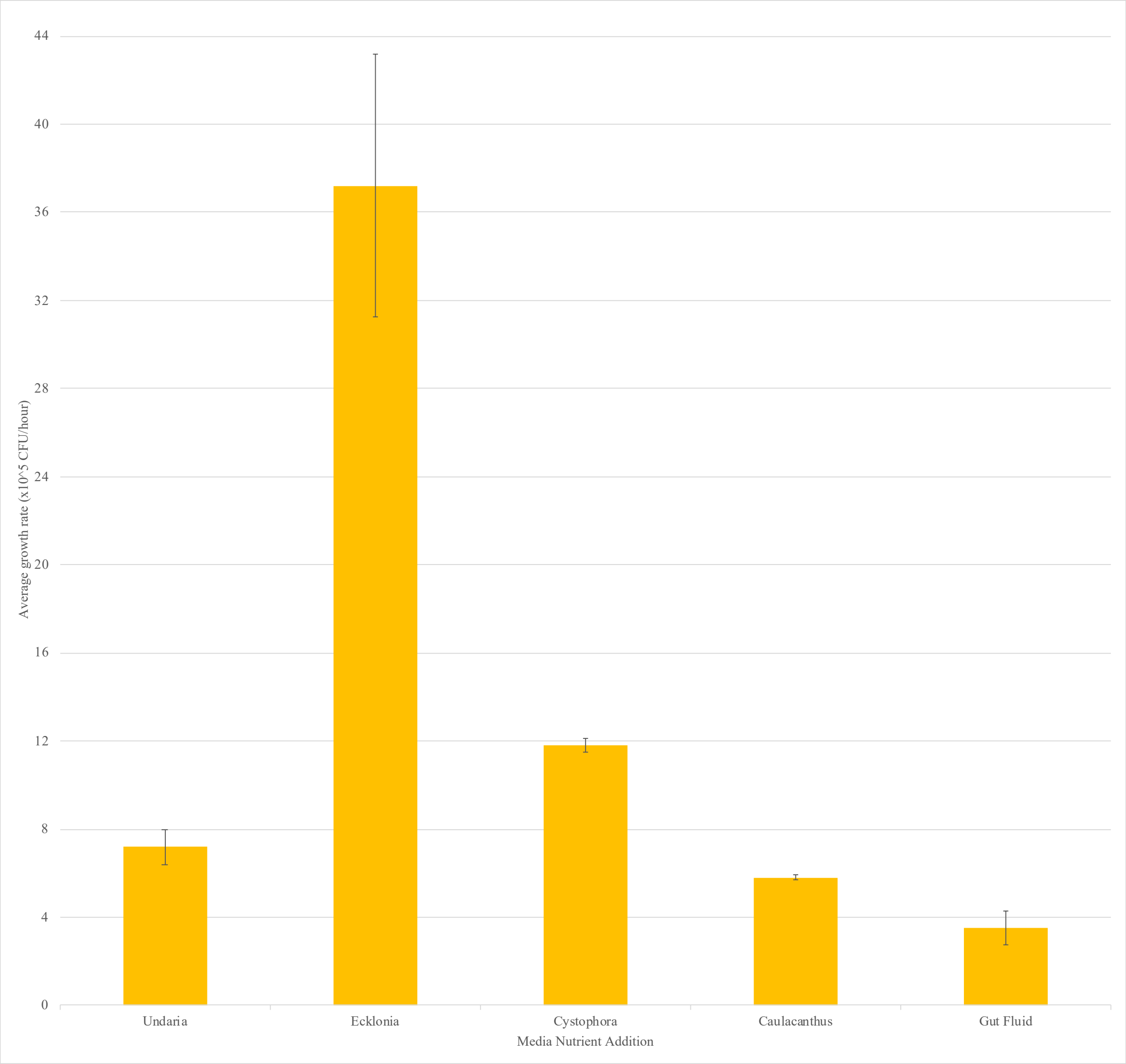


Figure 53: Average growth rate of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. With standard deviation.

The average growth rate of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 on YCFA media was shown in Figure 53 to increase compared to gut fluid with the nutrient additions of *Undaria, Ecklonia, Cystophora* and *Caulacanthus* extract (Figure 53). The significance of these differences will be further investigated using statistical tests. These results suggest that, for a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38, *Undaria, Ecklonia, Cystophora* and *Caulacanthus* extracts can be used as defined alternatives to gut fluid as a nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were significant differences in growth rates between some variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 in CFU per hour.

The one way ANOVA revealed a statistically significant difference in mean growth rate between at least two nutrients tested (F(4, 10) = [68.744], p = <0.001).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between *Ecklonia* and all four other nutrient additions: *Undaria* (p = <0.001, 95% C.I. = [22.248, 37.752]), *Cystophora* (p = <0.001, 95% C.I. = [17.681, 33.186]), *Caulacanthus* (p = <0.001, 95% C.I. = [23.648, 39.152]), and gut fluid (p = <0.001, 95% C.I. = [25.948, 41.452]).

The Tukey's HSD test for multiple comparisons also found that the mean value of growth rate was significantly different between *Cystophora* and gut fluid (p = <0.036, 95% C.I. = [0.515, 16.019]).

There was no statistically significant difference in growth rate between any of the other nutrient additions to YCFA medium: *Undaria* and *Cystophora* (p = 0.358), *Undaria* and *Caulacanthus* (p = 0.973), *Undaria* and gut fluid (p = 0.545), *Cystophora* and *Caulacanthus* (p = 0.159), *Caulacanthus* and gut fluid (p = 0.860).

The results of the one way ANOVA and post hoc Tukey test show that YCFA medium with *Ecklonia* extract added caused the fastest mean rate of growth for a mix of isolate BP38 with a significant increase of ≤0.001 compared to all four other nutrient additions. YCFA medium with *Undaria, Cystophora* or *Caulacanthus* were not significantly different from each other at the 0.05 level. YCFA medium with *K. sydneyanus* gut fluid had a significantly slower growth rate than the *Cystophora* nutrient addition, at a level of 0.036.

Summary

The results of the one way ANOVA and post-hoc Tukey test show that YCFA medium with *Ecklonia* extract added caused the fastest mean rate of growth for a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 with a significant increase of <0.001 compared to all four other nutrient additions. The average growth rate of the mix on YCFA with *Cystophora* extract was also significantly faster than that on YCFA with gut fluid ($p = <0.036$). The mean growth rates of YCFA with *Undaria, Caulacanthus* and *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level. However, the average growth of each of the alternative seaweed extracts was faster than that of the gut fluid. Therefore, for the mix of all three isolates the optimal defined medium tested is YCFA with *Ecklonia* aqueous extract, with an average growth rate of 37.191 (± 5.964) x 10^5 CFU/hour. However, *Ecklonia, Cystophora* and *Caulacanthus* seaweed extracts may also be used in an alternative YCFA media for this isolate.

All isolates seaweed extract utilisation

This summary allows comparison of all growth rate results for fish gut bacterial isolates cultured on YCFA media with the addition of one of four aqueous extracts of seaweed, or *K. sydneyanus* gut fluid. These results are displayed in Figure 54 and analysed for significance in further statistical tests. This analysis reveals multiple viable defined and simplified alternatives to the complex YCFA-gut fluid media for the *in vitro* culture of some fish gut bacterial isolates.

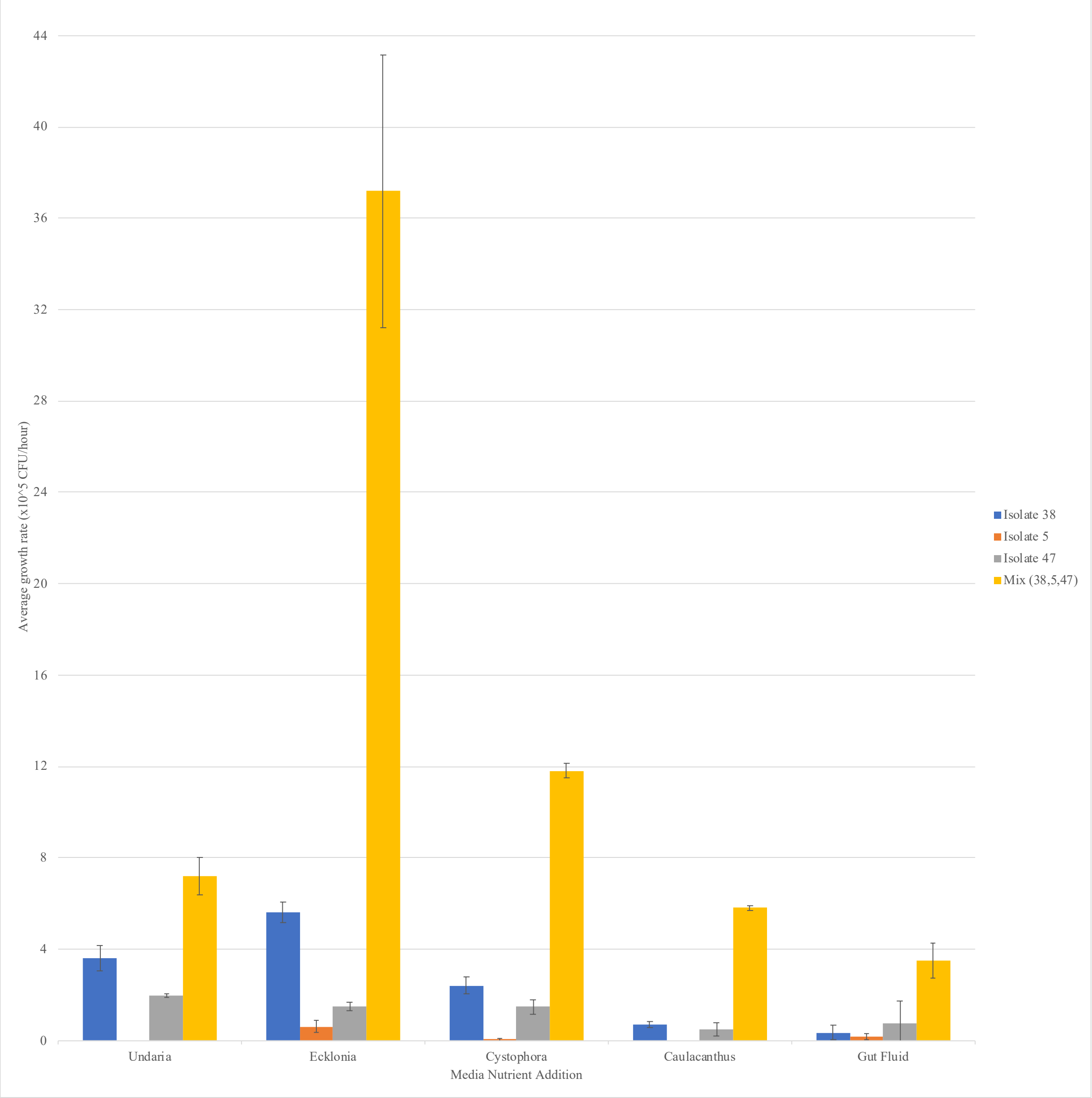


Figure 54: Average growth rates of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G, *Rikenellaceae* isolate BP38 and a mix of all three measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with *Undaria, Ecklonia, Caulacanthus, Cystophora* or gut fluid. With standard deviation.

Figure 54 allows the comparison of average growth rates on each variation of YCFA media for all isolates, including *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G,

Rikenellaceae BP38 and a mix of all three. This cross-isolate comparison reveals that the fastest average growth rate was achieved by a mix of all three isolates on YCFA with *Ecklonia* extract. The comparison in Figure 54 reveals that a mix of all three isolates produced the fastest average growth rate of all the isolates on all media. Isolate BP38 appeared to produce the second fastest rate of growth on all media except YCFA with gut fluid, with the pattern being the same as the mix as *Ecklonia* produced the fastest average growth, and *Undaria* the second. Isolate BP38 and a mix of all three isolates performed better than isolate BP47G and isolate BP5G on all media except YCFA with gut fluid, where isolate BP47G had a similar growth rate to BP38. Overall, Figure 54 shows that YCFA with *Ecklonia* presents the most successful media alternative to YCFA with *K. sydneyanus* gut fluid, as all isolates presented successful growth. This was the only medium to present strong successful growth for all isolates other than YCFA with gut fluid. Also, the highest average growth rate was met for all isolates with this medium, higher or equal to that seen on YCFA with gut fluid. The significance of these differences will be further investigated using statistical tests. These results suggest that for all isolates *Ecklonia* can be used as an alternative to the gut fluid nutrient addition to YCFA media.

Statistical significance of growth rate results

A two-way ANOVA was performed to analyse the effect of isolate and seaweed extract (or gut fluid) nutrient addition on mean value growth rate.

The two-way ANOVA revealed that there was a statistically significant interaction at the 0.05 level between the effects of isolate and nutrient addition on growth rate (F(12, 41) = [59.800], p = <0.001).

Simple main effects analysis showed that isolate had a significant effect on growth rate (p = <0.001). A post-hoc Tukey's HSD test for multiple comparisons found that the mean value growth rate was significantly different between: a mix of all three isolates and isolate BP5G (p = <0.001, 95% C.I. [11.517, 14.406]), a mix of all three isolates and isolate BP38 (p = <0.001, 95% C.I. [9.142, 12.031]), a mix of all three isolates and isolate BP47G (p = <0.001, 95% C.I. [10.458, 13.347]), isolate BP5G and isolate BP38 (p = <0.001, 95% C.I. [-3.819, -0.930]). There was no significant difference in mean value growth rate between isolate BP5G and isolate BP47G (p = 0.218) or isolate BP38 and isolate BP47G (p = 0.086).

Simple main effects analysis showed that nutrient addition also had a significant effect on mean growth rate across all isolates (p = <0.001). A post-hoc Tukey's HSD test for multiple comparisons found that the mean value growth rate was significantly different between *Ecklonia* and all other nutrient additions: gut fluid (p = <0.001, 95% C.I. = [8.317, 11.759]), *Undaria* (p = <0.001, 95% C.I. = [6.315, 9.758]), *Cystophora* (p = <0.001, 95% C.I. = [5.576, 9.018) and *Caulacanthus* (p = <0.001, 95% C.I. = [7.754, 11.196]). There was also a significant difference between gut fluid and *Undaria* (p = 0.016, 95% C.I. = [-3.723, -0.281]), gut fluid and *Cystophora* (p = <0.001, 95% C.I. = [-4.463, -1.021]) and *Cystophora* and *Caulacanthus* (p = 0.007, 95% C.I. = [0.457, 3.899]). There was no significant difference in mean value growth rate between gut fluid and *Caulacanthus* (p = 0.882), *Undaria* and *Cystophora* (p = 0.736), *Undaria* and *Caulacanthus* (p = 0.140).

Summary

Post-hoc Tukey's testing of the isolates in this experiment found that across all YCFA media variations the mix of all three isolates grew significantly faster than each individual isolate (p < 0.001). Of the individual isolates *Rikenellaceae* isolate BP38 grew significantly faster than

Lachnospiraceae isolate BP5G (p = <0.001). Whereas the average growth rates of *Oscillospiraceae* isolate BP47G did not vary significantly from *Lachnospiraceae* isolate BP5G or *Rikenellaceae* BP38.

Post-hoc Tukey's testing also found that YCFA with *Undaria, Ecklonia, Cystophora* or *Caulacanthus* aqueous extract can be used as alternative media to YCFA with gut fluid for the *in vitro* cultivation of these *K. sydneyanus* gut bacterial isolates as the average growth rate across all isolates in these simple and defined media alternatives is equal to or exceeding that of YCFA with gut fluid. The *Ecklonia* addition to YCFA media produced a significantly faster rate of growth than all other nutrient additions. *Undaria* produced a significantly faster growth rate than gut fluid, which was slower than that caused by *Ecklonia. Cystophora* produced a significantly faster growth rate than gut fluid, which was slower than that caused by *Ecklonia* or *Undaria*. The growth rate across all isolates on YCFA media with *Caulacanthus* aqueous extract was not significantly different to gut fluid. Therefore, these results show YCFA with *Ecklonia* aqueous extract produces the best growth for these bacteria, YCFA with *Undaria* the second best, YCFA with *Cystophora* the third best, and YCFA with *Caulacanthus* can also be used as an alternative to gut fluid. Therefore, these experiments have produced four new alternative defined media recipes that can be used for future experimental work on these *K. sydneyanus* isolates *in vitro*. Three of these media alternatives have the potential to improve the performance of these bacteria in laboratory experiments, therefore easing the difficulty of future laboratory experimentation.

Chapter 5 Section D: Variable vitamin utilisation by bacteria isolated from the same *Kyphosus sydneyanus* hindgut section

Rikenellaceae isolate BP38

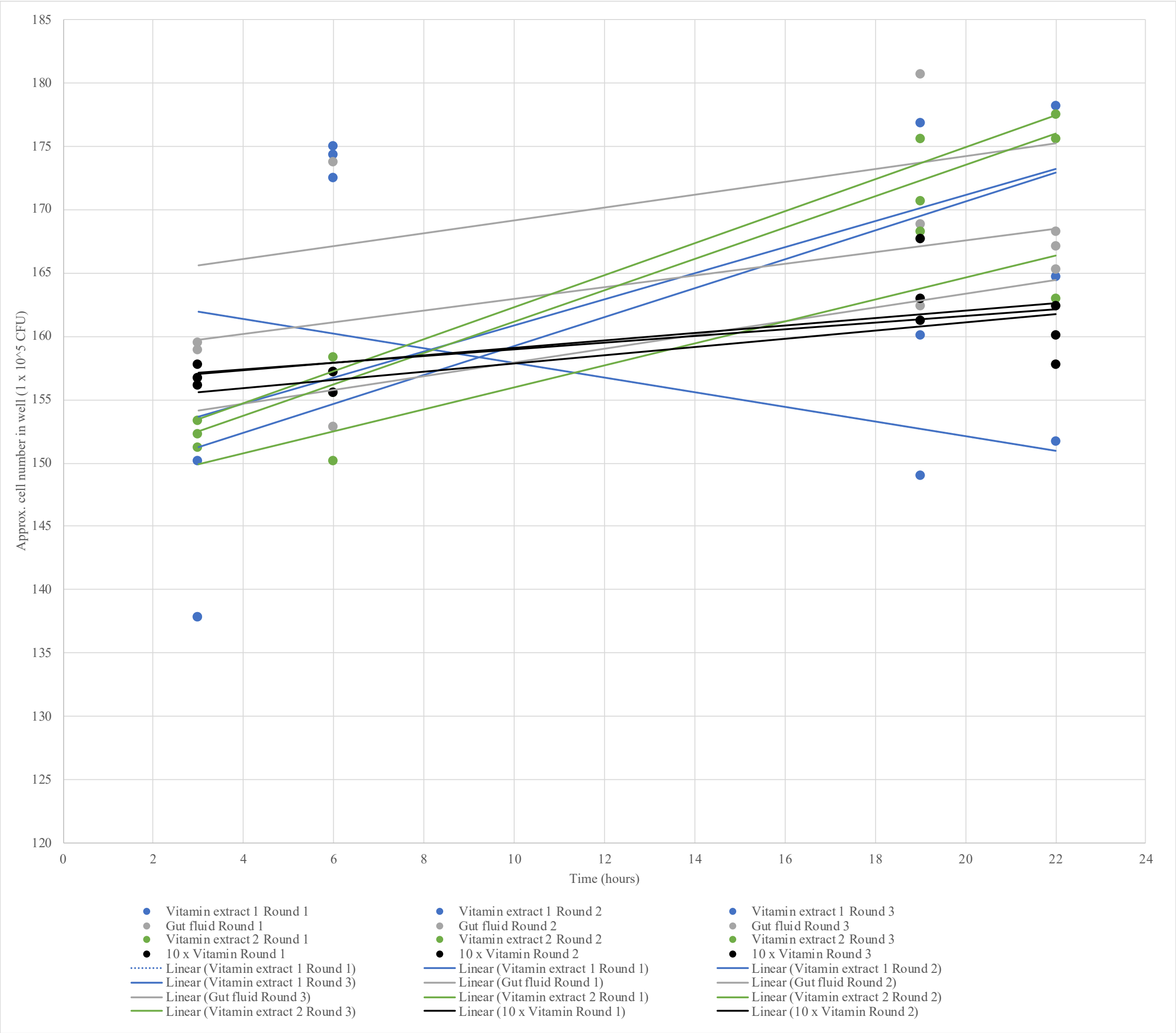


Figure 55: Growth of *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid.

The results presented in Figure 55 show that *Rikenellaceae* isolate BP38 presented the fastest rate of growth with the nutrient addition of the *Undaria* vitamin extract 1 or 2 to standard YCFA medium. The *K. sydneyanus* gut fluid or ten times normal amount of vitamin extract nutrient additions produced a similar rate of growth to each other, which was slower than that produced by the *Undaria* vitamin extracts.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 55. These results are presented in Table 19, along with average growth rates across all three rounds and associated standard deviations.

Table 19: Growth rates of *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Vitamin extract 1 of U. pinnatifida	1	0.000 [outlier]	0.722	0.628
	2	1.025		
	3	1.140		
Vitamin extract 2 of U. pinnatifida	1	0.866	1.120	0.220
	2	1.235		
	3	1.259		
YCFA medium with 10 times the usual amount of vitamin extract	1	0.322	0.293	0.031
	2	0.261		
	3	0.296		
Gut fluid	1	0.542	0.349	0.303
	2	0.505		
	3	0.000		

The results in Table 19 show a positive average growth rate for *Rikenellaceae* isolate BP38 on all media. The average growth rates across all three rounds for each media nutrient addition, calculated in Table 19, are presented in Figure 56 as a bar chart with standard deviation.

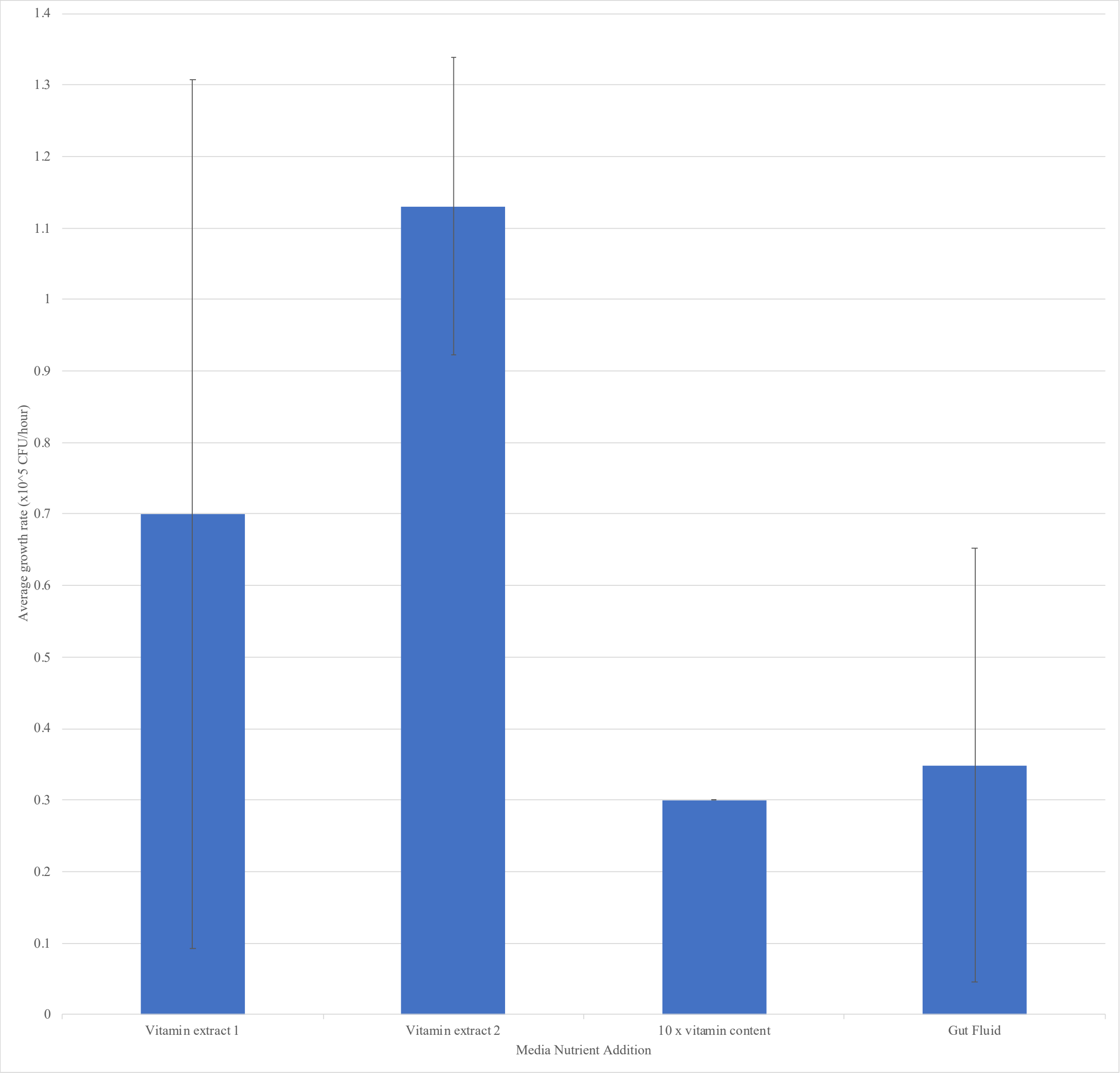


Figure 56: Average growth rate of *Rikenellaceae* isolate BP38, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. With standard deviation.

The average growth rate of *Rikenellaceae* isolate BP38 on YCFA media was shown in Figure 56 to increase compared to gut fluid with the addition of the vitamin extracts of *Undaria pinnatifida* (Figure 56). However, adding ten times the usual amount of vitamin mix to YCFA produced a similar average growth rate in *Rikenellaceae* isolate BP38 to YCFA with gut fluid. The significance of these differences will be further investigated using statistical tests. These results suggest that, for *Rikenellaceae* isolate BP38, the vitamin extract of *U. pinnatifida* can be used as defined alternatives to gut fluid as a nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were no significant differences in growth rates between any of the variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of isolate BP38 in CFU per hour.

The one way ANOVA revealed that there was not a statistically significant difference at the 0.05 level in mean growth rate between any of the nutrients tested ($F(3, 8) = [3.295]$, $p = 0.079$).

Summary

The results of the one way ANOVA and post-hoc Tukey test show that mean growth rates of YCFA with vitamin extracts 1 and 2, 10 x vitamin and *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level. However, the average growth of *Rikenellaceae* isolate BP38 on YCFA medium with either of the vitamin extracts was faster than that with gut fluid. Therefore, for *Rikenellaceae* isolate BP38 the optimal defined medium tested is YCFA with vitamin extract from *U. pinnatifida*, with an average growth rate of between 0.722 (± 0.628) x 10^5 and 1.120 (± 0.220) x 10^5 CFU/hour. However, all three vitamin nutrient additions can be used as an alternative to gut fluid in YCFA media for this isolate.

Lachnospiraceae isolate BP5G

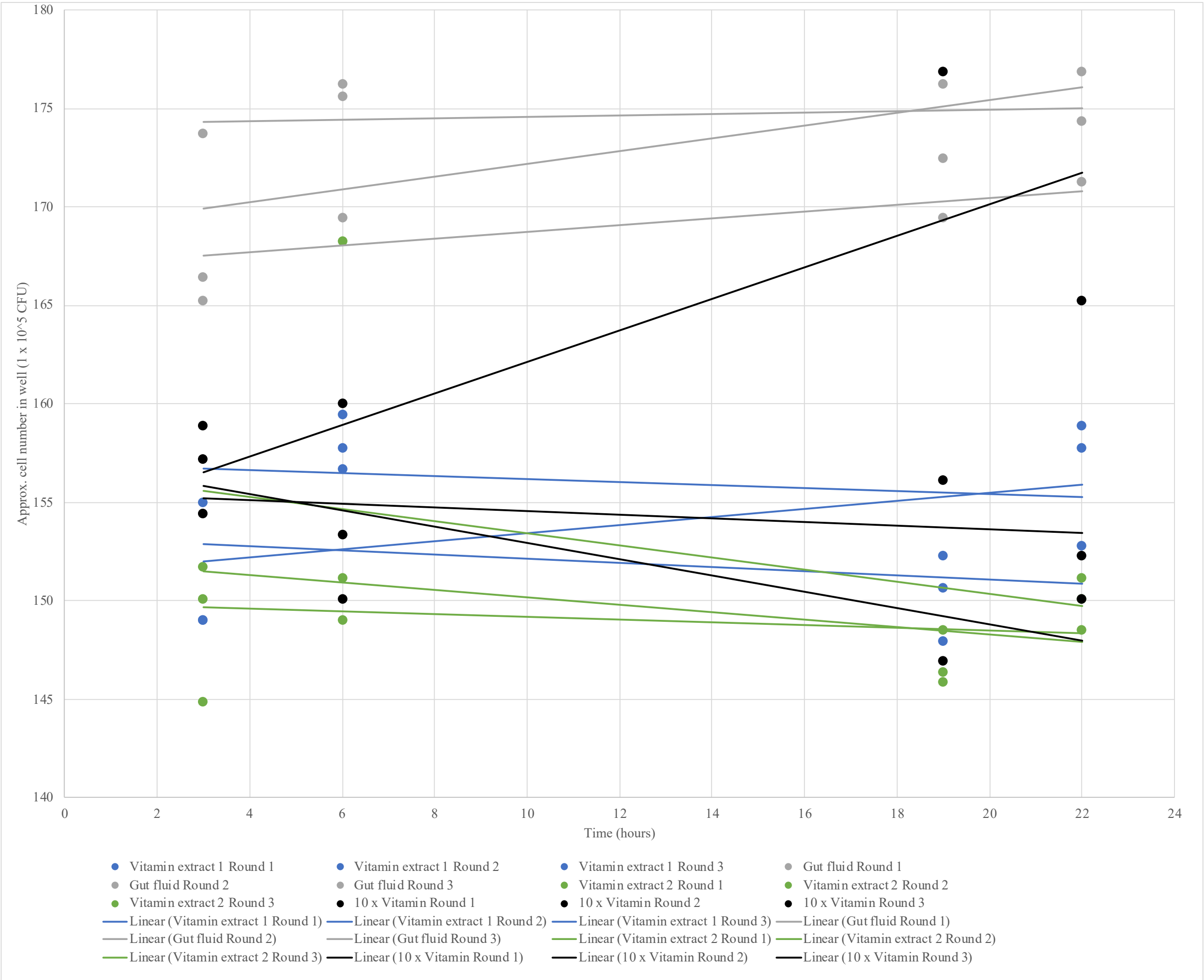


Figure 57: Growth of *Lachnospiraceae* isolate BP5G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid.

The results presented in Figure 57 show that *Lachnospiraceae* isolate BP5G presented a similar rate of growth with each of the five trialled nutrient additions to standard YCFA medium in this experiment. In all cases the rate of growth was very low or zero.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 57. These results are presented in Table 20, along with average growth rates across all three rounds and associated standard deviations.

Table 20: Growth rates of *Lachnospiraceae* isolate BP5G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Vitamin extract 1 of U. pinnatifida	1	0.206	0.069	0.119
	2	0.000		
	3	0.000		
Vitamin extract 2 of U. pinnatifida	1	0.000	0.000	0.000
	2	0.000		
	3	0.000		
YCFA medium with 10 times the usual amount of vitamin extract	1	0.800	0.267	0.462
	2	0.000		
	3	0.000		
Gut fluid	1	0.036	0.178	0.145
	2	0.325		
	3	0.173		

The results in Table 20 show a positive average growth rate for *Lachnospiraceae* isolate BP5G on YCFA media with the addition of *Undaria* vitamin extract 1, *K. sydneyanus* gut fluid, and ten times the usual concentration of vitamins. These was zero growth seen for the second vitamin extract of *Undaria*.

The average growth rates across all three rounds for each media nutrient addition, calculated in Table 20, are presented in Figure 58 as a bar chart with standard deviation.

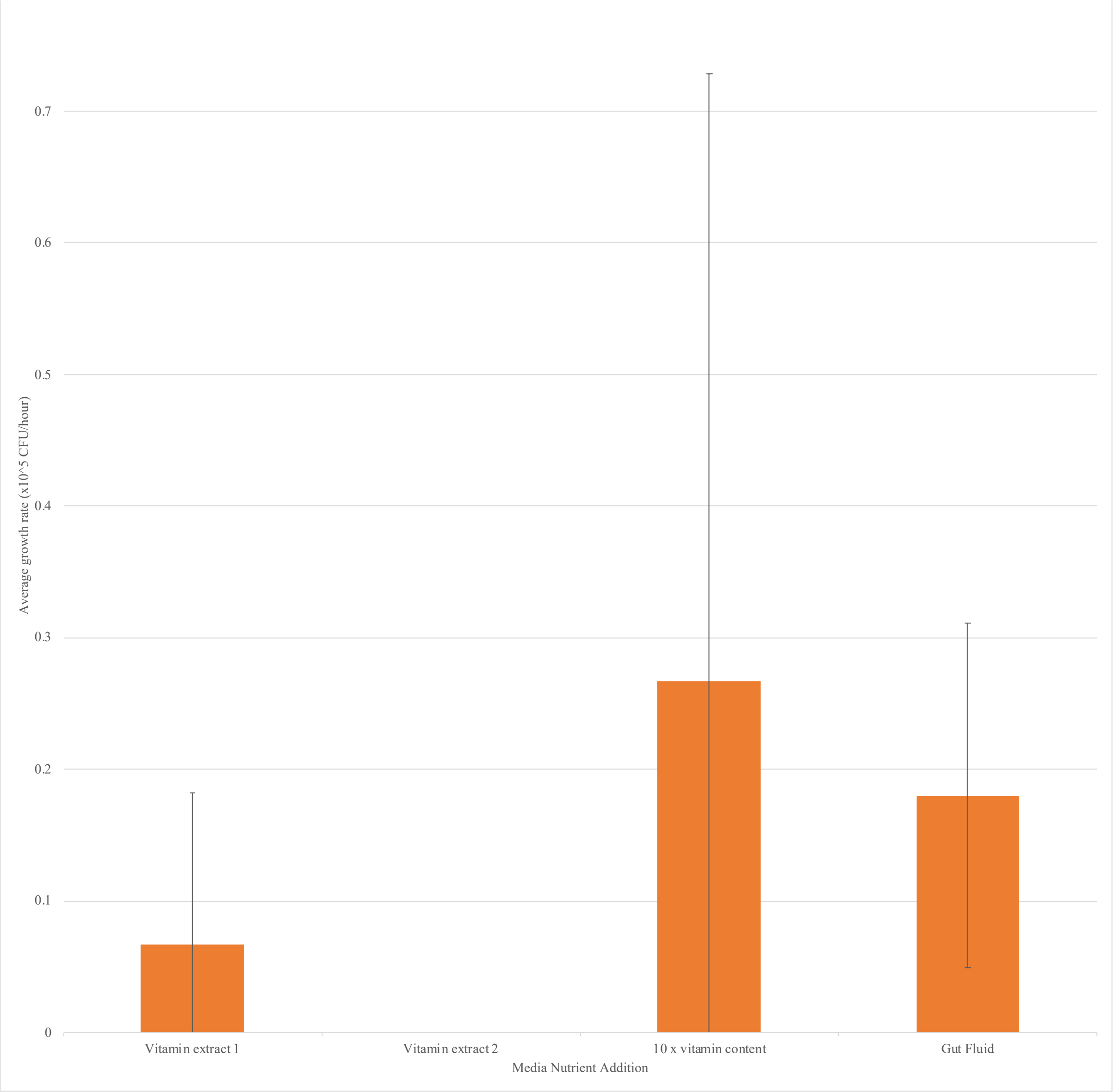


Figure 58: Average growth rate of *Lachnospiraceae* isolate BP5G, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. With standard deviation.

The average growth rate of *Lachnospiraceae* isolate BP5G on YCFA media was shown in Figure 58 to increase compared to gut fluid with the addition ten times the normal amount of vitamin mixture (Figure 58). However, there was a large amount of variation between replicates in these results. The significance of these differences will be further investigated using statistical tests. These results suggest that, for *Lachnospiraceae* isolate BP5G, a ten times increase in vitamin concentration can be used as a defined alternative to gut fluid in YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were no significant differences in growth rates between any of the variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of isolate BP5G in CFU per hour.

The one way ANOVA revealed that there was not a statistically significant difference at the 0.05 level in mean growth rate between any of the nutrients tested (F(3, 8) = [0.690], p = 0.583).

Summary

The results of the one way ANOVA and post-hoc Tukey test show that mean growth rates of YCFA with vitamin extracts 1 and 2, 10 x vitamin and *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level. However, the average growth of *Lachnospiraceae* isolate BP5G on YCFA media with 10 x vitamin was faster than that with gut fluid. Therefore, for *Lachnospiraceae* isolate BP5G the optimal defined medium tested is YCFA with 10 times the normal amount of vitamin mix, with an average growth rate of 0.264 (± 0.462) x 10^5 CFU/hour. However, vitamin extract 1 can also be used as an alternative to gut fluid in YCFA media for this isolate, as this media produces a similar average rate of growth to YCFA with gut fluid.

Oscillospiraceae isolate BP47G

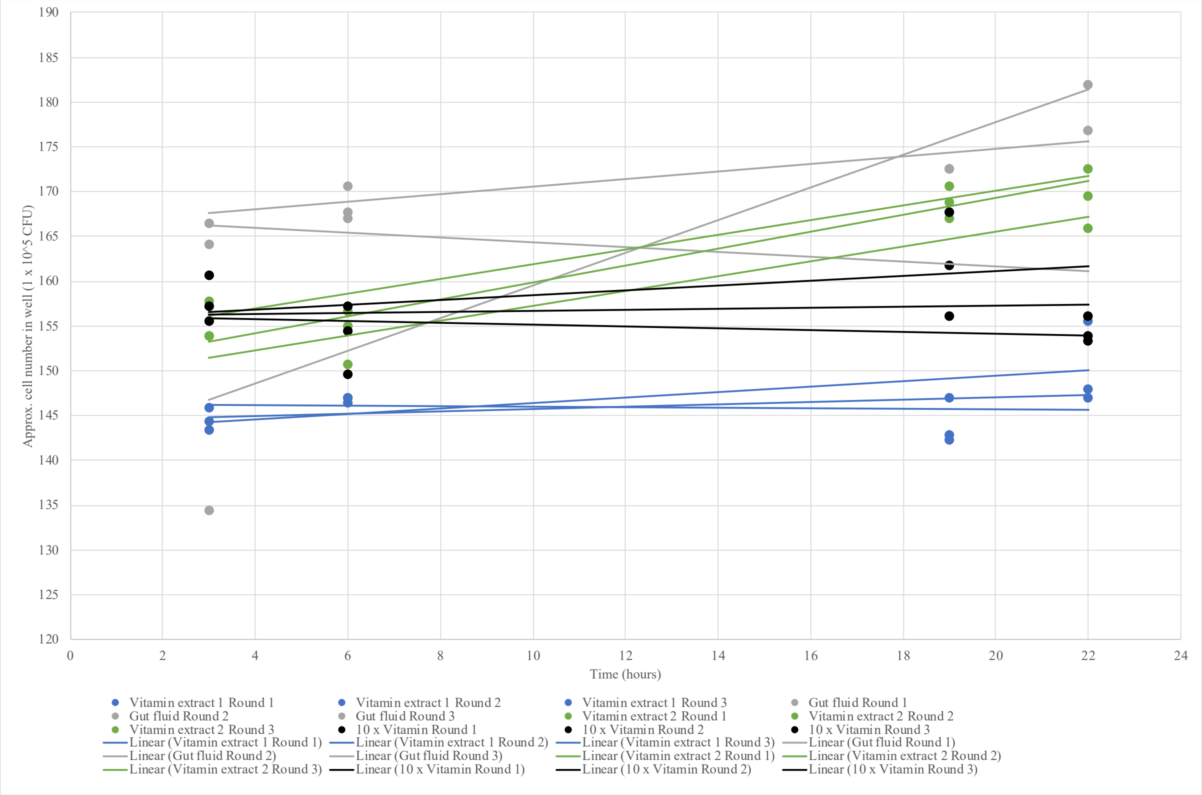


Figure 59: Growth of *Oscillospiraceae* isolate BP47G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid.

The results presented in Figure 59 show that *Oscillospiraceae* isolate BP47G a similar rate of growth with each of the five trialled nutrient additions to standard YCFA medium in this experiment. In all cases the rate of growth was very low.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 59.

These results are presented in Table 21, along with average growth rates across all three rounds and associated standard deviations.

Table 21: Growth rates of *Oscillospiraceae* isolate BP47G over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Vitamin extract 1 of U. pinnatifida	1	0.130	0.144	0.151
	2	0.301		
	3	0.000		
Vitamin extract 2 of U. pinnatifida	1	0.831	0.866	0.065
	2	0.941		
	3	0.826		
YCFA medium with 10 times the usual amount of vitamin extract	1	0.000	0.112	0.145
	2	0.060		
	3	0.275		
Gut fluid	1	0.000	0.747	0.955
	2	0.418		
	3	1.823		

The results in Table 21 show a positive average growth rate for *Oscillospiraceae* isolate BP47G on all media. The average growth rates across all three rounds for each media nutrient addition, calculated in Table 21, are presented in Figure 60 as a bar chart with standard deviation.

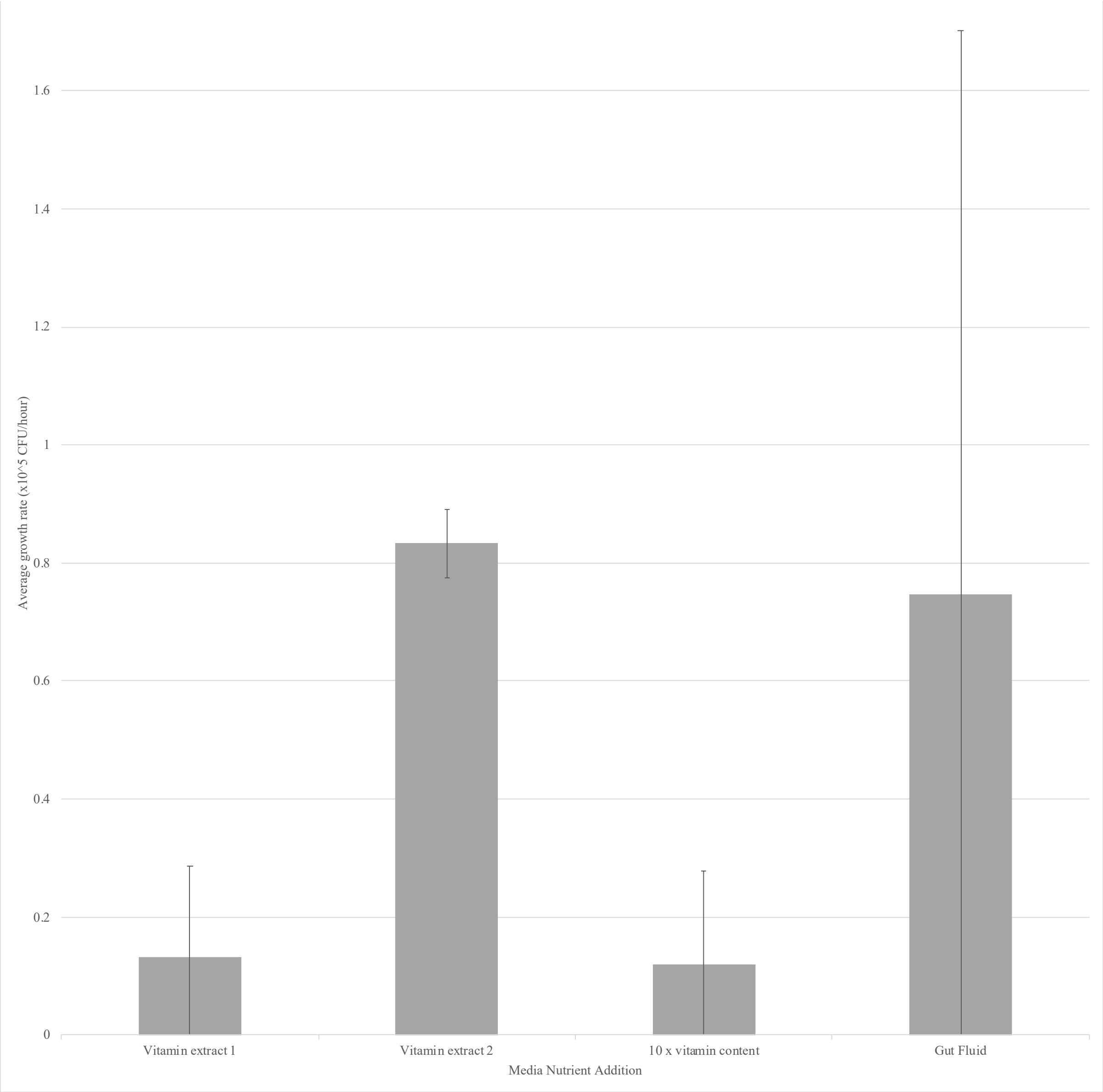


Figure 60: Average growth rate of *Oscillospiraceae* isolate BP47G, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. With standard deviation.

The average growth rate of *Oscillospiraceae* isolate BP47G on YCFA media was shown in Figure 60 to be similar or reduced when compared to gut fluid for all vitamin nutrient additions (Figure 60). The significance of these differences will be further investigated using statistical tests. These results suggest that, for *Oscillospiraceae* isolate BP47G, none of the vitamin additions trialled provide a viable alternative to gut fluid as a nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were no significant differences in growth rates between any of the variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of isolate BP47G in CFU per hour.

The one way ANOVA revealed that there was not a statistically significant difference at the 0.05 level in mean growth rate between any of the nutrients tested ($F(3, 8) = [2.183]$, $p = 0.168$).

Summary

The results of the one way ANOVA and post-hoc Tukey test show that mean growth rates of YCFA with vitamin extracts 1 and 2, 10 x vitamin and *K. sydneyanus* gut fluid were not significantly different from each other at the 0.05 level. However, the average growth of *Oscillospiraceae* isolate BP47G on YCFA media vitamin extract 2 was faster than that with gut fluid. Therefore, for *Oscillospiraceae* isolate BP47G the optimal defined medium tested is YCFA with vitamin extract 2, with an average growth rate of $0.866 (\pm 0.065) \times 10^5$ CFU/hour. However, vitamin extract 1 and 10 x vitamin extract can also be used as an alternative to gut fluid in YCFA media for this isolate, as this media produces a similar average rate of growth to YCFA with gut fluid.

Mixture of all three isolates (BP47G, BP5G and BP38)

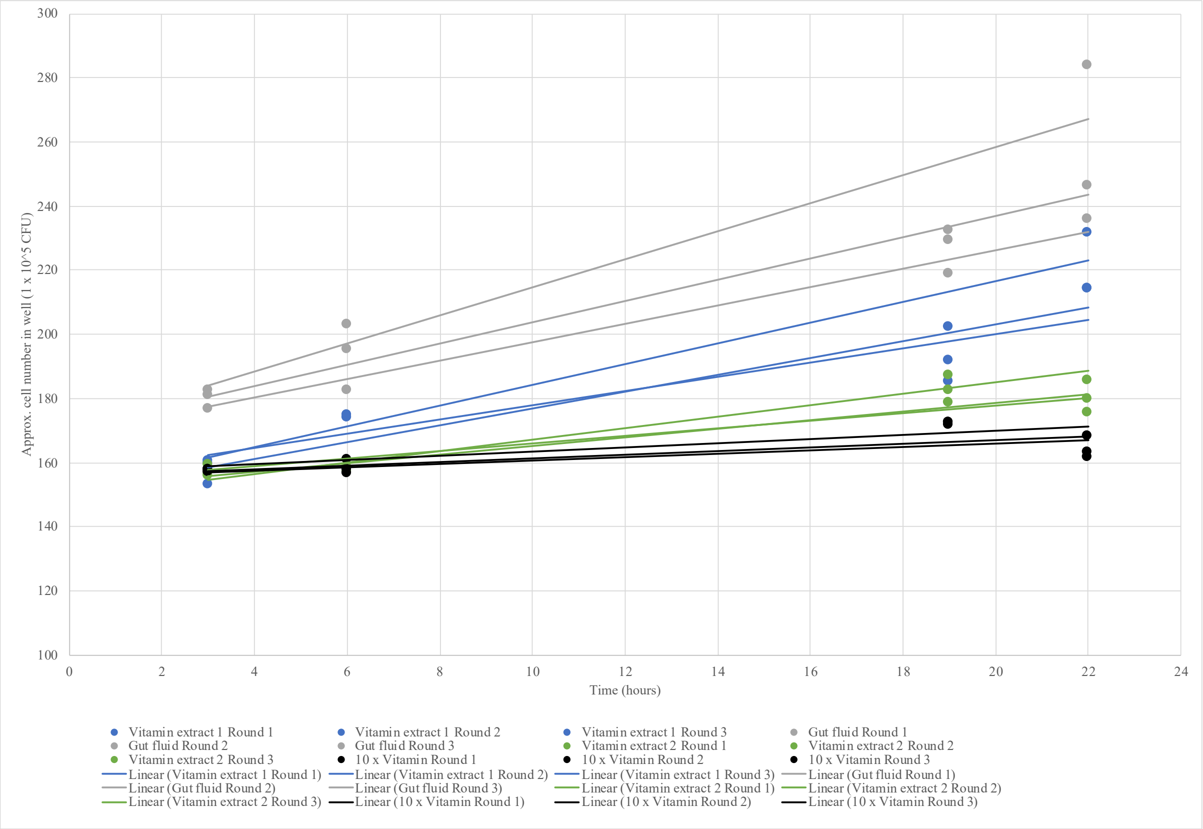


Figure 61: Growth of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22) with linear trendlines. Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid.

The results presented in Figure 61 show that a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 presented the fastest rate of growth with the nutrient addition of *K. sydneyanus* gut fluid to standard YCFA medium. The addition of the two *Undaria* vitamin extracts produced the second and third fastest growth rates. The nutrient addition of ten times the normal concentration of vitamins to YCFA media produced the slowest rate of growth out of all nutrient additions tested in this experiment.

The growth rates, in increase in colony forming units (CFUs) per hour, are calculated for each round from the gradients of the linear trendlines associated with the results in Figure 61. These results are presented in Table 22, along with average growth rates across all three rounds and associated standard deviations.

Table 22: Growth rates of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 over 22 hours. Growth measured by absorbance at 600 nm at four timepoints (3, 6, 19 and 22). Three rounds for each of five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. Growth rates calculated as an increase in colony forming units (CFUs) per hour. An average growth rate across the three rounds is presented, with standard deviation.

YCFA Media addition	**Round**	**Growth rates of each round (x 10^5 CFU/hour)**	**Average growth rate of each media (x 10^5 CFU/hour)**	**± STD**
Vitamin extract 1 of U. pinnatifida	1	2.619	2.687	0.510
	2	2.214		
	3	3.228		
Vitamin extract 2 of U. pinnatifida	1	1.803	1.433	0.329
	2	1.172		
	3	1.324		
YCFA medium with 10 times the usual amount of vitamin extract	1	0.516	0.585	0.068
	2	0.652		
	3	0.586		
Gut fluid	1	3.324	3.513	0.771
	2	4.360		
	3	2.854		

The results in Table 22 show a positive average growth rate for a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 on all media.

The average growth rates across all three rounds for each media nutrient addition, calculated in Table 22, are presented in Figure 62 as a bar chart with standard deviation.

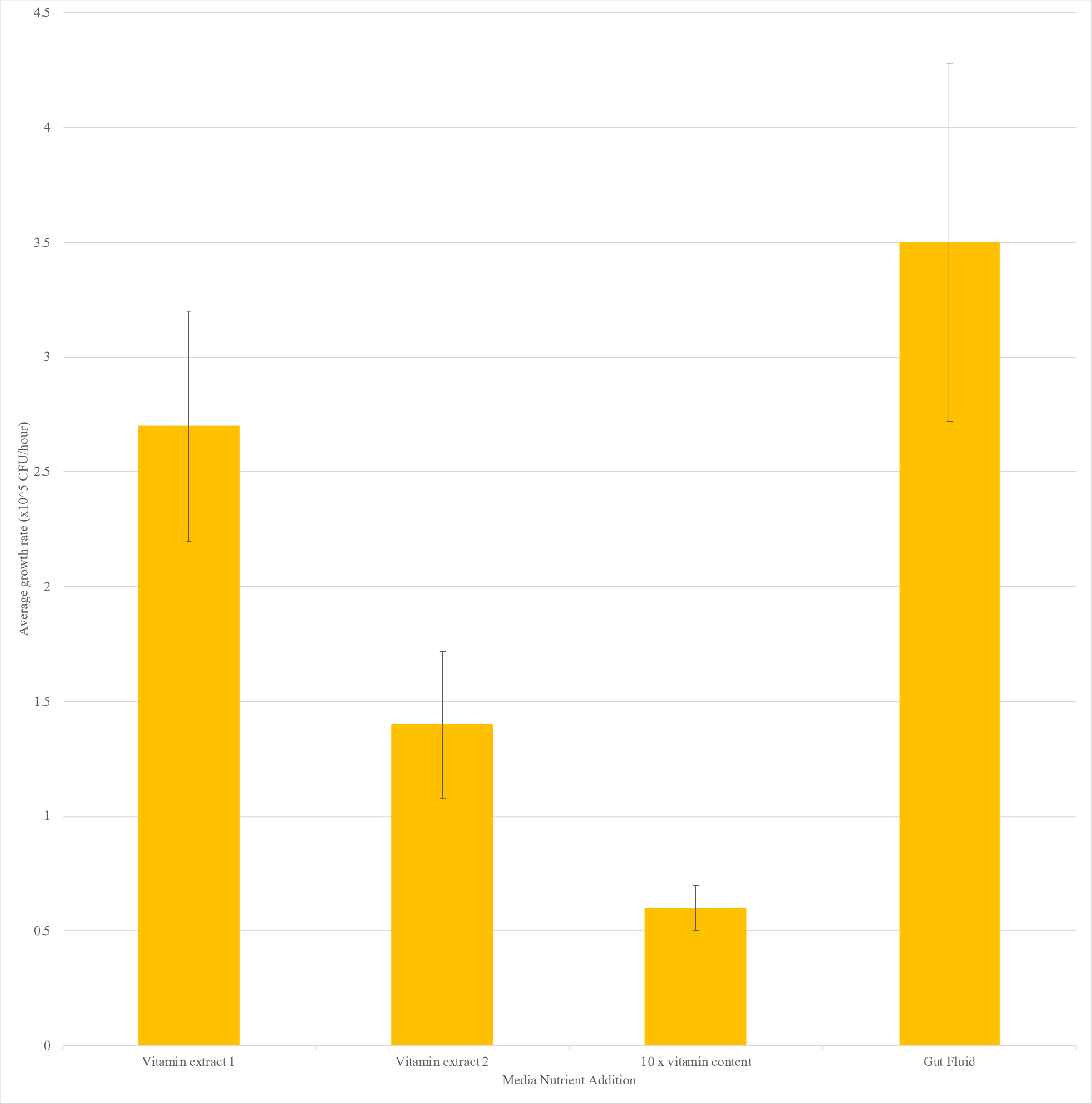


Figure 62: Average growth rate of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38, measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. With standard deviation.

The average growth rate of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 on YCFA media was shown in Figure 62 to be similar or reduced when compared to gut fluid for all vitamin nutrient additions (Figure 62). The significance of these differences will be further investigated using statistical tests. These results suggest that, for a mix of all three isolates, none of the vitamin additions trialled provide a viable alternative to gut fluid as a nutrient addition to YCFA media.

Statistical significance of growth rate results

A one way ANOVA, followed by a post hoc Tukey test, conducted in SPSS statistics, found there were significant differences in growth rates between some variables.

The one way ANOVA was performed to compare the effect of nutrient addition to YCFA medium on growth rate of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 in CFU per hour.

The one way ANOVA revealed a statistically significant difference in mean growth rate between at least two nutrients tested (F(3, 8) = [20.879, p = <0.001).

Tukey's HSD test for multiple comparisons found that the mean value of growth rate was significantly different between vitamin extract 1 and 10 x vitamin (p = 0.004, 95% C.I. = [0.779, 3.354]), vitamin extract 2 and gut fluid (p = 0.004, 95% C.I. = [-3.388, -0.812]) and 10 x vitamin and gut fluid (p = <0.001, 95% C.I. = [-4.211, -1.646]).

There was no statistically significant difference in growth rate between any of the other nutrient additions to YCFA medium: vitamin extract 1 and vitamin extract 2 (p = 0.060), vitamin extract 1 and gut fluid (p = 0.215), vitamin extract 2 and 10 x vitamin extract (p = 0.240).

Summary

The results of the one way ANOVA and post-hoc Tukey test show that mean growth rates of a mix of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G and *Rikenellaceae* isolate BP38 on YCFA media with *K. sydneyanus* gut fluid were significantly faster than vitamin extract 2 or 10 x vitamin ($p = \leq 0.004$). The average growth of the mix of three isolates on YCFA media with vitamin extract 1 was significantly faster than that on 10 x vitamin (p = 0.004), but not significantly slower than that on YCFA with gut fluid (0.215), and not significantly faster than that on vitamin extract 2 (p = 0.06). Therefore, for the mix of three isolates the optimal defined medium tested is YCFA with gut fluid with an average growth rate of 3.513 (± 0.771) x 10^5 CFU/hour, or vitamin extract 1 with an average growth rate of 2.687 (± 0.510) x 10^5 CFU/hour.

All isolates vitamin utilisation

This summary allows comparison of all growth rate results for fish gut bacterial isolates cultured on YCFA media with the addition of one of three vitamin nutrient additions, or *K. sydneyanus* gut fluid. These results are displayed in Figure 63 and analysed for significance in further statistical tests. This analysis reveals multiple viable defined and simplified alternatives to the complex YCFA-gut fluid media for the *in vitro* culture of some fish gut bacterial isolates.

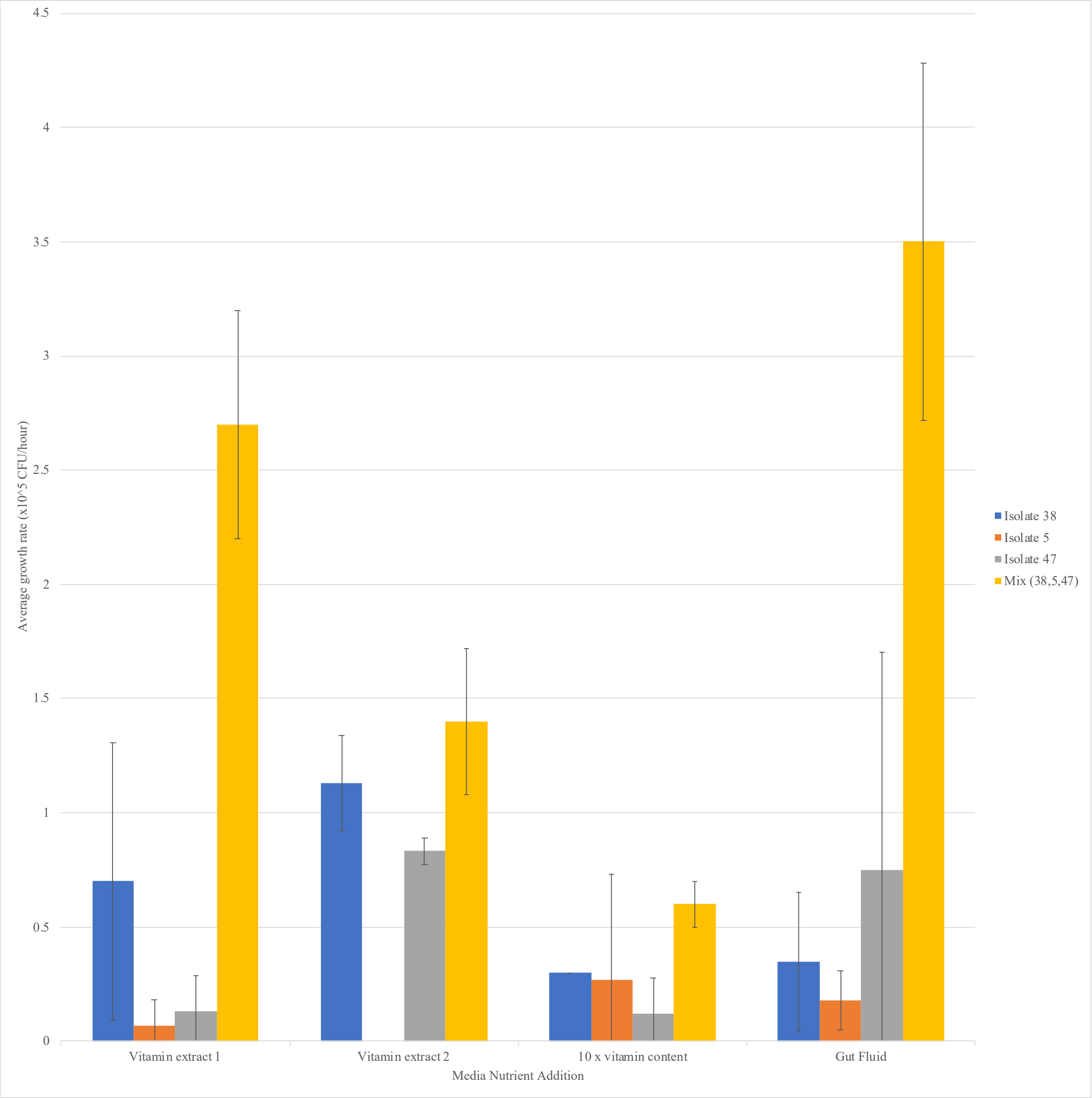


Figure 63: Average growth rates of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G, *Rikenellaceae* isolate BP38 and a mix of all three measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. With standard deviation.

Figure 63 allows the comparison of average growth rates on each variation of YCFA media for all isolates, including *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G,

Rikenellaceae BP38 and a mix of all three. This cross-isolate comparison reveals that the fastest average growth rate was achieved by a mix of all three isolates on YCFA with gut fluid. The comparison in Figure 63 reveals that a mix of all three isolates produced the fastest average growth rate of all the isolates on all media. Isolate BP38 appeared to produce the second fastest rate of growth on all media except YCFA with gut fluid, with the fastest average rates of growth for isolate BP38 occurring on YCFA with *U. pinnatifida* vitamin extracts one and two. Isolate BP38 and a mix of all three isolates performed better than isolate BP47G and isolate BP5G on all media except YCFA with gut fluid, where isolate BP47G had a faster growth rate than BP38. Overall, Figure 63 shows that either YCFA with vitamin extract 1, or with 10 x vitamin extract, present the most successful defined media alternatives to YCFA with *K. sydneyanus* gut fluid. All isolates presented successful growth on these media. However, these results show that YCFA with gut fluid presented the most reliable strong growth across all isolates. The significance of these differences will be further investigated using statistical tests. These results suggest that for all isolates none of the vitamin additions tested represent a good alternative to YCFA media with gut fluid.

Statistical significance of growth rate results

A two-way ANOVA was performed to analyse the effect of isolate and vitamin (or gut fluid) nutrient addition on mean value growth rate.

The two-way ANOVA revealed that there was a statistically significant interaction at the 0.05 level between the effects of isolate and nutrient addition on growth rate (F(9, 33) = [8.903], p = <0.001).

Simple main effects analysis showed that isolate had a significant effect on growth rate (p = <0.001). A post-hoc Tukey's HSD test for multiple comparisons found that the mean value growth rate was significantly different between: a mix of all three isolates and isolate BP5G (p = <0.001, 95% C.I. [1.481, 2.379]), a mix of all three isolates and isolate BP38 (p = <0.001, 95% C.I. [0.993, 1.890]), a mix of all three isolates and isolate BP47G (p = <0.001, 95% C.I. [1.177, 2.074]), isolate BP5G and isolate BP38 (p = 0.029, 95% C.I. [-0.937, -0.040]). There was no significant difference in mean value growth rate between isolate BP5G and isolate BP47G (p = 0.275) or isolate BP38 and isolate BP47G (p = 0.685).

Simple main effects analysis showed that nutrient addition also had a significant effect on mean growth rate across all isolates (p = <0.001). A post-hoc Tukey's HSD test for multiple comparisons found that the mean value growth rate was significantly different between 10 x vitamin and all other nutrient additions: gut fluid (p = <0.001, 95% C.I. = [-1.344, -0.447]), vitamin extract 1 (p = 0.006, 95% C.I. = [-1.041, -0.144]) and vitamin extract 2 (p = 0.011, 95% C.I. = [-0.999, -0.102]). There was no significant difference in mean value growth rate between any other nutrients, which included gut fluid and vitamin extract 1 (p = 0.277), gut fluid and vitamin extract 2 (p = 0.180) and vitamin extract 1 and vitamin extract 2 (p = 0.994).

Summary

Post-hoc Tukey's testing of the isolates in this experiment found that across all YCFA media variations the mix of all three isolates grew significantly faster than each individual isolate (p < 0.001). Of the individual isolates, *Rikenellaceae* isolate BP38, grew significantly faster than *Lachnospiraceae* isolate BP5G (p = 0.029). Whereas the average growth rates of

Oscillospiraceae isolate BP47G did not vary significantly from *Lachnospiraceae* isolate BP5G (p = 0.275) or *Rikenellaceae* BP38 (p = 0.685).

These results show YCFA with gut fluid produces the best growth for these bacteria, YCFA with vitamin extracts 1 or 2 the second best, and YCFA with 10 x vitamin the worst. Post-hoc Tukey's testing also found that YCFA with vitamin extract 1 and vitamin extract 2 can be used as alternative media to YCFA with gut fluid for the *in vitro* cultivation of these *K. sydneyanus* gut bacterial isolates as the average growth rate across all isolates in these media alternatives is not significantly different to that of YCFA with gut fluid (p = ≥ 0.18). The average rate of growth across all isolates produced by vitamin extracts 1 and 2 were very similar, with a significant difference close to one (p = 0.994). The 10 x vitamin addition to YCFA media produced a significantly slower rate of growth than all other nutrient additions (p = ≤ 0.011). Therefore, these experiments have produced a new alternative defined media recipe of YCFA with the vitamin extract of *U. pinnatifida*, which can be used for future experimental work on these *K. sydneyanus* isolates *in vitro*. However, this media alternative would decrease the performance of these bacteria in laboratory experiments, therefore increasing the difficulty of future laboratory experimentation.

Results Conclusion: All isolates nutrient utilisation

This summary allows comparison of all growth rate results for fish gut bacterial isolates cultured on YCFA media with the addition of eleven different potential growth factors, or *K. sydneyanus* gut fluid. These results are displayed in Figure 64 and analysed for significance in further statistical tests. This analysis reveals multiple viable defined and simplified alternatives to the complex YCFA-gut fluid media for the *in vitro* culture of some fish gut bacterial isolates.

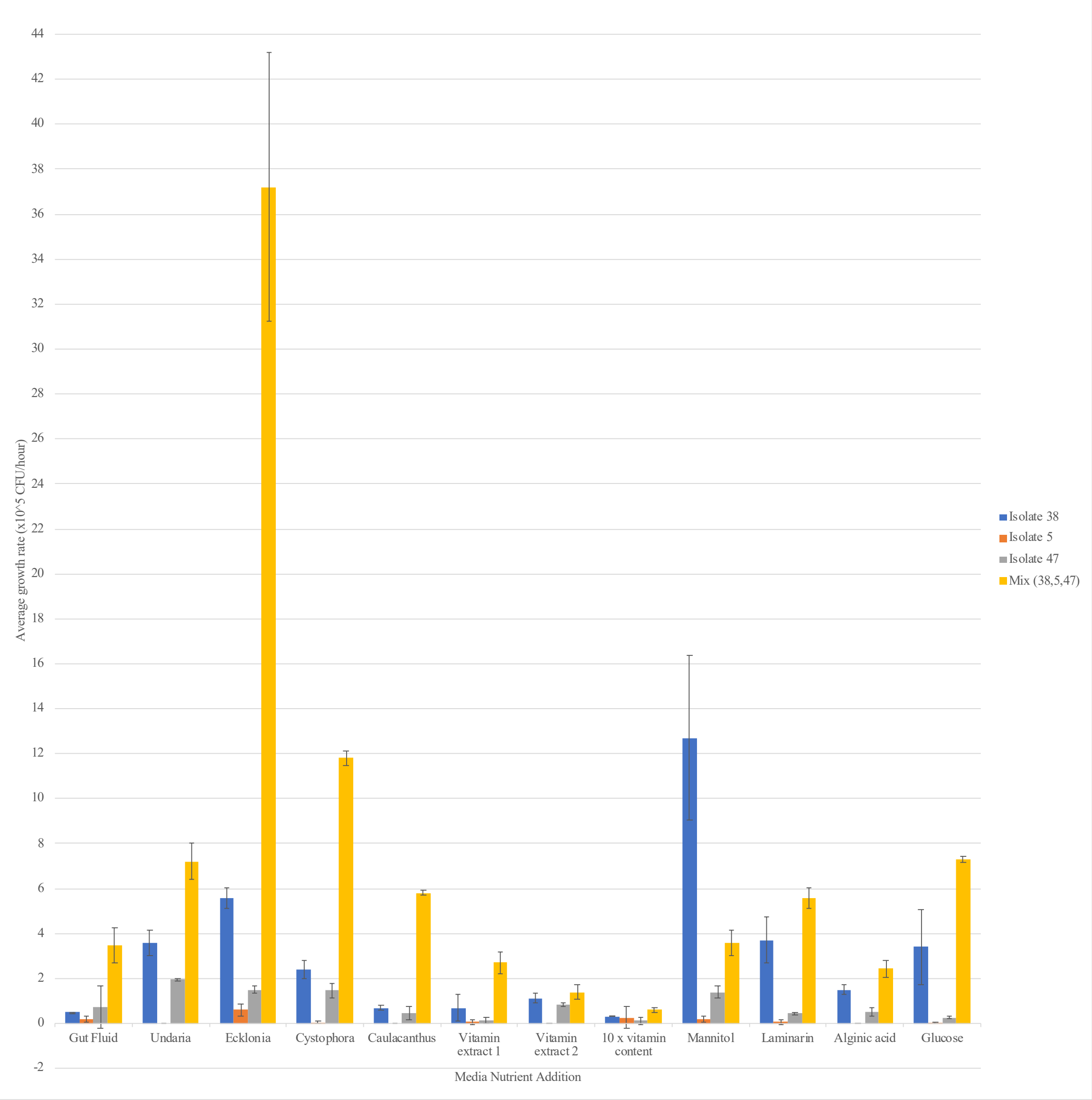


Figure 64: Average growth rates of *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G, *Rikenellaceae* isolate BP38 and a mix of all three measured by increase in number of colony forming units (CFUs) over time (hours). Growth measured on five media types, YCFA with vitamin extract 1, vitamin extract 2, 10 x vitamin or gut fluid. With standard deviation.

Figure 64 allows the comparison of average growth rates on each variation of YCFA media for all isolates, including *Oscillospiraceae* isolate BP47G, *Lachnospiraceae* isolate BP5G, *Rikenellaceae* BP38 and a mix of all three. This cross-isolate comparison for all nutrients trialled in this PhD reveals that the fastest average growth rate was achieved by a mix of all three isolates on YCFA with *Ecklonia*. The comparison in Figure 64 reveals that a mix of all three isolates produced the fastest average growth rate of on the majority of media trialled. Isolate BP38 appeared to produce the second fastest rate of growth on a majority of the media trialled, with the fastest average rates of growth for isolate BP38 occurring on YCFA with *mannitol*. This rate of growth drew with the growth of the mix on YCFA with *Cystophora* as the second fastest overall. Isolate BP38 and a mix of all three isolates appeared to perform better than isolate BP47G and isolate BP5G on all media except YCFA with gut fluid, where isolate BP47G had a faster growth rate than BP38. Overall, Figure 64 shows that YCFA with either *Ecklonia*, mannitol, *Undaria*, *Cystophora* or laminarin present the most successful alternatives to YCFA with gut fluid. All isolates presented successful growth on these media, with much of this growth exceeding the rate of that on YCFA with *K. sydneyanus* gut fluid. The significance of these differences will be further investigated using statistical tests. These results suggest that for all isolates there are multiple simplified media recipes that represent a good alternative to YCFA media with gut fluid.

Statistical significance of growth rate results

A two-way ANOVA was performed to analyse the effect of isolate and nutrient addition on mean value growth rate.

The two-way ANOVA revealed that there was a statistically significant interaction at the 0.05 level between the effects of isolate and nutrient addition on growth rate (F(33, 121) =

[69.925], p = <0.001). There was a statistically significant interaction at the 0.05 level between isolate and growth rate (F(3, 121) = [377.597], p = <0.001). There was a statistically significant interaction at the 0.05 level between nutrient and growth rate (F(11, 121) = [106.120], p = <0.001).

Simple main effects analysis showed that isolate had a significant effect on growth rate (p = <0.001). A post-hoc Tukey's HSD test for multiple comparisons found that the mean value growth rate was significantly different between all isolates: a mix of all three isolates and isolate BP5G (p = <0.001, 95% C.I. [6.140, 7.330]), a mix of all three isolates and isolate BP38 (p = <0.001, 95% C.I. [3.639, 4.828]), a mix of all three isolates and isolate BP47G (p = <0.001, 95% C.I. [5.468, 6.658]), isolate BP5G and isolate BP38 (p = <0.001, 95% C.I. [-3.096, -1.907]), isolate BP5G and isolate BP47G (p = 0.020, 95% C.I. [-1.266, -0.077]), isolate BP38 and isolate BP47G (p = <0.001, 95% C.I. [1.235, 2.424]).

Simple main effects analysis showed that nutrient addition also had a significant effect on growth rate (p = <0.001). A post-hoc Tukey's HSD test for multiple comparisons found that the mean value growth rate was significantly different between *Ecklonia* and all other nutrient additions: gut fluid (p = <0.001, 95% C.I. = [8.876, 11.200]), mannitol (p = <0.001, 95% C.I. = [4.389, 7.236]), laminarin (p = <0.001, 95% C.I. = [8.243, 11.090]), alginic acid (p = <0.001, 95% C.I. = [8.868, 11.715]), glucose (p = <0.001, 95% C.I. = [7.032, 9.878]), *Undaria* (p = <0.001, 95% C.I. = [6.613, 9.460]), *Cystophora* (p = <0.001, 95% C.I. = [5.873, 8.720]), *Caulacanthus* (p = <0.001, 95% C.I. = [8.052, 10.898]), vitamin extract 1 (p = <0.001, 95% C.I. = [8.918, 11.765]), vitamin extract 2 (p = <0.001, 95% C.I. = [8.960, 11.807]) and 10 x vitamin (p = <0.001, 95% C.I. = [9.511, 12.357]).

A post-hoc Tukey's HSD test for multiple comparisons found that the mean value growth rate was significantly different between mannitol and all other nutrient additions: gut fluid (p = <0.001, 95% C.I. = [3.064, 5.388]), laminarin (p = <0.001, 95% C.I. = [2.431, 5.277]), alginic acid (p = <0.001, 95% C.I. = [3.056, 5.902]), glucose (p = <0.001, 95% C.I. = [1.219, 4.066]), *Undaria* (p = <0.001, 95% C.I. = [0.801, 3.647]), *Ecklonia* (p = <0.001, 95% C.I. = [-7.236, -4.389]), *Cystophora* (p = 0.033, 95% C.I. = [0.061, 2.907]), *Caulacanthus* (p = <0.001, 95% C.I. = [2.239, 5.086]), vitamin extract 1 (p = <0.001, 95% C.I. = [3.106, 5.952]), vitamin extract 2 (p = <0.001, 95% C.I. = [3.148, 5.994]) and 10 x vitamin (p = <0.001, 95% C.I. = [3.698, 6.545]).

Nutrient additions that cause a significant increase in growth rate in comparison to gut fluid are: mannitol (p = <0.001, 95% C.I. = [3.064, 5.388]), glucose (p = <0.001, 95% C.I. = [0.421, 2.745]), *Undaria* (p = <0.001, 95% C.I. = [0.840, 3.164]), *Ecklonia* (p = <0.001, 95% C.I. = [8.876, 11.200]) and *Cystophora* (p = <0.001, 95% C.I. = [1.580, 3.904]).

There was no significant difference in mean value growth rate between gut fluid and any of the other nutrients, which included laminarin (p = 0.995), alginic acid (p = 1.000), *Caulacanthus* (p = 0.900), vitamin extract 1 (p = 0.999), vitamin extract 2 (p = 0.998) and 10 x vitamin (p = 0.309). Therefore, no nutrient addition produced a significantly slower average growth rate across all isolates than *K. sydneyanus* gut fluid.

Summary

Post-hoc Tukey's testing of the isolates across all YCFA media variations trialled in this PhD found that growth rates were significantly different between all isolates (p = ≤ 0.02). The mix of all three isolates had the fastest growth rate of all isolates trialled, with the average rate of growth across all media being significantly faster than each of the three individual isolates (p < 0.001). Of the individual isolates, *Rikenellaceae* isolate BP38, grew significantly faster than both *Lachnospiraceae* isolate BP5G (p = < 0.001) and *Oscillospiraceae* isolate BP47G (p = <0.001). The average growth rate of *Oscillospiraceae* isolate BP47G was significantly faster than that of *Lachnospiraceae* isolate BP5G (p = 0.020).

These results show YCFA with *Ecklonia* produces the best growth on average across all isolates, with significantly faster growth on this media than all eleven others trialled in this PhD (p = < 0.001). The second best average growth rate across all isolates was seen on YCFA with mannitol, which was significantly faster than all media other than YCFA with *Ecklonia* (p = ≤ 0.033). Post-hoc Tukey's testing also found that three other YCFA media nutrient additions trialled produced a faster average growth rate across all isolates than YCFA with gut fluid: glucose (p = < 0.001), *Undaria* (p = < 0.001) and *Cystophora* (p = < 0.001).

Therefore, across three *K. sydneyanus* gut bacterial isolates and a mix of all three, five media additions have been found within this PhD to produce a faster growth rate than the standard YCFA medium with *K. sydneyanus* gut fluid: *Ecklonia* extract, *Undaria* extract, *Cystophora* extract, mannitol and glucose. These novel media recipes can be used as alternative to YCFA with gut fluid for the *in vitro* cultivation of these *K. sydneyanus* gut bacterial isolates. These media alternatives would increase the performance of these bacteria in laboratory experiments, increasing the ease of future laboratory experimentation.

There were a further six nutrient additions to YCFA media trialled within this PhD: laminarin, alginic acid, *Caulacanthus* extract, vitamin extract 1, vitamin extract 2 and 10 x vitamin. The average growth rate across all isolates in these media alternatives were not significantly different to that of YCFA with gut fluid at the 0.05 level. Therefore, these alternative media recipes may also be used in place of YCFA media with experiments have produced a new alternative defined media recipe of YCFA with *K. sydneyanus* gut fluid. However, these alternatives would not be as reliable in producing strong isolate growth as the five media found to produce significantly faster growth than YCFA with gut fluid.

Chapter 6: Discussion

Introduction

The results of the experimental work of this PhD research, detailed in **Chapter 5**, reveal the functional digestion of various nutrients by bacteria isolated from the gut of the marine herbivorous fish *Kyphosus sydneyanus.* This chapter discusses these results in the context of the **Chapter 2** literature review. The bacteria used in these experiments were isolated from *K. sydneyanus* gut Section IV, in the hindgut where most bacterial fermentation takes place (Clements, et al., 1994). Despite gut bacteria of the herbivorous fish not being as closely associated with the host as that of terrestrial vertebrates, *K. sydneyanus* have evolved a dependency on gut bacteria for digestion, with the evolution of a specialised hindgut, in which a large amount of bacteria can perform fermentation of brown algae to SCFAs for the nutritional benefit of the host (Clements & Choat, 1993; Rimmer & Wiebe, 1987; Clements, 1994). This PhD explores the concepts of bacterial co-evolution, niche partitioning and consortial mutually beneficial digestion among the *K. sydneyanus* gut microbiota.

There was an overall significant difference in growth rate between each of the three bacteria species, and the mix. The best performing inoculum overall was the mixture of all three species. This shows that the *Rikenellaceae* isolate (BP38), *Oscillospiraceae* isolate (BP47G) and *Lachnospiraceae* isolate (BP5G) can be successfully cultured together. This result also suggests that mutually beneficial digestive mechanisms are shared between these three isolates. This is a major finding of this PhD, providing functional evidence that cross-feeding between bacterial species is essential for optimal microbial metabolism of complex substrates in the fish gut, agreeing with predictions by Seeto, et al., (1996) that the gut microbiota of herbivorous marine fish is consortial in nature.

Functional evidence of the co-evolution of *K. sydneyanus* and their gut bacteria, is a clear preference for the *in vitro* culture conditions most similar to the natural environment of the *K. sydneyanus* gut. By far the fastest rate of growth overall being on YCFA with *E. radiata* extract, above that achieved by any single species. This is a brown algae that makes up the majority of the *K. sydneyanus* diet off the Northeastern New Zealand coast (Clements & Choat, 1997; Moran & Clements, 2002). These results show that *K. sydneyanus* gut bacteria perform well *in vitro* when provided with a nutrient source similar to that seen in their natural environment. Further evidence for co-evolution between *K. sydneyanus* and their hindgut bacteria is the increase in growth rate of the bacteria on the more difficult to digest brown algae compared to the easily accessible red algae.

The mix of all three bacteria also grew faster on the complex algal extracts than on simple sugars, suggesting there is niche partitioning of *K. sydneyanus* gut bacteria. These results show that each species tested is able to access different nutrients for growth within the seaweed that makes up the majority of the diet of their host organism.

A significant difference in bacterial growth rate was also seen when certain nutrients were added to the culture media. Of the eleven alternative YCFA recipes, none produced a significantly slower pan-isolate growth rate than YCFA with gut fluid. Therefore, this work has produced eleven simpler alternatives to the complex undefined YCFA media with gut fluid that can be relied upon to replace YCFA with gut fluid to culture *K. sydneyanus* gut bacteria *in vitro*. This culture based work enables future researchers working on these bacteria to be confident in their use of these alternative recipes.

This discussion has produced four reliable and replicable novel media recipes for the optimal *in vitro* culture of *K. sydneyanus* gut bacteria. These media are YCFA with mannitol, laminarin, glucose or alginic acid. The best performing media across all isolates was YCFA with mannitol or glucose. Use of these defined alternatives to YCFA media with gut fluid will increase accuracy and reliability of future research on these bacteria. These novel media may then be used in functional applications, such as large scale seaweed fermentation.

This discussion will also provide information on the functional digestive pathways possessed by these *K. sydneyanus* gut bacteria species. These pathways have been analysed in herbivorous fish with genomic tools (Seeto, et al., 1996). However, this PhD reveals which pathways are active *in vitro*, and can therefore be reliably utilised for real world industrial applications. These findings represent an important guide for scientific partners in industry with an interest in utilising the natural digestive abilities of fish gut bacteria for the development of high value products.

Discussion of bacterial isolates used

Methodology used to measure growth rates of the four variations in *K. sydneyanus* gut bacteria inoculum

The bacteria used in this PhD was isolated from Section IV of the *K. sydneyanus* gut. Section IV is the second to last section of the *K. sydneyanus* gut. By Section IV algae has been digested to a paste made up of small particles. Simple and easy to access energy sources present in algae are quickly digested by bacterial fermentation or endogenous enzymes in the early gut sections. Bacteria in Section IV of the *K. sydneyanus* gut must make use of the remaining nutrients. The results of growth rate experiments in **Chapter 5** agree with White, et al., (2010), that simple carbohydrates and sugar alcohols such as mannitol are fermented by the fish gut bacteria. This study found that Section IV bacteria can utilise these simple energy sources. This was shown when simple sugars such as mannitol produced good rates of growth were provided *in vitro*. These results are further analysed in the discussion of carbohydrate and sugar alcohol nutrient additions later in this chapter.

The main aim of this PhD was to produce a defined and replicable media that may be used in place of YCFA with *K. sydneyanus* gut fluid. As can be seen from the growth experiments within this PhD, the results of which are detailed in **Chapter 5**, YCFA with gut fluid produced a large amount of variation in growth rate between bacteria species. This unreliable growth success among the different species that make up the *K. sydneyanus* gut microbiome hinders experimental reproducibility across all bacteria of interest and delays research work. The large variation in growth success of *K. sydneyanus* gut bacteria on this media is likely caused by a variation in nutrient load between *K. sydneyanus* gut fluid samples. Gut fluid is extracted from the gut contents of spear caught *K. sydneyanus* fish off the Northeastern New Zealand coast, then processed into gut fluid for addition to media, as detailed in **Chapters 3 and 4**. Therefore, it is impossible to maintain a consistent media recipe and subsequent bacterial culture success when culturing on YCFA media with fish gut fluid.

There are many bacteria present in each section in the *K. sydneyanus* gut. To find if these isolates benefit from coexisting in the same niche, three isolates were grown together in a mix during these growth rate experiments. The cross-media significant increase in growth rate of the mix compared to any individual isolate suggests that these bacteria have a mutually beneficial relationship, where complementary nutrient digestion pathways allow a greater total amount of energy to be accessed from a range of substrates when multiple bacteria species are present. This finding will be explored further in the mixture of isolates sections within this discussion.

Growth experiments carried out in this PhD reveal the varying *in vitro* growth success of three individual *K. sydneyanus* gut bacteria isolates and a mix of all three together. The three bacterial species examined in this study were all isolated from the same ecological niche of Section IV of the *K. sydneyanus* gut. Despite this, these results revealed significant differences in the growth rates of these isolates when cultured on 12 different media. This significant difference in growth between species on the same nutrients suggests that there are species specific nutritional preferences. These results lead into a case for niche partitioning at the family level for *K. sydneyanus* gut bacteria. This finding will be explored further in the individual isolate sections within this discussion.

Difference in growth rates seen between the four variations in *K. sydneyanus* gut bacteria inoculum

As detailed in **Chapter 5,** the average growth rate of each of the three isolates and the mix across all media were significantly different from each other at the 0.05 level. Overall, the mix produced the fastest growth, *Rikenellaceae* (BP38) second fastest, *Oscillospiraceae* (BP47G) third fastest and *Lachnospiraceae* (BP5G) the slowest.

The following is a further breakdown of the results detailed in **Chapter 5**. Across the seaweed extracts the *Rikenellaceae* (BP38) isolate grew significantly faster than the *Lachnospiraceae* (BP5G) at the 0.05 level, but not significantly faster than the *Oscillospiraceae* (BP47G). The mix grew significantly faster than all three individual isolates across all seaweed extracts. Across the simple carbohydrates and sugar alcohols, isolate

Rikenellaceae (BP38) grew significantly faster than both *Oscillospiraceae* (BP47G) and *Lachnospiraceae* (BP5G). The rate of growth of isolate *Rikenellaceae* (BP38) was not at significantly different to that of the mix across the simple carbohydrates and sugar alcohol. Across the vitamin additions the *Rikenellaceae* isolate (BP38) also grew significantly faster than the *Lachnospiraceae* (BP5G), but not the *Oscillospiraceae* (BP47G). The mix grew significantly faster than all three individual isolates across the vitamin additions.

There were three groups of nutrient addition types: seaweeds, carbohydrates and vitamins. The *Oscillospiraceae* (BP47G) and *Lachnospiraceae* (BP5G) isolates generally displayed greater similarity in growth response with each other than with the *Rikenellaceae* (BP38) or the mix. Statistical comparison within the three smaller groups of seaweed, carbohydrate and vitamin nutrient addition results revealed further differences in growth response between isolates. There was no significant difference at the 0.05 level between the growth rates of the *Oscillospiraceae* (BP47G) and the *Lachnospiraceae* (BP5G) in each of these three groups (**Chapter 5**). Whereas the growth of the *Rikenellaceae* (BP38) isolate was significantly faster than either one or both of the Clostridia isolates (BP47G and BP5G), within all three nutrient test groups. These results reflect the closer phylogenetic relatedness between the *Oscillospiraceae* (BP47G) and *Lachnospiraceae* (BP5G) isolates of the Clostridia order. These results show that the genetic relatedness of the isolates have an impact on their nutritional preference *in vitro*. This is the first *in vitro* evidence that demonstrates similarity in the digestive abilities of fish gut bacteria with a shorter phylogenetic distance between them.

Discussion of seaweed nutrient additions

Background on the four seaweed nutrient additions

Bacteria from the *K. sydneyanus* hindgut grow significantly faster on extracts of any of the three tested brown algae (*Undaria pinnatifida, Cystophora torulosa, Ecklonia radiata*) than a red algae common in the diet of adult *K. sydneyanus* (*Caulacanthus ustulatus)*. The faster rates of growth were seen on both brown algae common in the *K. sydneyanus* diet (*E. radiata*) and those rarely encountered in their natural environment (*U. pinnatifida, C. torulosa*).

The metabolomic activities of *K. sydneyanus* gut bacteria are specialised to provide energy to the host from a food source, brown algae, that these organisms cannot otherwise access. Whereas these bacteria are less likely to have the digestion pathways necessary to access nutrients from food sources that these fish can digest endogenously. These findings agree with a previous study by Flint, et al. (2005) demonstrating that the environment precipitates a co-evolutionary relationship between herbivorous fish and some gut bacteria.

Pan-isolate seaweed performance

Three of the seaweed extracts (*U. pinnatifida, E. radiata* and *C. torulosa*) improved the pan-isolate performance of the *K. sydneyanus* gut bacteria *in vitro*, with faster average growth rates than gut fluid media. This success allows future experimentation on these fish gut bacteria to be conducted on a replicable media, increasing the reliability of results.

Comparing growth rates produced by brown vs. red algae

All three brown algae species (*U. pinnatifida, E. radiata* and *C. torulosa*) produced significantly faster pan-isolate growth rates than the red algae *C. ustulatus*. This is despite the fact that red algae *C. ustulatus* has been found by the Clements, et al., research group to sometimes make up 100% of the stomach contents of adult *K. sydneyanus* caught off the Northeastern coast of New Zealand [unpublished]. This finding suggests that red algae provide limited energy to the *K. sydneyanus* gut microbiota. These findings match with previous studies which revealed that, unlike brown algae, some red and green algae carbohydrates can be digested by *K. sydneyanus* endogenous enzymes (Moran & Clements, 2002). Therefore, most nutrients of red algae are taken up by the host fish before they reach the later gut sections, where the density of microbiota is greatest.

The bacteria used in this PhD were isolated from gut Section IV, and as expected grew faster on brown algae than red. The significantly increased growth rates caused by the three brown algal extracts show that there is a high efficiency in the rate of conversion from these seaweeds to energy in the form of bacterial growth. These results are an *in vitro* demonstration that *K. sydneyanus* gut bacteria possess digestive pathways tailored to brown algae as an energy source. These growth results are as expected as brown algae makes up the majority of the adult *K. sydneyanus* diet.

Comparing growth rates produced by each algal extract

The most reliable pan-isolate seaweed extract, producing the fastest growth rate, was *E. radiata* extract, with an average pan-isolate growth rate significantly faster than gut fluid. This result was to be expected as *E. radiata* makes up a majority of the stomach contents found in most of the *K. sydneyanus* fish spear caught off the Northeastern coast of New

Zealand by the Clements laboratory group. Therefore, *E. radiata* algae is the main substrate that these *K. sydneyanus* gut bacteria isolates would encounter in their natural environment. These results indicate that these bacteria can derive a large amount of energy from the nutrients present in *E. radiata* when cultured in the *in vitro* environment.

The two brown algae native to Northeastern New Zealand, *E. radiata* and *C. torulosa*, both produced significantly faster pan-isolate growth rates than the non-native brown algae *U. pinnatifida*. Whereas the non-native brown algae *U. pinnatifida* and the native red algae *C. ustulatus* had growth rates that were not significantly different from each other. The faster growth rates caused by the native brown algae suggest that *K. sydneyanus* gut bacteria are specifically adapted to their niche of a diet dominated by these species of algae. While the poor growth generated by the native red algae reflects the endogenous digestion of this substrate, as explained earlier in this discussion. These results show that the nutritional differences between native and non-native brown algae are great enough to significantly impact the amount of energy derived from digestion of these substrates by gut bacteria. These results show that the digestive pathways present in *K. sydneyanus* gut bacteria function at a higher rate of energy conversion for native than non-native algae. These results show that bacteria isolated from the fish gut can better access energy from brown algae common in the host *K. sydneyanus* diet. These findings support the theory of a consistent fish gut microbiome, which is specifically adapted to diet and retained for long periods. This supports the theory of a commensal relationship between herbivorous fish and their gut bacteria.

Along with *U. pinnatifida*, *C. torulosa* is another brown algae not commonly seen in the diet of *K. sydneyanus* caught off the Northeastern coast of New Zealand. The difference in the two brown algae being that *U. pinnatifida* is a recent invasive species, only discovered in the last three decades growing off of the Northeastern coast of New Zealand (South, 2017). Whereas *C. torulosa* is a brown algae native to other parts New Zealand, mainly located on the Eastern Coast of the South Island, and is rarely seen on the North Eastern coast (Stewart, 2004). However, although there was no significant pan-isolate difference in bacteria growth rate between these two brown algae, the average growth rate on media with the native *C. torulosa* was faster. This faster average growth rate demonstrates that *C. torulosa* algae, which has a longer history of growth in New Zealand than *U. pinnatifida*, is better digested by *K. sydneyanus* gut bacteria. This is despite *C. torulosa* making up a smaller portion of the adult *K. sydneyanus* diet than *U. pinnatifida* (Moran & Clements, 2002). These results suggest co-evolution of *K. sydneyanus* gut bacteria with their host as these gut bacteria are better adapted to digest algae commonly consumed by their *K. sydneyanus*.

Individual isolate performance on seaweed extracts

Looking at the growth results for each bacterial isolate reveals a more detailed picture of species-specific seaweed preferences. These results show that for most of the *K. sydneyanus* gut bacteria tested, *E. radiata* produced the fastest rate of growth. The fastest overall growth rate was seen from the mix of all three isolates on YCFA with *E. radiata*, with the mix of all three isolates performing better than each individual isolate on all media trialled. This result suggests that in the fish gut environment this mix of species would work quickly to digest *E. radiata* into energy for growth. For the *Oscillospiraceae* isolate (BP47G) growth was not significantly different across the seaweed extracts trialled. The only growth failures were produced by *Lachnospiraceae* isolate (BP5G) on YCFA with *U. pinnatifida* and *C. ustulatus* extracts, with a poor rate of growth seen across all media for the *Lachnospiraceae* isolate (BP5G).

There was a surprisingly positive result seen for the growth of *K. sydneyanus* gut bacteria on the brown algae *C. torulosa* as this seaweed is not known to be a dietary substrate of choice for the host fish *K. sydneyanus*. Whereas the red algae *C. ustulatus* gave a lower rate of growth, despite making up 100% of the *K. sydneyanus* gut contents on occasion. This result was not the case across all isolates, with the *Oscillospiraceae* isolate (BP47G) growing better in the presence of the red algae *C. ustulatus* than the brown algae *C. torulosa*. This variation in substrate preference between isolates suggests that a shift in *K. sydneyanus* diet is made possible by the variation in nutrient preferences in their gut microbiota. A secondary flexible group of bacteria is usually present in the gut, modulated by environmental diversity and external conditions (Shapira, 2016). As discussed later in the chapter, the various differing nutritional preferences and digestive abilities of *Oscillospiraceae* isolate (BP47G) suggest that this isolate is part of this secondary group of bacteria in the *K. sydneyanus* gut. Interspecific competition between bacterial species in the fish hindgut niche reduced by specialised and divergent nutrient specialisation. This variation also reflects the metabolic diversity of the fish hindgut microbiota.

Mixture of isolates performance on seaweed extracts

The mix of three bacteria grew significantly faster than each individual isolate on each seaweed media. This result suggests that the presence of multiple isolates improves bacterial digestive abilities and increases the rate of conversion of the nutrients in these extracts to energy for bacterial growth. A greater efficiency in seaweed digestion is achieved when multiple species are present. This contrasts with the carbohydrate growth rate experiment results which instead suggest that inter-species competition in the mix reduced the overall rate of growth of these bacteria. The difference between these results is due to the complexity of seaweed extracts allowing niche partitioning to occur, reducing the competition between bacterial species and increasing the overall biomass supported. These results are discussed further later in this chapter in an in depth comparison between the seaweed and carbohydrate results.

Pan-seaweed isolate performance

There was also a significant difference between the pan-media growth rates of some of the isolates tested. These results reveal the differing functional digestive abilities of *K. sydneyanus* gut bacteria species. Across all YCFA media variations, the mix of all three isolates grew significantly faster than each of the three individual isolates. Also, the *Rikenellaceae* isolate (BP38) grew significantly faster than the *Lachnospiraceae* isolate (BP5G). However, although the average pan-seaweed growth rate of the *Rikenellaceae* isolate (BP38) was higher than the *Oscillospiraceae* isolate (BP47G) this difference was not significant. The average growth rates of *Lachnospiraceae* (BP5G) and *Oscillospiraceae* (BP47G) were not significantly different from each other. These results suggest that the *Rikenellaceae* isolate (BP38) and the mix of all three are best equipped with digestion pathways to gain the largest energy benefit from the digestion of these four seaweed extract nutrient additions.

Seaweed findings

There was an overall pan-isolate growth increase on the three brown algae species compared to the red algae species *Caulacanthus ustulatus*. The major finding of these seaweed nutrient addition experiments was that on average the isolates tested grew significantly faster on YCFA with *E. radiata* than YCFA with any of the other nutrient additions trialled. Therefore, the *K. sydneyanus* gut bacteria grew best in nutrient conditions most similar to their natural environment.

Successful growth was achieved in most isolates on all four media types, despite the brown algae *U. pinnatifida* and *C. torulosa* not being common in the diet of the host fish. This suggests that a similar nutrient profile is present in the three brown algae.

The growth rates of individual isolates across all media recipes were more similar to each other than to the mixture. The mixture showed a large and significant increase in growth compared to all individual isolates. These results show that individual isolates struggle to make full use of the energy available in these extracts. This leads to the conclusion that bacteria of the *K. sydneyanus* gut possess varying and complementary digestion pathways to gain the greatest nutritional benefit from algae. These bacteria work together in mutually beneficial relationships to access the largest amount of energy for growth. Within the natural environment of the *K. sydneyanus* gut these bacteria have a symbiotic relationship with other commensal species in the gut microbiome. These results show that bacteria of the *K. sydneyanus* gut microbiome have co-evolved to complement each other. This co-evolution has likely led to a loss of certain digestion pathways required for individual isolates to completely digest these algae alone, as seen in retained co-evolved gut microbiomes (Engel & Moran, 2013; Shapira, 2016). These findings are further evidence for a retained gut microbiota that has co-evolved with the *K. sydneyanus* host.

Discussion of carbohydrate (and sugar alcohol) nutrient additions

Background on the simple carbohydrates in brown algae

Brown algae is digested to laminarin, mannitol, glucose and alginic acid in the gut (Painter, 1983; Seeto, et al., 1996). These carbohydrate and sugar alcohol products of algae digestion are resistant to endogenous fish enzymes, so cannot be directly taken up by the host *K. sydneyanus* through the gut wall (Zemke-White & Clements, 1999; Moran & Clements, 2002). Instead, these carbohydrates and sugar alcohols are used as substrates for bacteria fermentation, mainly to SCFAs, which are then taken up by the fish and contribute to fulfilling their energy requirements. (Mountfort, et al., 2002). Therefore, it would be expected that the fish hindgut bacteria isolates used in these experiments would be able to utilise the simple carbohydrates and sugar alcohols of laminarin, mannitol, glucose and alginic acid as energy for growth.

Mannitol

Mannitol was found to be present in *E. radiata* at a much higher concentration than the simple carbohydrates (Seeto, et al., 1996). Despite this, it is likely that Section IV *K. sydneyanus* gut bacteria will rarely encounter mannitol in their natural environment as mannitol is used up in earlier gut sections, being mostly removed by Section III (Figure 1) (White, et al., 2010). However, as section IV of the hindgut is not separated from the earlier sections by a sphincter, unlike Section V, bacteria from earlier sections may be found in this later section.

The growth rate experiments of this PhD found a significantly faster average growth rate of Section IV bacteria on mannitol compared to laminarin, glucose or alginic acid. This result suggests that these bacteria retain the ability to adapt to variations in host diet and nutritional supply. Therefore, the fast rates of growth on mannitol are likely to reflect the large input of mannitol into the *K. sydneyanus* gut system. This high level of mannitol makes possession of a mannitol digestion pathway energetically beneficial to these bacteria, as they move through the *K. sydneyanus* gut.

Glucose

In contrast to mannitol, there is little free glucose in *E. radiata*, as most is laminarin-derived (Seeto, et al., 1996). Also, the combined action of two different enzymes is required to release this glucose from laminarin. (Russo, 1993). The two required enzymes are mainly present in the hindgut of *K. sydneyanus* (Russo, 1993). Therefore, bacteria able to break down laminarin to release glucose, or to utilise free glucose, are likely to be present in hindgut where most of this enzymatic activity occurs. The Section IV isolates used in this study are therefore likely to possess the ability to utilise both laminarin and glucose as an energy source, providing good rates of growth on these two nutrients *in vitro*. The results of this PhD concur with this prediction as they show good growth of Section IV *K. sydneyanus* gut bacteria with both the nutrient additions laminarin and glucose.

Alginic acid

All isolates tested were found to grow well on alginic acid. Pan-isolate growth on alginic acid was at a similar or faster rate than that seen on media with gut contents. Therefore, the hindgut bacteria tested in these experiments can utilise alginic acid from the cell walls of brown algae. Along with earlier findings, this demonstrates the large breadth of carbohydrate digestion abilities possessed by the bacteria of the *K. sydneyanus* gut. The hindgut bacteria of *K. sydneyanus* provide a large pool of digestive abilities to the host. Consequently, the gut bacteria are able to digest brown algae into a form that can be utilised by the host *K. sydneyanus*, so that this fish can access all components of this brown algae for energy.

The poor pan-isolate growth results seen with the nutrient addition of alginic acid in this PhD agree with predictions by Seeto, et al., (1996) that the catabolism of macroalgae structural polysaccharides is not important for microbial metabolism. This prediction is based on there being no remaining energy in the rate limiting glycolysis conversion to acetate for additional substrates such as alginic acid, which would be prepared for glycolysis by alginase activity (Seeto, et al., 1996). This study found that alginic acid produced some of the lowest average growth rates among all isolates. These results indicate that alginic acid is not a major energy source for these bacteria in the natural environment of the *K. sydneyanus* hindgut.

Effect of carbohydrate complexity on pan-isolate growth rate results

Pan-isolate growth was significantly faster on media with the very simple carbohydrate and sugar alcohol additions of mannitol or glucose than on media with the complex addition of gut fluid. In contrast, the more complex carbohydrates of laminarin and alginic acid produced growth rates that were not significantly different to that seen on gut fluid media. Simpler sugar alcohols, such as mannitol, are an easier source of energy for growth, as less energy is lost by the bacteria in digesting these nutrients. Therefore, these *K. sydneyanus* gut bacteria possess digestive pathways that make faster use of the simplest products of brown algae digestion for growth than complex carbohydrates.

Gut bacteria in Section IV were found to digest the more complex carbohydrates of laminarin and alginic acid to the simple carbohydrate and sugar alcohol of mannitol and glucose and then use this digestion as an energy source for growth *in vitro* (Montgomery & Gerking, 1980; Skea, et al., 2005). This dynamic is reflected in these results, as the *K. sydneyanus* gut bacteria were shown to have the ability to digest both the complex carbohydrates of laminarin and alginic acid. Previous research has found that simple carbohydrates are digested to SFCAs that are released by the bacteria and taken up by the host fish (Clements & Choat, 1993; Rimmer & Wiebe, 1987; Titus & Ahern, 1988).

Bacteria of the *K. sydneyanus* hindgut have been shown to be specialised to the specific nutritional environment of their hindgut niche. Bacteria isolated from Section IV of the *K. sydneyanus* gut can utilise carbohydrates common in this environment as energy for growth *in vitro*. However, the maximum energy for growth was derived from mannitol, which is likely to only be present in small quantities in Section IV the hindgut. This retention of a lesser used digestive pathway benefits the host organism by increasing their adaptive ability to varying environments and food sources. This result reflects an important balance between adaption to a specific niche and maintenance of flexibility in metabolome. This balance improves survivability when the nutritional composition of a niche is liable to changes. The *K. sydneyanus* diet is varied, as discussed earlier in this chapter, this variation in diet is reflected in these growth results, with these gut bacteria able to grow well on all simple carbohydrates and sugar alcohol tested.

Background on carbohydrates in the red algae

Red and green algae are digested to starch, floridean starch, carrageenan and agar (Clements & Choat, 1997; Rimmer & Wiebe, 1987; Painter, 1983). The amount of energy gut bacteria is able to salvage from ingested green and red algae in the adult *K. sydneyanus* gut is limited by the action of endogenous enzymes. Endogenous *K. sydneyanus* enzymes such as amylase allow breakdown of the intracellular polysaccharides from green and red algae to SCFAs for use in bioactivities and as energy for growth by the host fish (Montgomery & Gerking, 1980; Barrington, 1957; Kapoor, et al., 1975; Moran & Clements, 2002; Mountfort, et al., 2002).

Growth of *K. sydneyanus* hindgut bacteria on glucose was significantly faster than that on media with gut fluid. The mix of all three bacteria isolates grew best on glucose, despite this polysaccharide not being the preferred carbon source of any of the three isolates alone. These results reflect the high likelihood of encountering glucose in Section IV of the *K. sydneyanus* gut due to this carbohydrate being present in large quantities in both brown and red algae (Seeto, et al., 1996).

Pan-isolate performance of each carbohydrate

Investigation into the effect of four carbohydrates and sugar alcohol (mannitol, laminarin, alginic acid and glucose) on the growth rates of *K. sydneyanus* gut bacteria isolates revealed a significant pan-isolate preference for mannitol and glucose, above gut fluid. The significantly increased growth rates seen on both nutrients suggests greater efficiency in the rate of conversion from these carbohydrates and the sugar alcohol mannitol to energy in the form of bacteria growth.

Interestingly, none of the alternative YCFA recipes tested produced a significantly slower pan-isolate growth rate than YCFA with gut fluid. Therefore, this work has produced four simple and defined alternatives to the complex undefined YCFA media with gut fluid that may be used to culture *K. sydneyanus* gut bacteria *in vitro*. Two of these media alternatives have the potential to improve the performance of these bacteria *in vitro*. This success will allow future experimentation on these fish gut bacteria to be conducted in a replicable media, increasing reliability of culture and results.

This study found that mannitol is the most reliable pan-isolate carbohydrate growth factor, producing the highest average pan-isolate growth rate, significantly above that produced by YCFA with gut fluid. This finding supports the findings by White, et al., (2010) that mannitol from brown algae is an important dietary constituent for *K. sydneyanus*, where it is utilised as an indirect nutrient after fermentation by the gut microbiota. This study confirms the ability of *K. sydneyanus* hindgut bacteria to ferment mannitol and use this fermentation process as a source of energy for their own growth. This fermentation of mannitol was previously predicted by White et al., (2010) who found that mannitol is removed by Section III. Fermentation by gut bacteria salvages energy from mannitol which can then be to the host fish (Seeto, et al., 1996; Choat & Clements, 1998). These results were also surprising, as White, et al., (2010) also found that mannitol concentration decreased along the gut, these Section IV bacteria however still preferably utilised mannitol for growth. This result reflects the importance of mannitol to maintain a thriving *K. sydneyanus* gut microbiota, even in the hindgut where concentrations of this carbohydrate are much lower than in the foregut. These results also reflect the importance of mannitol as an energy source for these gut bacteria.

It is likely that the concentrations of other simple and easily digestible nutrients including laminarin, glucose and alginic acid have decreased by Section IV. The findings of positive pan-isolate growth in the presence of all of these nutrients show that Section IV *K. sydneyanus* gut bacteria still possess the digestive pathways needed to make use of these carbohydrates and sugar alcohol. The ability of these Section IV bacteria to use simple sugars as an energy source may be due to these same pathway steps being used in the final breakdown of larger particles to easily digestible molecules in the hindgut. In the hindgut these bacteria ferment algae, converting larger molecules into these simple sugars then to energy for growth, so require the ability to digest these simple sugars.

Mannitol produced a faster growth rate among these *K. sydneyanus* hindgut bacteria than the three carbohydrates trialled: laminarin, alginic acid and glucose. These findings show that mannitol provides the largest amount of energy to these gut bacteria upon fermentation.

However, it is not known if mannitol also produces the largest amount of SCFAs for energy and nutrient transfer to the host fish. This information is needed to correctly understand the benefits of microbial fermentation on fish digestion.

These findings also show that *K. sydneyanus* gut bacteria are specifically adapted to the nutritional environment of the host gut. These pan-isolate findings suggest a consistency in the digestive abilities of successful bacteria species in the herbivorous fish gut, rather than a coincidental and transitory relationship between fish and their gut bacteria. It is possible that these bacteria species are ingested along with brown algae and then move through the gut in a transitory manner. It is also possible that once these bacteria are ingested, they are retained, creating a consistent gut microbiome for efficient digestion of brown algae.

Pan-carbohydrate (and sugar alcohol) isolate performance

The was also a significant difference in the pan-carbohydrate growth rates of each isolate used. The variation found in this study reveals the differing functional digestive abilities of the *K. sydneyanus* gut bacteria. Across all YCFA media variations the *Rikenellaceae* isolate BP38 and a mix of all three isolates grew significantly faster than both *Oscillospiraceae* isolate BP47G and *Lachnospiraceae* isolate BP5G. These results show that the *Rikenellaceae* isolate (BP38) and the mix of all three are better equipped with pathways to gain the largest energy benefit from the digestion of these carbohydrate (and sugar alcohol) nutrient additions.

Carbohydrate findings

The major finding of these carbohydrate (and sugar alcohol) nutrient addition experiments was that all isolates grew at their fastest average rates on YCFA with mannitol. Looking at the growth results for each individual isolate tested reveals a more detailed picture of isolate specific nutrient preferences. For some isolates other carbohydrates produced an equal rate of growth to mannitol. For the mix of all three isolates the fastest rate of growth on YCFA with mannitol was equal to that on YCFA with glucose. For the *Lachnospiraceae* isolate (BP5G) this fastest rate of growth on YCFA with mannitol was equal to that seen on YCFA with gut fluid. For all other isolates YCFA with mannitol produced faster growth than YCFA with gut fluid. The only growth failure was seen by the *Lachnospiraceae* isolate on alginic acid.

As touched on in the introduction to this chapter, the findings in **Chapter 5** show that all three of the individual *K. sydneyanus* gut bacteria isolates can utilise mannitol, laminarin, alginic acid and glucose. The three individual isolates showed positive growth with all four of these nutrient additions. The *Rikenellaceae* isolate (BP38) had a particularly high rate of growth on mannitol, suggesting that in the fish gut environment the *Rikenellaceae* isolate (BP38) will work quickly to catabolise any mannitol present as a preferred carbon source.

The mix of three isolates produced a significantly faster growth compared to individual isolates in the majority of conditions. These results suggest that greater energy efficiency in carbohydrate digestion is achieved when multiple species are present. The mix of three bacteria grew significantly slower on mannitol than the *Rikenellaceae* isolate (BP38) alone, but this was still at a faster rate than the isolates *Oscillospiraceae* (BP47G) and *Lachnospiraceae* (BP5G). The massive increase in growth by the *Rikenellaceae* isolate (BP38) is tempered by the presence of other bacteria species in the system. This competition reduces the overall rate of growth of these bacteria. The mix of all three bacteria isolates grew best on glucose, despite this carbohydrate not being the preferred carbon source of any of the three isolates alone. This suggests that the breakdown of glucose by the three isolates together produces the largest amount of energy, this energy is transferred to growth processes in these bacteria.

Comparison between seaweed and carbohydrate results

Across the seaweed and carbohydrate results, the fastest average growth of the three individual bacteria was on YCFA media with mannitol. Whereas the mixture of all three isolates grew significantly faster on media with *E. radiata* aqueous extract. These results show that there is no novel growth factor present in *E. radiata* aqueous extract, as this extract only increased growth above known growth factors for the mix, not for the individual isolates. These results also show that there is no novel growth factor present in the *K. sydneyanus* gut fluid. Despite being the best replication of the natural environment *in vitro*, Gut fluid from Section IV produced slower growth than *E. radiata* aqueous extract. Instead, these results indicate that there is a complex and mutually beneficial relationship between these isolates that enables the mixture of three species to make grow at the fastest rate on *E. radiata* aqueous extract, causing elevated growth in comparison to various defined media additions. This elevated growth can only be accessed in the presence of the specific composition of nutrients that are present in the natural environment of these *K. sydneyanus* gut bacteria.

Unlike the seaweed extract results, overall cross carbohydrate rate of growth of *Rikenellaceae* isolate (BP38) was not significantly different to the mix. This result indicates that *Rikenellaceae* isolate (BP38) possesses all the pathways necessary to produce the maximum amount of energy from the digestion of these carbohydrates. The *Rikenellaceae* isolate (BP38) can utilise these carbohydrates as energy for growth in a highly efficient way. In this case, in contrast to the seaweed results, the addition of further isolates in the mix had no pannutrient growth rate effects above *Rikenellaceae* isolate (BP38) alone. This suggests that the most efficient isolate dominated this mix and that the other two isolates, when competing to digest the same carbohydrate, did not possess alternative digestion pathways that could make use of discarded nutrients from this process, or by-products of carbohydrate digestion.

The similarity in digestive pathways possessed by the three individual bacteria species for the four trialled carbohydrates causes these bacteria to directly compete for access to these simple nutrients. The maximum growth rate of the mix across the simple carbohydrates is equal to that of the most efficient individual isolate. This contrasts with the results for seaweed extracts, which contain a complex mix of nutrients. In the case of complex seaweed extracts, isolates can preferentially digest different nutrients from the seaweed mix using the various pathways they each possess. This niche partitioning in complex media explains the increase in growth rate seen with the mix of isolates on YCFA with seaweed extracts in comparison to simple carbohydrates.

In summary, the simple carbohydrates are digested in a similar way by all bacteria tested and therefore in a mix these bacteria are competing for resources and do not benefit from a commensal relationship with one another in terms of increased overall energy gain from substrate digestion. However, in the complex mix of nutrients present in the aqueous extract of seaweeds each isolate possessed digestion pathways that varied sufficiently to make use of different nutrients within the extracts, or possibly of certain by-products of the digestion processes performed by other isolates. In this situation the mix of three isolates had a mutually beneficial commensal relationship where overall rate of growth was improved by the addition of more than one isolate to the fermentation of these complex seaweed extracts.

These results are culture based proof of niche partitioning in the *K. sydneyanus* hindgut microbiota.

Discussion of vitamin nutrient additions

Pan-isolate vitamin performance

Investigation into the effect of water-soluble vitamins extracted from the brown algae *Undaria pinnatifida* on the growth rates of *K. sydneyanus* gut bacteria isolates revealed that there was no significant difference between growth rates with these vitamin extracts or with *K. sydneyanus* gut fluid. These results show that YCFA media with *U. pinnatifida* vitamin extract can be used as an alternative to the more nutritionally complex YCFA gut fluid media. None of the vitamin nutrient additions in this experiment exceeded the growth produced by gut fluid, and there was an overall slower growth rate on these vitamin media than that seen by the seaweed and simple carbohydrate nutrient additions. For this experiment gut fluid was a more reliable pan-isolate growth factor than the vitamin extracts.

The pan-isolate performances produced by the two tested vitamin extracts of Undaria were not significantly different. This suggests that the extraction process was sufficiently replicable and produced a vitamin extract that was not significantly nutritionally different with each extraction procedure. These results confirm the quality of these extracts and the accurate application of extraction methods in each case.

Interestingly, a ten times increase in the amount of vitamin mixture added to normal YCFA media produced a significantly slower growth rate than YCFA with *K. sydneyanus* gut fluid or YCFA with vitamin extract of Undaria. This makes ten times the vitamin mix the only nutrient addition trialled within this PhD that produced a slower growth rate than YCFA with gut fluid. These results show that there are unique vitamin growth factor(s) present in *U. pinnatifida* that contribute to the increased rate of *in vitro* growth with the addition of brown algae to YCFA media. However, further non-vitamin growth factors from the aqueous extract of *U. pinnatifida* are also required in order to reach the optimal rate of growth seen with the addition of total aqueous extract of *U. pinnatifida*. These results show that there are vitamin growth factors present in *U. pinnatifida*, but these vitamins are not solely responsible for the accelerated rate of growth seen on *U. pinnatifida* extract in previous experiments.

Vitamin findings

The significant increase in growth rate seen with the addition of the two vitamin extracts of *U. pinnatifida* to YCFA media compared to the growth rate seen on a ten times increase in the vitamin mix suggests that some of the essential growth factor(s) present in brown algae are vitamins. These results suggest that there are likely growth factor(s) in this *U. pinnatifida* vitamin extract that are not present in the industry sourced vitamin solution.

The growth experiments performed on the vitamin nutrient addition media reveal that vitamins, either from *U. pinnatifida* or industry, are not the limiting factors to *K. sydneyanus* gut bacteria growth *in vitro*. The rates of growth achieved by the *K. sydneyanus* gut bacteria isolates on the two vitamin extracts of *U. pinnatifida* were significantly slower than those achieved by total *U. pinnatifida* extract in the previous seaweed extract growth rate experiment. This result suggests that there are other non-vitamin essential nutrients present in brown algae required for optimal growth of *K. sydneyanus* gut bacteria *in vitro*.

One conclusion of this work is that there is no single essential growth factor present in brown algae that produces optimal growth of *K. sydneyanus* gut bacteria *in vitro*, but multiple. This conclusion was also supported in the seaweed extract experiments by the different growth rates seen on the three brown algae tested. The seaweed aqueous extracts are nutritionally complex additions that will have many similarities and many differences. The differences in growth rates between each seaweed extract shows that there is no pan-algae nutrient responsible for accelerated growth, but an essential mix of many factors.

The finding that *U. pinnatifida* likely contains vitamins essential to *K. sydneyanus* gut bacteria growth that are not present in vitamin mix sourced from industry is of great interest to microbiologists aiming to isolate gut bacteria *in vitro*. The use of an aqueous extract from a dietary substrate is shown in these experiments to be the best method of ensuring culture success *in vitro*.

Discussion summary

Pan-isolate overall media performance

The fastest pan-isolate growth rate was produced by YCFA with *E. radiata* aqueous extract, with a significantly faster growth on this media than all eleven others trialled in this PhD. The second best average pan-isolate growth rate was seen on YCFA with mannitol, which was significantly faster than all media other than YCFA with *E. radiata*. The YCFA with mannitol medium is a simple, defined and replicable recipe so can be used to improve the accuracy and replicability of future *in vitro* experimental work on these bacteria.

Three of the other YCFA media nutrient additions trialled also produced a significantly faster average growth rate across all isolates than YCFA with gut fluid, these were glucose, *U. pinnatifida* and *C. torulosa*. These three nutrient additions provide a good alternative recipe for the *in vitro* culture of *K. sydneyanus* bacteria and were found to produce varying growth success on different isolates which lends insights to the nutritional preferences of these bacteria.

The pan-isolate growth of all eleven alternative nutrients were found to match or exceeded the growth produced by gut fluid at the 0.05 significance level. Therefore, all of these media recipes can be used for *in vitro* culture as alternatives to the more nutritionally complex YCFA with gut fluid media. Six of these media alternatives; laminarin, alginic acid, *C. ustulatus* extract, vitamin extract 1, vitamin extract 2, and 10 x vitamin, produced growth that was not significantly different to YCFA with gut fluid.

These pan-isolate results reveal that all eleven of the novel replicable media recipes developed within this PhD can be used as alternatives to YCFA with gut fluid for the *in vitro* cultivation of *K. sydneyanus* gut bacteria isolates. These eleven alternative media recipes may be used in place of YCFA media to increase the accuracy and replicability of laboratory experimentation. Five of these media recipes, which used additions of *E. radiata*, mannitol, *C. torulosa*, *U. pinnatifida* and glucose, were found to produce a significantly faster bacterial growth rate than YCFA with gut fluid. These highly performing media are of particular interest as they can be used to improve the *in vitro* performance of these bacteria, easing future laboratory experimentation.

Pan-media overall isolate performance

There was also a significant difference in the pan-media growth rates of all of the isolates used. These results reveal the differing functional digestive abilities of *K. sydneyanus* gut bacteria. Across all twelve YCFA media variations, the mix of all three isolates grew significantly faster than each of the three individual isolates. Of the individual isolates, the *Rikenellaceae* (BP38) grew significantly faster than both the *Lachnospiraceae* (BP5G) and the *Oscillospiraceae* (BP47G). Also, average growth rate of the *Oscillospiraceae* (BP47G) was significantly faster than that of the *Lachnospiraceae* (BP5G). These results suggest that isolate BP38 and the mix of all three are better equipped with digestion pathways to gain the largest energy benefit from the digestion across these twelve different nutrient additions.

Major Findings

Individual nutrient addition findings

The best performing nutrient addition overall was the aqueous extract of the brown algae *E. radiata*. This seaweed makes up the majority of *K. sydneyanus* diet. Therefore, it is not surprising that addition of an extract from this algae produced the fastest average growth overall across individual bacteria isolated from the *K. sydneyanus* gut and the mix of all three together.

There was a large and significant increase in growth rate of the mix of three isolates on *E. radiata* in comparison to all individual isolates. However, the growth rates of the three individual isolates on *E. radiata* was not dramatically increased in comparison to the other nutrient additions. In fact, *E. radiata* only produced the fastest growth for the *Lachnospiraceae* isolate (BP5G). These results do not point to a unique growth factor present in *E. radiata* causing this improved growth *in vitro*. The presence of a single highly effective growth factor would produce dramatically increased rates of growth for at least one of the individual isolates, similar to that seen on the mix. Instead, this dramatic increase in growth on *E. radiata* is only seen when multiple bacteria are in culture together. These results suggest that individual isolates can work together to degrade nutrients, while benefiting energetically from this in commensal relationships. The aqueous extract of *E. radiata* provides multiple energy rich niches in which each isolate can thrive. These findings point towards the presence of multiple growth factors in the aqueous extract of *E. radiata* that are not present in the simple YCFA medium recipe and are also not present in the other nutrient additions trialled within this PhD. Multiple *K. sydneyanus* gut bacteria may have co-evolved to digest and make the most of the complex mixture of nutrients in *E. radiata*.

Another important finding is the similar growth success seen on YCFA with *U. pinnatifida* extract and YCFA with glucose. The growth rates of both the *Rikenellaceae* isolate (BP38) and the isolate mix on glucose was very similar to those seen on aqueous *U. pinnatifida* extract. The strong growth of all isolates on YCFA with glucose suggests that this simple carbohydrate addition provides an equal energy benefit to the nutritionally complex and undefined *U. pinnatifida* aqueous extract. Alongside this finding is the superior pan-isolate growth seen on YCFA with mannitol. There results show that YCFA media with these simple carbohydrates provide an increased amount of energy for bacterial growth than the complex nutrient source of *U. pinnatifida* extract. The equal or superior pan-isolate growth produced by multiple simple and defined media in this PhD allows these novel media to be used as reliable alternatives in future *in vitro* experimentation on these *K. sydneyanus* gut bacteria.

Individual isolate findings

The pan-media growth rate of the *Rikenellaceae* isolate (BP38) was significantly faster than either the *Oscillospiraceae* (BP47G) or *Lachnospiraceae* (BP5G) isolates. This growth was significantly faster across both the simple carbohydrate and the seaweed extract additions. These results suggest that this isolate possesses efficient digestive pathways that allow this bacteria to convert these varied substrates to energy in the form of growth at a faster rate than that of the other two isolates tested. This efficiency of *Rikenellaceae* isolate (BP38) may be caused by the possession by the of a wider range of digestive pathways that are not possessed by the *Oscillospiraceae* (BP47G) or *Lachnospiraceae* (BP5G) isolates.

The gut microbiota is usually made up of two parts; a core group of species under direct genetic and immune control by the host, and a secondary flexible group modulated by environmental diversity and external conditions (Shapira, 2016). Although *K. sydneyanus* is not considered to have a core microbiota (Stevenson, et al., 2022), The *Rikenellaceae* isolate (BP38), performed significantly better than either the *Oscillospiraceae* (BP47G) or *Lachnospiraceae* (BP5G) isolates in most conditions.

The short generation times and fast evolution of gut bacteria increase the adaptiveness of the host fish to changing external environments (Dillon & Dillon, 2004). The flexibility provided by adaption of the gut microbiota supports host organism dispersal and survival in new habitats (Macke, et al., 2017). The relative abundance of the various bacteria species within the pools can also be modulated by host genes (Bordenstein & Theis, 2015). The gut microbiota influence host reproduction and range, thereby affecting the genetic diversity and population dynamics of these host organisms (Macke, et al., 2017). Therefore, although alone the *Oscillospiraceae* (BP47G) or *Lachnospiraceae* (BP5G) isolates do not perform as well as the *Rikenellaceae* isolate (BP38), when all three are cultured together *in vitro*, much faster growth was achieved than otherwise possible individually. This is functional proof that the herbivorous fish gut bacteria are consortial nature, which benefits growth success of these isolates *in vitro*.

The faster growth rates of the *Rikenellaceae* isolate (BP38) on most media additions trialled would suggest that in the *K. sydneyanus* gut environment these bacteria will be quick to metabolise these nutrients and multiply, dominating the microbiome, over the *Oscillospiraceae* (BP47G) or *Lachnospiraceae* (BP5G). The *Rikenellaceae* isolate (BP38) had a particularly high rate of growth on mannitol, suggesting that in the fish gut environment this *Rikenellaceae* will work quickly to catabolise any mannitol present as a preferred carbon source. This appears to be what occurs for the mixture of isolates on the single carbohydrate additions, where the mix grew at a slower or similar rate to the *Rikenellaceae* isolate in most cases. For these simple carbohydrates, the large increase in growth by the *Rikenellaceae* (BP38) seems to be tempered by the presence of other bacteria species in the system. This competition reduces the overall rate of growth of the microbiome. In contrast, the mix grew significantly faster than the *Rikenellaceae* isolate BP38 on most of the YCFA media with additions of algae aqueous extracts. These results suggest that greater energy production from simple carbohydrate digestion is achieved when multiple species are present in these cultures.

These findings point towards an interesting relationship between different species of bacteria in the *K. sydneyanus* gut. The *K. sydneyanus* gut bacteria benefit from community digestive action when supplied with complex algae aqueous extracts that are undefined. However, these bacteria do not benefit from community digestive action when they are supplied with simple carbohydrates. Therefore, it is likely that these bacteria possess various different digestion pathways which are able to make use of multiple nutrients present in the brown algae aqueous extracts, allowing niche partitioning and a larger total number of bacteria cells to survive on a more complex medium. Whereas, in order to digest a single carbohydrate source, all these isolates are likely to possess the same or similar digestion pathways. During simple carbohydrate digestion these three spacies are in direct competition for the same nutrient source. In the case of YCFA media with a simple carbohydrate addition, overall bacteria number is limited by this competition for resources. This is functional culture based evidence for niche partitioning. There are also significant differences in growth between isolates exposed to the same nutrient addition, suggesting that there are isolate specific nutritional preferences.

Findings on the functional digestive abilities of *K. sydneyanus* gut bacteria

This study is the first to investigate the functional digestive pathways possessed by *K. sydneyanus* gut bacteria. Across the twelve media trialled, the average growth rate of each isolate and the mix was found to be significantly different. Therefore, a major finding of this study is that the growth rates of these bacteria are significantly different at the Family level. These findings suggest that these are Family level differences in the functional digestion of the eleven nutrients by these isolates.

Analysis of the metabolome of *K. sydneyanus* gut bacteria provides valuable insight into the digestive pathways possessed by these bacteria. However, these pathways are not necessarily functional *in vitro*. To recognise functional digestive abilities bacteria must be cultured in the lab (Stewart, et al., 2012).

The findings of this PhD include the first analysis of functional digestion by *K. sydneyanus* gut bacteria *in vitro*. As a result, these bacteria can be reliably utilised to perform these digestive functions in real-world industrial applications. These findings represent an important guide for scientific partners in industry with an interest in utilising bacterial digestive abilities. For example, the use of these fish gut bacteria for the fermentation of seaweed to produce high value products such as fish feed and fertiliser.

Interpretation of major findings

Co-evolution of *K. sydneyanus* and their gut bacteria

Gut microbiota co-evolution with a host is likely to consist of a loss of gene function and loss of ability to synthesise costly metabolites if they can easily be taken up from the gut environment of the host fish (Engel & Moran, 2013; Shapira, 2016). This loss of microbiota function is seen in the growth results of this PhD, suggesting co-evolution between *K. sydneyanus* and their gut microbiota.

There was an increase in the growth rate of *K. sydneyanus* gut bacteria on extracts of three brown algae (*Undaria pinnatifida, Cystophora torulosa, Ecklonia radiata*) than a red algae common in the diet of adult *K. sydneyanus* (*Caulacanthus ustulatus)*. The *K. sydneyanus* gut bacteria are specialised to provide energy brown algae, that must be fermented. Rather than red algae, which is less resistant to digestion. These gut bacteria allow the host organism, *K. sydneyanus*, to access nutrients from brown algae.

Red algae are digested endogenously by enzymes early in the host fish gut, making fermentation by bacteria in the hindgut unnecessary (Montgomery & Gerking, 1980). Faster growth was seen on brown algae common in the *K. sydneyanus* diet (*E. radiata*) than those rarely encountered in their natural environment (*U. pinnatifida, C. torulosa*) (Clements & Choat, 1997; Moran & Clements, 2002). Therefore, these bacteria are less likely to possess the digestion pathways necessary to make use of food sources that the host fish can digest endogenously.

Gut bacteria from North-Eastern New Zealand caught *K. sydneyanu*s grew significantly faster on algae dominant in the diet of their host fish (*Ecklonia radiata)*, than algae less often seen in this diet (*Undaria pinnatifida, Cystophora torulosa* and *Caulacanthus ustulatus*) (Clements & Choat, 1997; Moran & Clements, 2002). The dramatic increase in culture success under these conditions is evidence that these bacteria have specifically adapted to their natural environment.

Therefore, this study suggests a level of recruitment, retention, and selection among the herbivorous fish gut bacteria. Although this recruitment and co-evolution is likely limited, with core microbiome unlikely to be present in these marine organisms due to their strong ontogenic morphological and dietary variation (Stevenson, et al., 2022).

Consortial nature of *K. sydneyanus* gut bacteria

This study found that mutually beneficial digestive mechanisms allow for a mixture of isolates to grow faster than each individual isolate. This study also goes further to provide evidence of niche partitioning in the fish gut environment. The mixture of all three bacteria grew faster on complex algal extracts than it grew on the simple sugars. This result suggests that niche partitioning is occurring among the three bacteria in the mixture. This is functional evidence that the ability to access nutrients for growth within the complex seaweed extract differs at the Family level among the *K. sydneyanus* gut bacteria. These results also suggest that that consortial digestive action significantly increases the rate of growth.

When individual nutrient additions are examined, there are some findings of particular interest for understanding of the digestive preferences of these *K. sydneyanus* gut bacteria. The mix of three bacteria grew faster than each individual species in most conditions and grew at the fastest rate overall on media with *E. radiata* extract. An inability to access certain nutrients is likely to be limiting the growth of individual bacteria in comparison to the mix. Media with *E. radiata* extract best represents the nutritional profile of the *in vivo* environment that these bacteria are sourced from, as *E. radiata* is the main food source for *K. sydneyanus*. These individual bacteria must therefore rely on the digestive processes of other bacteria in the *K. sydneyanus* gut to release certain nutrients essential for optimal growth. Over many years these bacteria have adapted a symbiotic relationship not only with the host fish but with each other. These results are consistent with the belief of Seeto, et al., (1996), that the gut microbiota of herbivorous marine fish is consortial in nature, where competition and cross-feeding between bacterial species are essential for optimal microbial metabolism in the fish gut. Therefore these results support the common theory in published literature on this subject.

Another interesting finding of this PhD is that the mix of all three bacteria isolates grew best on glucose out of all of the simple carbohydrates tested. This result is despite this carbohydrate not being the preferred carbon source of any of these three isolates when cultured alone. This result shows that the breakdown of glucose by these three isolates together produces an increased total amount of energy for growth compared to each individual isolate. This is the clearest evidence of consortial digestion in this study.

This increased growth rate of multiple isolates on glucose in comparison to individual isolates is consistent with Hungate's 1966 hypothesis that interspecies transfer of electrons increases total energy release from glucose fermentation in the gut. In a monoculture, a single cellulolytic isolate can ferment glucose to produce ethanol, acetate, H_2 and CO_2. The addition of a second isolate with the ability to oxidise reduced substances removes ethanol and H_2. This is because the second isolate oxidises H_2, producing electrons that can reduce fumerate to succinate (Hungate, 1966). This is an interspecies transfer of electrons away from ethanol production by the first isolate and towards succinate production by the second isolate. This transfer is performed by the second isolate which oxidises H_2, producing electrons. These electrons are then used by the second isolate to reduce fumerate to succinate. There is an increase in acetate carbon and a reduction in ethanol carbon in the mixed culture compared to the monoculture. In a study testing Hungate's hypothesis, Iannotti, et al (1973) cultured the cellulolytic bacterium *Oscillospiraceae. albus*, and the oxidising and reducing bacterium *Vibrio succinogenes* together. Iannotti, et al., (1973) found that *V. succinogenes* could not survive in a monoculture, but there was good growth of both isolates in the mixed culture. In the mixed culture no ethanol or H_2 was produced, and the only acid products were acetic and succinic acids (Iannotti, et al., 1973). These findings show that the presence of multiple isolates enables complete carbon recovery, which was not achieved in a monoculture. Hungate's 1966 hypothesis illustrates the mutually beneficial digestive mechanisms that produce the superior performance of the mix of three isolates in comparison to any single isolate in this PhD.

This is the first culture based evidence of inter-species cooperation in digestion of substrate the *K. sydneyanus* gut. These methods, published in Wornell, et al (2022), may be used to design inoculum for optimal anaerobic digestion of various substrates in industry applications. These results also further confirm there are mutually beneficial digestive mechanisms active in the consortium of symbiotic bacteria in the herbivorous anaerobic gut, as hypothesised by Seeto, et al., (1996) and Hungate (1966).

Growth factors and media development

Due to the findings of this PhD, multiple defined media have been developed to replace the complex and undefined YCFA media with gut fluid that is currently used for the anaerobic *in vitro* culture of *K. sydneytanus* gut bacteria. Novel bacteria strains can be tested for nutrient preference in a high throughput manner using the novel methodology published within this PhD. These methods can also be utilised to enable the development of bespoke media for the culture of bacteria of interest from any environment. Five novel media recipes were developed that produced significantly faster pan-isolate growth than YCFA with gut fluid, the standard media used to culture these gut bacteria.

These media developments inform large scale batch fermentation work occurring in this research group, with the ability to adjust batch fermentation conditions based on these results to include a mixture of isolates known to be present in large numbers in the natural environment of the *K. sydneyanus* gut. Species shown in this PhD to growth well in culture together were *Oscillospiraceae* (BP47G), *Lachnospiraceae* (BP5G) and *Rikenellaceae* (BP38).

On the simple carbohydrates and sugar alcohols of mannitol, alginic acid or laminarin one isolate (the *Rikenellaceae* BP38) dominates the culture, as this digestion cannot be partitioned to provide separate niches for each isolate. This shows that where a single niche is available, competition between multiple isolates restricts overall growth. These results show the importance of nutritional complexity in the cultural environment to mimic the natural *K. sydneyanus* gut environment of these bacteria, which allows for niche partitioning among multiple isolates and an improved overall growth rate. In the case of the simple carbohydrates and sugar alcohols the most efficient isolate dominates the culture. This study showed that the *Rikenellaceae* isolate BP38 is the most efficient bacterium used in this study. This work found that complexity in nutrient profile is required to support the optimal growth of multiple bacterial isolates. Therefore, when culturing a single isolate one of the previously mentioned simple defined media can be used, but when culturing multiple isolates and performing batch fermentation a complex nutrient addition of brown algae extract is recommended.

The main aim of this PhD was to find a novel growth factor for these isolates. It was thought that there may be a novel growth factor present in brown algae. However, the growth results show that there are multiple nutrients present in brown algae that produce good growth *in vitro* and may be used as growth factors. We found that simple carbohydrates and sugar alcohols are good alternatives to gut fluid as additions to YCFA media, producing similar growth rates to brown algae in the individual isolates. Therefore, these alternatives can be used in culture media *in vitro* as *K. sydneyanus* gut bacterial growth factors.

Of these alternatives, four were chosen as the most reliable and replicable media recipes for the optimal *in vitro* culture of *K. sydneyanus* gut bacteria. These media are YCFA with mannitol, laminarin, glucose or alginic acid. The best performing media across all isolates was YCFA with mannitol or glucose. These novel media recipes increase the performance of fish gut bacteria *in vitro*, easing further laboratory experimentation. Use of these defined alternatives to the undefined YCFA media with gut fluid will also increase the accuracy and reliability of experimental work. These novel media can also be used in functional applications, such as large-scale seaweed fermentation.

General Conclusions and Future Work

This study utilised *in vitro* experiments to provide new insight into the functional digestive mechanisms possessed by certain *K. sydneyanus* gut bacteria. The reliable and replicable media recipes for the optimal *in vitro* culture of *K. sydneyanus* gut bacteria, YCFA with mannitol, laminarin, glucose or alginic acid, can be used in future work to further investigate functional metabolism of these bacteria.

Discussion of the results revealed a preference for complex media most similar to their natural environment, even over easier to digest nutrient sources. There was higher growth rate seen for the mix of all three bacteria on the complex algal extracts than simple sugars. By far the fastest growth rate was seen on media with *E. radiata*, the nutrient source most ingested by the host fish. This suggests a level of recruitment, retention, and selection among the herbivorous fish gut bacteria, along with co-evolution between the host fish and their gut bacteria. This is a subject of interest to recent genetic based studies on this subject (Stevenson, et al., 2022).

This work includes functional evidence that agrees with Seeto, et al., (1996), that the gut microbiota of herbivorous marine fish are consortial in nature. The significantly faster average growth rate of the mixture of all three bacteria than any individual isolate on across all seaweed-based media is evidence that cross-feeding between bacterial species is essential for optimal microbial metabolism of complex substrates in the fish gut.

The strongest evidence of consortial digestion is that the mix of all three bacteria grew best on glucose out of all the simple carbohydrates. Despite this carbohydrate not being the preferred carbon source of any of these isolates individually. This shows that the breakdown of glucose by these three isolates together produces an increased total amount of energy for growth compared to individual growth. This result is consistent with Hungate's 1966 hypothesis that interspecies transfer of electrons increases total energy release from glucose fermentation in the gut. Proving these bacteria are capable of functioning in a consortial manner in the fish gut microbiota.

There also appears to be niche partitioning among fish gut bacteria. Each species can access different nutrients rather than directly competing, allowing for a faster overall growth rate in a mixed culture. Results on simple media also revealed that the *Rikenellaceae* isolate BP38 as the most efficient bacterium tested, with the fastest overall individual growth rate. The mixture of all three bacteria grew faster on complex algal extracts than it grew on the simple sugars. This is functional evidence that the ability to access nutrients for growth within the complex seaweed extract differs at the Family level among the *K. sydneyanus* gut bacteria. Or that consortial digestive action significantly increases the rate of growth when these three bacteria are grown together.

Taken together, the conclusions of this work provide useful insight into the level of co-evolution between herbivorous fish and their gut bacteria, along with their consortial nature. These concepts, common to terrestrial herbivores, may play a larger role in the herbivorous fish gut than expected considering the challenges to intergenerational transfer of bacteria (Stevenson, et al., 2022). The detailed picture of *in vitro* growth preferences of these bacteria provides a useful guide to those investigating the gut bacteria of herbivorous fish for their functional digestive abilities. Future work should use this novel methodology to further investigate functional digestive abilities of these organisms. This study, and future work, will improve growth conditions of fish gut bacteria used in industrial processes.

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Appendix 1: Published Paper

CURRENT PROTOCOLS
A Wiley Brand

ARTICLE TITLE:
High-throughput method of novel media development for the culture of anaerobic gut bacteria

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ABSTRACT:

Here we present a high-throughput protocol for comparing growth rates of fastidiously anaerobic bacteria on different media. These protocols can be used to develop a solid media made up of commercially sourced ingredients, providing replicable growth conditions for previously uncultured anaerobic bacteria. Gut microbiota play important roles in the health of their host and detailed investigation of these organisms requires in vitro culture. Culturing strictly anaerobic bacteria can be a challenge as the gut environment they inhabit is nutritionally complex. Use of complex media containing nutritionally rich but undefined gut fluid reduces accuracy of physiological and metabolomic studies. As many fastidious bacteria grow poorly in a liquid broth, these protocols measure bacterial growth rate on solid media. These protocols speed up and simplify the growth rate measurement process with use of a multi-well format and of equations in place of physical McFarland standards to calculate approximate cell density. Bacterial strains belonging to the families Erysipelotrichaceae and Lachnospiraceae (Phylum Firmicutes) isolated from the hindgut of *Kyphosus sydneyanus* were used to demonstrate the efficacy of these protocols. Bacterial growth rates were compared between a nutritionally rich medium with gut fluid, versus a novel replicable medium with mannitol.

Basic Protocol 1: Preparing solid YCFA growth media
Basic Protocol 2: Sample collection from the fish gut and plating to single isolates
Basic Protocol 3: Genetic identification of single isolates with colony PCR and 16S rRNA gene sequencing
Basic Protocol 4: Measurement of bacterial growth rates on solid media

KEYWORDS:
Anaerobic culture, laboratory growth, in vitro cultivation, gut microbiota, growth factor

INTRODUCTION:

Culturing gut bacteria in vitro is a great challenge as the gut environment is anaerobic and nutritionally complex. Bacteria isolated from this environment are obligately anaerobic (Hungate, 1975), requiring the use of specialized techniques and equipment to ensure that all oxygen is removed from the system during in vitro culture. Gut bacterial isolates sourced from the same environment will have varying and species-specific nutritional requirements (Tramonatano, et al., 2018). Predicting the likely essential growth factors of a gut bacterial isolate is difficult. Optimizing the growth rate of a chosen isolate on replicable media is therefore a challenge, with many possible limiting factors.

The difficulties in isolation and maintenance of viable gut bacteria culture makes genomic sequencing the preferred alternative to in vitro analysis of these organisms. Genomic studies of environmentally isolated bacteria can suggest the presence of metabolic pathways (Béjà, et al., 2000; Béjà, et al., 2002). However, to understand the physiology of novel microbes and the functional processes they engage in, cultivation is necessary (Zengler, et al., 2002; Stewart, et al., 2012). The protocols outlined in this present study address the current challenge for microbiologists in achieving successful culture of environmentally isolated microorganisms, to improve understanding of their metabolic properties and potential (Zengler, et al., 2002). These protocols (Figure 1) are robust, reproducible and easily adaptable.

Despite innovations to culture methods, many bacteria species are still classed as 'viable non-culturable' (Barer & Harwood, 1999), with sequencing studies revealing that less than 1% of extant bacterial species have been grown in the laboratory (Barer & Harwood, 1999; Bomar, et al., 2011). This 'great plate count anomaly' is a decades old problem in microbiological research, as originally described by Staley and Konopka (1985). These as-yet uncultivated microorganisms are a large unexplored reservoir of biological compounds, with great potential for structural diversity and for use in novel drug development (Demain & Sanchez, 2009; Stott, et al., 2008). The considerable time and energy it takes to produce a primary bacteria culture in vitro has led to a lack of investigators, one of the reasons for the large number of uncultivated bacteria (Lagier, et al., 2015).

There is not a standard process used to isolate anaerobic microbes in vitro for the first time. Many gut bacteria of interest are still cultured in rich media with additions such as host gut fluid or blood (Lagier, et al., 2015; Tramonatano, et al., 2018). These rich media recipes that attempt to replicate the nutrient composition of the gut environment are highly nutritionally complex, but problematically, chemically undefined. Use of media containing gut fluid reduces experimental reproducibility as the nutrient profile varies between individual host organisms, including fish (Egerton, et al., 2018), so is subject to nutrient variation from batch to batch. The use of rich media with gut fluid or blood limits the accuracy of metabolic and physiological investigation of microbes in culture (Tramonatano, et al., 2018).

Development of an alternative chemically defined culture medium requires trial and error experiments measuring the effect of nutrient additions on bacterial growth rate. For fastidious anaerobic microbes this process is laborious and time consuming due to long incubation times and strict culture conditions. The obligate anaerobes isolated from the gut environment require specific bacteriological culture techniques (Bryant, 1972; Hungate, 1969) and media containing a variety of specific growth factors (Hensgens, et al., 1994; Jarrell & Kalmokoff, 1988; Scherer & Sahm 1981; Schönheit, et al., 1979; Tanner & Wolf, 1988). Many gut bacteria species that have already been successfully cultured in vitro will still take weeks to grow to detectable levels (Leadbetter, 2003). We found that our fish gut bacteria isolates did not grow exponentially, unlike most bacterial growth (Hall, et al., 2013). The linear growth of our isolates demonstrates the greater effect of limiting factors on the growth of these anaerobic bacteria. With a lower survival rate of the daughter cells generated by bacterial fission. Doubling time (hours) and first order growth rate constant (α) (minutes^{-1}) are the two most common ways to represent bacterial growth rate. These cannot be used in this case as they are calculated based on an assumption of exponential bacterial growth. Therefore, in these protocols we use the increase in colony forming units (CFU) over time (hours) to represent bacterial growth.

Here we present a laboratory protocol for high-throughput microbial growth evaluation on a solid medium in strictly anaerobic conditions. This protocol details bacterial growth assessments beginning with the isolation of bacteria from gut samples and preparation of inoculum for multi-well cultures and provides instructions for anaerobic culture maintenance and culture transfer to a microplate for growth rate measurement by optical density on a standard microplate reader. This approach is appropriate for the efficient investigation of microbial growth rates on media with variable nutrient additions. With the outcome of developing a novel media with defined ingredients, replicable in future work.

Importantly, these protocols have been designed for maximum recovery of viable anaerobic bacteria in in vitro culture. The results of Protocols 1 to 3 are single bacterial isolates of known genetic identity, cultured on a rich media containing host gut fluid. Solid media is used in all protocols as many anaerobic bacteria are unable to grow in a liquid culture (Speers, et al., 2009).

Protocol 4 presents a method of high-throughput growth rate analysis on solid media. The usual way of measuring growth on a solid media, colony counting, cannot be used as bacteria isolated from the gut may grow in mats. These bacteria may also grow in colonies invisible to the naked eye (Tramonatano, et al., 2018). This protocol is used to compare bacterial growth rates on novel replicable media to those on rich media. Resulting in the development of an optimal replicable medium for an isolate of interest.

Basic Protocol 4 sets out the calculations used to convert absorbance readings to growth rates. If bacterial growth is linear, growth rates are calculated as increase in colony forming units over time in units of CFU/hour. If bacterial growth is exponential, growth rates are calculated as a doubling time in units of hours, or a first order growth rate constant (α) in units of minutes^{-1}. These results allow direct comparison of isolates growing on different media, revealing the growth effects of various media additions.

These protocols describe how to isolate and identify bacteria from the fish gut while maintaining anaerobic culture conditions. They also set out a high-throughput and replicable method of measuring the effect of various media additions on bacterial growth rate. Results of these protocols may be used to develop an optimal replicable medium for gut bacteria isolates of interest from the fish gut, or other organisms including humans. This replicable media may then be used during metabolomic analysis of the isolate. These protocols can be used to improve the in vitro culture conditions of both previously isolated and novel gut bacteria.

STRATEGIC PLANNING:

Equipment

Many bacteria isolated from the gut environment are strictly anaerobic and cannot tolerate any exposure to oxygen (Hungate, 1975). Excluding all oxygen from an in vitro culture system requires specialized equipment and techniques. Detailed information on how to set up the Hungate gassing apparatus, used in Basic Protocols 1, 2 and 4, is outlined in Hungate (1975) and Bryant (1972). This method requires the use of Hungate gassing apparatus with the ability to deliver nitrogen, carbon dioxide, and hydrogen gas to the system. The technique, first introduced by Hungate (1969), enables aseptic and anaerobic transfer of ingredients between containers for generation of sterile anaerobic bacterial culture media (Hungate, 1969). Gas is passed via a pressure reducing valve through a hot reduced copper wire column, removing all trace oxygen present in the source gas (Moore, 1966). The gas passes out of this column through a glass Y tube, then through rubber tubing and out of 16-18 gauge bent gassing needles through 2 mL sterile cotton-plugged Luer-Lok syringe barrels (Bryant, 1972). A reducing agent (e.g. cysteine-HCL) is also added to the media to ensure all oxygen is removed (Bryant, 1972).

Other specialized equipment, used throughout the culture process, are required to maintain in vitro culture of strictly anaerobic bacteria, including anaerobic jars, an anaerobic hood, and an incubator. The anaerobic jar, first introduced by McIntosh and Fildes (1916), has long been used for the cultivation of anaerobic microorganisms. The anaerobic jar used in this protocol (Figure 2) was custom built for us by Mason Tool and Engineering Limited (Auckland, New Zealand). This anaerobic jar is filled with N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) to 5 psi using the Hungate gassing apparatus. Anaerobic hoods and incubators can be purchased from many suppliers and will interchangeably perform the same function. The anaerobic hood must be large enough to fit the anaerobic jar through the air lock. To ensure maintenance of anaerobic conditions in the hood, a catalyst (palladium) and a desiccant (calcium chloride) are used. To ensure maintenance of anaerobic conditions in the jars a catalyst (palladium) and activated charcoal are present. Activated charcoal absorbs hydrogen sulfide produced by the bacteria, preventing it from affecting catalyst function. The catalyst, desiccant, and charcoal are activated by heating at 150^0C for 1 hour. An anaerobic monitor allows quick detection of any oxygen entering the system. The incubator must also be large enough to fit the anaerobic jar and must have an adjustable temperature control that can be adjusted to the body temperature of the host organism from which the gut bacteria were isolated. In our protocol the gut bacteria are isolated from the gut of a silver drummer (*Kyphosus sydneyanus*), and incubated at 20°C, within the 17-23°C range of ambient seawater temperature used as *K. sydneyanus* body temperature in Mountfort, et al., (2002).

Bacteria of interest

Proteobacteria, Firmicutes and Bacteroidetes comprise 90% of the fish intestinal microbiota across species (Ghanbari et al., 2015; Egerton, et al., 2018). These protocols were verified using two bacterial isolates derived from section IV the K. *sydneyanus* gut (Figure 4). Isolate BP52G in the Erysipelotrichaceae family, from the Firmicutes phylum, 16S rRNA gene sequence accessed from: NCBI prokaryotic genome annotation pipeline (Tatusova, et al., 2016). Accession number: MG827409. Isolate BP5G in the Lachnospiraceae family, from the Firmicutes phylum, 16S rRNA gene sequence accessed from: NCBI prokaryotic genome annotation pipeline (Tatusova, et al., 2016). Accession number: CP092441

Media

YCFA (**Y**east extract-**c**asein hydrolysate-volatile **f**atty **a**cids) medium was designed by Dr Flint's lab, the specific recipe for this medium was later outlined in Duncan, et al. (2002). The work by Duncan, et al. (2002) proved the effectiveness of YCFA as a base medium for the culture of anaerobic bacteria. Here we use an adapted version of YCFA medium with reduced yeast extract and tryptone in comparison to Duncan, et al., (2002) (see Reagents and Solutions). Reducing nutrient richness reduces confounding variables during bacterial growth rates experiments, allowing the effect of nutrient additions to be seen clearly for all isolates. Also, this ensures that any novel media developed is as minimal as possible.

The addition of gut fluid to this medium provides an unknown quantity and unknown variety of nutrients. Using the older anaerobic M2GSC medium recipe (Miyazaki et al., 1997), Barcenilla *et al.* (2000) found gut fluid was necessary to cultivate the obligately anaerobic bacteria *Fusobacterium prausnitzii* from human feces (Duncan, et al., 2002). However, Duncan, et al., (2002) found that the nutrients present in a YCFA medium with added glucose contained all the nutrients required to culture *F. prausnitzii* in vitro. YCFA medium with *K. sydneyanus* gut fluid is used in these protocols as a standard against which the growth effects of alternative nutrient additions can be compared. However, our research showed that YCFA with glucose did not support the growth of strains BP5G and BP52G isolated from the hindgut of the herbivorous (seaweed eating) fish *Kyphosus sydneyanus*. This was not unexpected given the environment from which it was isolated. Members of the gut microbiota of *K. sydneyanus* are likely under selection to use the abundant carbon substrates available in the gut of the host. Mannitol is a common sugar alcohol present in brown algae, which is the main food source of *K. sydneyanus* (Clements and Choat, 1997), the host organism of our isolates. Also, mannitol concentration decreases along the gut of *K. sydneyanus,* correlating with an increase in gut microbiota density (White, et al. 2010). This suggests that mannitol is fermented by the gut microbiota. For these reasons mannitol was chosen as the trial growth factor in these protocols. Mannitol was used at an overall 0.2% (w/v) or ~10 mM concentration in YCFA-M. This is based on recommendations by Holdeman, et al., (1977), and Browne, et al., (2016), who used a 0.2% (w/v) concentration of sugars in their anaerobic media.

The growth rate of the chosen isolates (Erysipelotrichaceae and Lachnospiraceae) was measured on both YCFA medium with mannitol (YCFA-M) and YCFA medium with gut fluid (YCFA-GF) in controlled conditions to allow comparison. Apart from gut fluid, the remainder of YCFA medium ingredients were commercially sourced. These protocols provide a pathway to develop a commercially sourced alternative to YCFA-GF for any gut bacteria isolate of interest.

Reproducibility

All parameters in this experiment, qualitative and quantitative, should be reported to ensure reproducibility. Each timepoint for one isolate in two conditions is replicated in triplicate on a single 48-well plate. Triplicate repeat timepoints are separately converted from absorbance readings to growth rates. These growth rates are averaged to generate a reliable average growth rate of each isolate on each media.

Data analysis

Reproducible and efficient data analysis methods are required for the analysis of growth measurements from high-throughput methods. This protocol details the equations required to convert microplate reader absorbance measurements over time to approximate growth rates in the units of colony forming units (CFU) over time (hours), using McFarland standards (Casciato, et al., 1974; McFarland, 1907). Analysis was conducted in R v.4.1.2 (R Core Team, 2014) producing figures using the package ggplot2 v.3.3.2 (Wickham, 2016).

BASIC PROTOCOL 1

Basic protocol title:

Preparing Solid YCFA Growth Media

Introduction:

This protocol (Figure 5) has two parts, with Part A detailing the method used to create a version of YCFA (**Y**east extract-**c**asein hydrolysate-volatile **f**atty **a**cids) medium, adapted from Duncan, et al., (2002) (see Reagents and Solutions). Part B uses this method to create 48-well plates with two different media types. Example layouts are provided (Figure 6) that allow trial of possible bacterial growth factors, for example mannitol in YCFA+M medium, against a standard of growth rate on YCFA medium with gut fluid (YCFA+GF). Throughout these protocols, strict adherence to Hungate technique is required to maintain anaerobic conditions (Hungate, 1969).

Materials:

Reagents for media (see Reagents and Solutions)
Stak-Pak, Catalyst (Palladium) (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6501000)
Stak-Pak, Dessicant (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6502000)
Charcoal, Technical, ActIVated for Gas Adsorption (Fisher Chemical, cat no. 7440-44-0)
Mass scale
Weigh paper or trays
Spatula (for weighing out reagents)
Hungate flasks (100 ml) (Custom made by Mr. Alistair Mead, Senior Technician, Faculty of Science, University of Auckland)
Butyl rubber stoppers for Hungate flasks (Thomas Scientific, cat no. 1208Z83).
Bunsen burner
Autoclave, autoclave tape
Water bath (at 60 °C)
Sterile 90 mm petri dishes
Sterile 48 well culture plates with lids
Serological pipette controller
Serological pipettes (10 ml)
Laminar flow hood
Anaerobic jar with pressure gauge (Custom made by Mason Tool and Engineering Ltd, Auckland, New Zealand)
Hungate gassing apparatus delivering N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere
Materate Flowmeters for Air – Bench standing (Glass Precision Engineering Ltd., England, cat no. MFA1001-BS)
Sterile water (37 ml) (deionized or autoclaved)

Protocol steps:

Part A Creating 100 ml of anaerobic YCFA medium

15. Measure the 'Part 1' ingredients from 'Reagents and Solutions' to quantities specified.

These reagents are prepared in advance as individual stock solutions of minerals to speed up the process of media making.

16. Add to a Hungate flask with 74 ml sterile water.

    Deionized or autoclaved water can be used.

17. Gas flask with N_2 over an open flame until medium turns a pink colour, and agar has melted. The gas delivered to flask by a gassing needle from the Hungate gassing apparatus, held in place with butyl rubber stopper using the Hungate technique.

    Hungate flask should be constantly swirled while held with the base one inch away from the top of the Bunsen burner flame.

    Hungate gassing apparatus, developed by Hungate (1969) and described by Bryant (1972), enables the aseptic and anaerobic transfer of ingredients between containers outside of an anaerobic hood. This allows generation and manipulation of sterile anaerobic bacterial culture media without the restrictions associated with the use of an anaerobic hood (Bryant, 1972; Hungate, 1969). The details of setting up and using the Hungate apparatus are outlined in Bryant (1972).

18. Once gassed, remove the gasing needle and stopper the flask using the Hungate technique (Hungate, 1969; Bryant, 1972).

    The Hungate technique of aseptic removal of the Hungate gassing needle and replacement of the stopper into the flask is outlined in Bryant (1972). This technique enables aseptic and anaerobic conditions to be maintained throught the culture or media making processes.

19. Secure stopper in place with using two pieces of aluminum wire twisted around the stopper and the neck of the Hungate flask.

20. Autoclave for 15 min at 121°C.

    Move onto the next step immediately before medium cools and agar solidifies.

21. Secure the 100 ml Hungate flask in a 60°C water bath. Remove the aluminium wire.

22. Adjust the gas canisters of the Hungate apparatus to deliver a N_2, CO_2 and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere.

    Three pure gas cylinders separately containing N_2, CO_2 and H_2 are attached to the Hungate apparatus prior to a copper column, as outlined in Bryant (1972). The ratio of gasses provided through the Hungate apparatus to the gassing needles can be adjusted using the dials on the cylinders, these adjust the pounds per square inch (psi) released from each cylinder. The volume delivery of each gas to the Hungate gassing apparatus can further be checked using flow meters. The flow meters are used to measure the standard cubic centimeter per meter (sccm) for each gas. This allows a precise N_2, CO_2 and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere to be provided by the gassing needles.

23. Insert a needle of the Hungate gassing apparatus, held in place with a butyl rubber stopper using the Hungate technique (Hungate, 1969; Bryant, 1972).

    Remove wire, then insert needle of Hungate gassing apparatus into the flask alongside the bung using the Hungate technique.

24. Add 'Part 2' ingredients from 'Reagents and Solutions' to quantities specified, these are added anaerobically under a N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere using the Hungate apparatus.

Between new ingredient additions return the bung into place alongside the needle, maintaining anaerobic conditions.

These reagents are prepared as stock solutions of minerals to speed up the process of media making.

25. For YCFA-GF medium add Gut fluid; volumes required are detailed in Part 3A from 'Reagents and Solutions'. For YCFA-M medium add Mannitol; volumes required are detailed in Part 3B from 'Reagents and Solutions'. For YCFA negative control medium add nothing at this stage.

    Swirl after all ingredients added to ensure even distribution of ingredients, keep in the 60°C water bath until ready to add to plates.

26. For 90 mm petri dishes, pour 16 mL media into each petri dish inside a sterile hood.

    Leave in the sterile hood to solidify and cool for 30 mins.

    This step of the process is performed in aerobic conditions. Any dissolved oxygen is removed from the media during Step 13.

    These petri dishes are used for plating to single isolates, and for whole plate streaking for the growth rate experiments that are outlined later in this paper.

27. Store anaerobically at 3 - 5°C for up to 90 days in sealed anaerobic jars, gassed with N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure. Storage of the petri-dishes in the anaerobic jar removes any dissolved oxygen from the media, to completely deplete the media of oxygen.

    Gas jars using Hungate apparatus with tube attachment that can be inserted into jar (Figure 3). Copper tube can be inserted into one of the clampable rubber tubes attached to the jar.

    Each jar should contain one autoclave tape-sealed petri-dish full of Stak-Pak Catalyst, and one full of activated charcoal. These petri-dishes should be placed at the bottom of the petri-dish stack inside each jar. This helps maintain anaerobic conditions inside the jar. Ensure catalyst and charcoal are freshly activated by heating at 150°C for 1 hour.

28. For 48 well plates, refer to Part B of this protocol.

    These plates are used for growth rate experiments, outlined later in this protocol.

Part B: Creating 48-well plates containing different possible growth factors for use in growth rate analysis

The purpose of this protocol is to discover the optimal medium for in vitro growth of a specific anaerobic bacteria of interest. When culturing bacterial species in the lab for the first time the optimal medium must be found by trial and error. For fastidious anaerobic bacteria, this process can take a long time. This protocol outlines a high-throughput method of optimizing in vitro growth of anaerobic bacteria. In this protocol each 48-well plate is loaded with two different solid agar media, YCFA-GF and YCFA-M, allowing direct comparison of isolate growth rate. These plates are poured in aerobic conditions, then depleted of any dissolved oxygen by storage in sealed anaerobic jars, gassed with N2, CO2, and H2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure, stored at 3 – 5 °C. To ensure plates are completely depleted of oxygen, store new plates in the anaerobic jar for at least two days.

6. Prepare 48-well plates in a sterile hood, ready for media addition

7. Take the prepared YCFA media variants, contained in Hungate flasks, from the 60ºC water bath and into the sterile hood
8. Decide layout of media in 48-well plates. An example, used in our work, is Figure 6.

   Growth rate experiment requires three replicates of each condition at each timepoint, with a no isolate control. A minimum of four timepoints during the growth phase are required to calculate the bacterial growth rate (Hall, et al., 2013). A zero-hour timepoint may also be useful to check the inoculum concentration is consistent across wells. There may also be an optional stationary phase timepoint, taken after an isolate has reached maximum growth, this informs study of optimal culture conditions, as it suggests when growth stopped, and the stationary phase began.
9. Use sterile serological pipettes to add 500 µl of prepared medium into each well of the 48-well plates inside the sterile hood.

   This step must be performed quickly as soon as the medium leaves the 60ºC water bath it begins solidifying. Leave the poured 48-well plates in the sterile hood to solidify and cool for 30 mins.

   Use of a serological pipette allows a large amount of medium to be taken up at once in a sterile fashion, then quickly pipetted into many wells. A multichannel pipette or a 1000 µl pipette may be used instead, as long as this method can still be performed quickly and in sterile conditions.
10. Store anaerobically at 3 – 5 ºC for up to 90 days in sealed anaerobic jars, gassed with N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure. Storage in sealed anaerobic jars for two days or more guarantees reduction, for completely anaerobic plates.

    Gas jars using Hungate apparatus with copper tube attachment (Figure 3). Copper tube can be inserted into one of the clampable rubber tubes attached to the jar.

    Each jar should contain one autoclave tape-sealed petri-dish full Stak-Pak Catalyst, and one full of activated charcoal. These petri-dishes should be placed at the bottom of the petri-dish stack inside each jar. This helps maintain anaerobic conditions inside the jar. Ensure catalyst and charcoal are freshly activated by heating at 150⁰C for 1 hour.

BASIC PROTOCOL 2:

Basic protocol title:

Sample Collection from the Fish Gut and Plating to Single Isolates

Introduction:

This protocol consists of two parts (A) sample collection and (B) culture to single isolates. For our experiment, bacteria were isolated from the hindgut of *K. sydneyanus* (Silver drummer). Specimens were collected off the Northeastern coast on New Zealand, where *K. sydneyanus* is a common herbivorous fish (Mountfort, et al., 2002). The gut was removed by dissection and allocated into five sections (Figure 4) following the guidelines of Johnson and Clements (2022). Microbial collection can take place from segments III, IV and V, as microbial activity is highest in these hindgut sections (Clements, et al., 1994). This was shown by Clements, et al., (1994), where three herbivorous fish were found to have significantly higher concentrations of short chain fatty acids, a microbial fermentation product, in gut segments III, IV and V than in the earlier segments I and II. Increased short chain fatty acid concentration is a sign of bacterial fermentation (Clements, et al., 1994). We sampled from gut sections IV and V in this protocol. We then went on to use novel bacteria species isolated from gut section IV in our example results from Basic Protocols 3 and 4. YCFA media containing fish hindgut fluid (YCFA-GF) was used to culture these bacteria to single isolates. The bacteria isolated in this protocol can be used in the following Basic Protocols 3 and 4.

Materials:

Solid media petri dishes
Dissection equipment
Falcon tubes (50 ml) (Fisher Scientific, cat no. 14-432-22)
Disposable inoculating loops (disposable loops recommended as it is difficult and tedious to sterilize loops in the anaerobic hood using the incandescent flaming device) (Thomas Scientific, cat no. 1230Z30)
Anaerobic jar with pressure gauge (Custom made by Mason Tool and Engineering Ltd, Auckland, New Zealand)
Vinyl Anaerobic Chamber Type A Glove Box (Coy Laboratory Products Inc., Grass Lake, Mich., cat no. 7000000)
Stak-Pak, Catalyst (Palladium) (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6501000)
Stak-Pak, Dessicant (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6502000)
Charcoal, Technical, ActIVated for Gas Adsorption (Fisher Chemical, cat no. 7440-44-0)
CAM-12 Anaerobic monitor (COY Laboratory Products Inc., Grass Lake, Mich., cat no. 6250000)
Hungate gassing apparatus delivering N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere
Hungate tubes (10 ml glass test tubes)
Butyl rubber stoppers for Hungate tubes (Thomas Scientific, cat no. 1208Z83)
Incubator

Protocol steps:

Part A: Sample Collection

5. Remove gut from fish, divide gut into five sections, numbered I-V, as in Figure 4**.**

   This step is performed at the collection site.

6. Remove gut contents from gut sections by squeezing cntents from the gut into a Hungate tube

7. Transport the contents of gut sections IV and V to the laboratory in Hungate tubes, stoppered under anaerobic conditions.

   A transportable version of the Hungate apparatus can be used on the boat at the collection site. Hungate gassing apparatus is connected to a small cylinder containing 90% N_2 *and 10%* CO_2 *to purce the Hungate tubes of oxygen before sealing with a sterile butyl rubber stopper.*

   Contents of gut sections IV and V to be stored separately so as to later enable precise in vivo location information to be found for a bacteria isolate of interest

8. Place the tube of gut contents inside the anaerobic chamber

Anaerobic chamber maintained at an atmosphere of N_2:H_2:CO_2 (87.5:7.5:5) (H_2 levels at 3% inside the chamber) using gas cylinders. The absence of oxygen inside the anaerobic chamber is confirmed throughout the process using the CAM-12 anaerobic monitor. Stak-Pak Catalyst and Dessicant should be present inside the anaerobic chamber. These paks are made from wire mesh to allow air to be circulated through the material.

Allow solids to settle before moving onto the next part of this protocol. As the gut contents are collected anaerobically, they can be streaked straight away after the solids have settled (5 minutes standing time).

Everything is done at room temperature until this stage, but if there is a need to store these gut contents until streaking can be done, they need to be stored at 19 – 20 °C.

Part B: Culture to Single Isolates

7. Streak 100 µl crude gut contents onto a YCFA-GF petri dish inside the anaerobic chamber.

    Streak using a sterile disposable inoculating loop.

    This is 'culture one' of the isolate of interest.

8. Bring anaerobic jar into the anaerobic chamber, place streaked plates inside jar and seal.

    Anaerobic chamber maintained at an atmosphere of N_2:H_2:CO_2 (87.5:7.5:5) (H_2 levels at 3% inside the chamber) using gas cylinders. The absence of oxygen inside the anaerobic chamber is confirmed throughout the process using the CAM-12 anaerobic monitor. Stak-Pak Catalyst and Dessicant should be present inside the anaerobic chamber. These paks are made from wire mesh to allow air to be circulated through the material.

    Each jar should contain one autoclave tape-sealed petri-dish full of Stak-Pak Catalyst, and one full of activated charcoal. These petri-dishes should be placed at the bottom of the petri-dish stack inside each jar. This helps maintain anaerobic conditions inside the jar. Ensure catalyst and charcoal are freshly activated by heating at 150^0C for 1 hour.

9. Remove anaerobic jar from anaerobic chamber and gas with gas mix of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure, using the Hungate gassing apparatus.

10. Incubate anaerobic jar at 20 °C. Incubation period of 3 days, or until single colonies are visible.

    Place sealed anaerobic jar inside incubator. As in Mountfort, et al., (2002), bacterial incubation should be maintained at the ambient seawater temperature of 17 to 23 °C, because the gut environment of K. sydneyanus will also be within this temperature range.

11. Pick a well isolated colony and re-streak on YCFA-GF once they grow to maximum cell number (about 3 days).

    This is 'subculture one' of the isolate of interest.

12. Re-streak two more sub-cultures of each colony of interest to produce pure single isolate cultures, ready for identification using colony PCR followed by Sanger sequencing using 16S V3-V4 primers.

    Label cultures after each streak to ensure consistency in colony picking.

BASIC PROTOCOL 3:

Basic protocol title:

Genetic Identification of Single Isolates with Colony PCR and 16S Sequencing

Introduction:

This protocol describes how to genetically identify the single isolates that were cultured in Basic Protocol 2. This protocol consists of two parts: (A) colony PCR amplification with purification, and (B) 16S sequencing with analysis. Genetic identification of these isolates enables the results of Basic Protocol 4 to be analyzed in relation to previous studies on the same species, or close genetic relatives.

Materials:

PCR tubes and lids
PCR SuperMix (Invitrogen, cat no. 10572014)
Vinyl Anaerobic Chamber Type A Glove Box (Coy Laboratory, cat no. 7000000)
Toothpics (sterilised)
16S V3-V4 region primers: e.g. 341F forward primer and 785R reverse primer (Klindworth, et al., 2013)
PCR hood (Grant Instruments, cat no. 7.970 866)
Thermal cycler (Thermo Fisher Scientific, cat no. 4375305)
QIAquick PCR Purification Kit (Qiagen, cat no. 28104)
Water, PCR grade (Roche, cat no. 03315843001)
IMPLEN NanoPhotometer N60 Micro-Volume UV-VIS Spectrophotometer (Fisher Scientific, cat no., 15442203)
Eppendorf Safe-Lock Tubes, 1.5 mL (Eppendorf, cat no. 0030120086)
Geneious Prime 2019.2.1 bioinformatics software (Kearse, et al., 2012) (https://www.geneious.com) (see ‘Internet Resources’)
NCBI BLAST: Basic Local Alignment Search Tool) (nih.gov) (Altschul, et al., 1990) (see ‘Internet Resources’)
Permanent marker pen

Protocol steps:

Part A: Colony PCR

9. Prepare the following PCR master mix to amplify each sample:

Primer F341	1 µl	x no. of samples
Primer R806	1 µl	x no. of samples
2x concentrated PCR SuperMix	12.5 µl	x no. of samples
H_2O	10.5 µl	x no. of samples

Forward primer (F341) sequence: 5' CCTACGGGNGGCWGCAG 3'. Reverse primer (R806) sequence: 5' GACTACHVGGGTATCTAATCC 3'. These primers cover the V3 – V4 region. Primer concentration of 25 μM. The volume of each reaction will be 25 μl.

10. Take the anaerobic jar containing the third sub-culture of single isolates from the incubator into the anaerobic chamber.

    These culture plates from Basic Protocol 2 have been incubating in an anaerobic jar at 20 °C N2, CO2, and H2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere at 5 psi pressure for a period of 3 days, or until single colonies were visible

    Anaerobic chamber maintained at an atmosphere of N_2:H_2:CO_2 (87.5:7.5:5) (H_2 levels at 3% inside the chamber) using gas cylinders. The absence of oxygen inside the anaerobic chamber is confirmed throughout the process using the CAM-12 anaerobic monitor. Stak-Pak Catalyst and Dessicant should be present inside the anaerobic chamber. These paks are made from wire mesh to allow air to be circulated through the material.

11. Take the PCR tubes, lids, and toothpicks into the anaerobic chamber.
12. Touch a single colony with a toothpick and rub onto the wall of a PCR tube. Circle colony on plate using a permanent marker pen. Using the marker pen, label the circle on the plate to match the PCR tube. The culture of this isolate can then remain linked to the genomic identification during subsequent re-streaking.
13. Attach PCR lids and take out of anaerobic hood and into PCR hood. Add 25 μl PCR mix into each PCR tube, pipette up and down to mix.
14. Perform PCR with the following thermal cycler settings:

1 cycle:	95^{0}C	5 min
25 cycles:	95^{0}C	30 sec
	55^{0}C	30 sec
	72^{0}C	30 sec
1 cycle:	72^{0}C	30 min
Final step:	12^{0}C	(hold)

15. Use the QIAquick PCR Purification Kit to purify DNA and elute with 30 μl PCR grade water.
16. Measure DNA concentration using the NanoPhotometer then send for sequencing.

Part B: 16S Sequencing

5. Use the NanoPhotometer to quantify the DNA.

   Optimum DNA concentration is 5-20 ng/μl, can adjust concentration using PCR grade water.

   Optiumum purity (A260/280) of 1.8 or above.

6. Send purified samples, along with appropriate V3-V4 primers, for sequencing of the 16S rDNA gene at a sequencing facility, using Sanger sequencing.

   Forward primer (F341) sequence: 5' CCTACGGGNGGCWGCAG 3'. Reverse primer (R806) sequence: 5' GACTACHVGGGTATCTAATCC 3'. These primers cover the V3

– V4 region. Primer concentration of 25 μM. The volume of each reaction will be 25 μl.

7. Perform species identification using BLAST computer algorithm, accessed on the NCBI website. BLAST is used to compare and align these 16S sequences to the BLAST public database (Altschul, et al., 1990) (see 'Internet Resources').

   If the isolate is a known species, then there will be a 16S rRNA genetic sequence match in the NBCI database. In this case further experimental work will add to the current knowledge of this species.

   If the isolate is a novel genus or species, then relatedness to known species can be found using BLAST and Geneious analysis. Currently, the cutoff for a novel species and genus is a sequence identity of 98.7% and 94.5% respectively (Yarza, et al., 1996). Further experimental work on these novel isolates will provide new information on these previously uncultured species.

8. Perform computational analysis of 16S sequencing data using Geneious bioinformatics software. This software is used to generate phylogenetic trees (Kearse, et al., 2012) (see 'Internet Resources').

   Phylogenetic trees should include 16S sequences from the public NBCI database, along with any unpublished sequences that have previously been identified using these methods.

   Generation of a phylogenetic tree informs future work on the isolate. If close relatives are found within the NBCI database, then previously published studies will inform media choice for culture of these bacteria.

   Phylogenetic trees generated from many bacteria isolated from the same environment will give insight to the microbiome of this environment.

BASIC PROTOCOL 4

Basic protocol title:

Measurement of Bacterial Growth Rates on Solid Media

Introduction:

This is a high-throughput protocol measuring the growth rate of fastidious anaerobic gut bacteria on different solid media. Growth effects of various nutrient additions can be compared to develop an optimal medium. In this protocol YCFA medium (Duncan, et al., 2002), with the addition of gut fluid (YCFA-GF), is used as a standard against which novel media recipes are compared. YCFA-GF often produces optimal growth rates of bacteria in vitro as gut fluid replicates the natural nutrient environment of gut bacteria in vivo. However, as the nutrient content of fish gut fluid is variable (Egerton, et al., 2018), this is an un-replicable media recipe. This protocol allows a preferable replicable medium, with commercially sourced ingredients, to be developed for any gut bacteria of interest. Importantly, strict anaerobic conditions are maintained at all times. Fish hindgut bacteria, cultured to single isolates and genetically identified using Basic Protocols 2 and 3, were tested for growth rate on two solid media: YCFA-GF and YCFA-M. Here YCFA-M is being tested as an alternative to YCFA-GF media. Mannitol was chosen as a media ingredient of interest for reasons explained in Basic Protocol 1.

Determination of bacterial growth rate on solid media in this protocol is based on methods developed for anaerobic bacteria culture by Casciato, et al., (1974). Casciato, et al., (1974) used water containing 0.1% gelatin (water-gel) to wash bacteria from the surface of solid media in a petri dish. Approximate cell density in this water-gel suspension was measured by conversion of the optical density at 600 nm to approximate colony forming units per ml (CFU/ml) with the widely used McFarland standards (Casciato, et al., 1974; McFarland, 1907). In this protocol we adjusted the inoculum to the cell density of McFarland standard 4 (approximately 12.4 x 10^8 CFU/ml) for inoculation into each well of the 48-well plates created in Basic Protocol 1 (Figure 6). Any of the four McFarland standards can be chosen, this choice is based on the growth success of the isolate of interest in the preculture stage. If the isolate grows poorly a lower McFarland standard can be used. The purpose of the choice of a single McFarland standard for all inoculums within an experiment is to maintain consistency, producing directly comparable results between isolates and conditions.

This protocol applies principles from Casciato, et al., (1975) to a high-throughput method, with 48-well plates allowing triplicate replicates and testing of multiple factors under the same conditions. Another novel development in this protocol is that there is no need for physical McFarland standards. These have been replaced with Equations 1 and 2, which we generated by graphing McFarland standards on an exponential curve against their approximate CFU/ml concentrations. These equations speed up and simplify the bacterial growth rate calculation process.

As explained in the introduction, these previously uncultured anaerobic bacteria are often slow growing, in which case the usual exponential growth profile does not apply. If bacterial growth is linear, as with fish gut bacteria isolates BP5G and BP52G, equations are provided within this protocol to calculate bacterial growth rate as an increase in colony forming units over time (CFU/hour). If bacterial growth is exponential, this protocol provides equations to calculate growth rates as either a doubling time in units of hours, or a growth rate constant (α) in units of minutes^{-1}, both of which are long established ways to express bacterial growth rate, outlined in Hall, et al., (2013). All of these growth rates allow the direct comparison of the growth effects of various media ingredients in order to develop an optimal medium.

Materials:

Gelatin from Porcine Skin (Sigma-Aldrich, cat no. G2625)
Sterile water (50 ml) (deionized or autoclaved)
Eppendorf Safe-Lock Tubes, 2 mL (Sigma-Aldrich, cat no. EP022363344)
96-well plate, flat bottom (Sigma-Aldrich, cat no. CLS3340)
Absorbance microplate reader (Parkin Elmer, EnVision 2104 Multilabel Plate Reader)
Centrifuge (that can accommodate 2 ml tubes)
Disposable sterile inoculating loops (Thomas Scientific, cat no. 1230Z30)
Disposable sterile delta cell spreader (Fisher Scientific, cat no. 50-751-5036)
Falcon tubes (50 ml) (Fisher Scientific, cat no. 14-432-22)
Mass scale
Weigh paper or trays
Spatula (for weighing out reagents)
Pipette controller
Vortex (that can accommodate 50 ml tubes)
Vinyl Anaerobic Chamber Type A Glove Box (Coy Laboratory Products Inc., Grass Lake, Mich., cat no. 7000000)

CAM-12 Anaerobic monitor (COY Laboratory Products Inc., Grass Lake, Mich., cat no. 6250000)

Stak-Pak, Catalyst (Palladium) (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6501000)

Stak-Pak, Dessicant (COY Laboratory Products Inc., Grass Lake, Mich. SKU: 6502000)

Charcoal, Technical, ActIVated for Gas Adsorption (Fisher Chemical, cat no. 7440-44-0)

Hungate gassing apparatus delivering N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) gas atmosphere

Hungate tubes (10 ml glass test tubes)

Butyl rubber stoppers for Hungate tubes (Thomas Scientific, cat no. 1208Z83)

Protocol steps:

Part A: Prepare the primary growth culture

11. Streak chosen bacteria from single colony isolates onto individual 90 mm petri dishes containing solid YCFA-GF medium (generated in Basic Protocol 1). Perform this step inside the anaerobic hood using disposable inoculating loops.

    One isolate of interest per petri dish. Streak over entire plate.

12. Place inoculated petri dishes into an anaerobic jar inside the anaerobic chamber. Remove sealed jar from anaerobic chamber and gas jar with gas mix of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) to 5 psi using the Hungate apparatus with the copper tube attachment to insert into jar (Figure 3).

    Anaerobic chamber kept at 20 ^oC and maintained at N_2:H_2:CO_2 (87.5:7.5:5) (H_2 levels at 3% inside the chamber). The absence of oxygen inside the anaerobic chamber is confirmed throughout the process using the CAM-12 anaerobic monitor. Stak-Pak Catalyst and Dessicant should be present inside the anaerobic chamber. These paks are made from wire mesh to allow air to be circulated through the material.

    Each jar should contain one autoclave tape-sealed petri-dish full of Stak-Pak Catalyst, and one full of activated charcoal. These petri-dishes should be placed at the bottom of the petri-dish stack inside each jar. This helps maintain anaerobic conditions inside the jar. Ensure catalyst and charcoal are freshly activated by heating at 150^0C for 1 hour.

13. Incubate anaerobic jar containing the preculture at 20 ^oC for 3 days, or to peak growth phase of chosen isolate under these conditions.

    Place sealed anaerobic jar inside incubator. Incubation temperature chosen as in Mountfort, et al., (2002): bacterial incubation should be maintained at the ambient seawater temperature of 17 to 23^oC because the gut environment of K. sydneyanus will also be within this temperature range.

14. Inside the anaerobic chamber, pipette 2000 µl water-gel (see Reagents and Solutions) onto petri dish with isolate culture. Resuspend bacteria into the water-gel using a disposable cell spreader, or alternatively using a disposable inoculating loop.

    Gently rub colonies with disposable cell spreader or disposable inoculating loop to resuspend them into the water-gel.

15. Transfer preculture water-gel suspension from petri dish to 2 ml Eppendorf tube.

Tip petri dish to gather all water-gel at one side. Pipette water-gel suspension from just above the agar layer into 2 ml Eppendorf tube.

16. Transfer 100 µl sample of this preculture water-gel suspension into a 96-well plate. Remove from the anaerobic chamber and measure absorbance at 600 nm in an absorbance microplate reader. In addition, transfer 100 µl of sterile water-gel to another well to serve as a blank reference for the absorbance reading.

    The volume of sample in the 96-well plate must remain consistent as the distance the light has to travel affects the resulting absorbance readings. The same volume is used for absorbance readings in the 96-well plate at all stages of the protocol.

17. Calculate preculture cell concentration (x 10^8 CFU/ml) in the 100 µl sample from its absorbance at 600nm using Equation 1.

$$\text{Cell density in sample } (x\ 10^8 \text{ CFU/ml}) = 1.1449\ e^{3.5633\ x\ (\text{Absorbance at } 600\ nm)}$$

Equation 1

Equation 1 explains the exponential relationship between approximate cell density and absorbance at 600 nm among the widely used McFarland standards (Casciato, et al., 1974; McFarland, 1907). Equation 1 is the equation of the exponential trendline from a graph plotting these two variables against one another for each of the four McFarland Standards.

18. Calculate amount of water-gel needed to reconstitute a cell pellet from the 2 ml water-gel suspension sample to McFarland standard 4 (cell density approximately 12.4 x 10^8 CFU/ml) using Equation 2. Equation 2 finds the numerical ratio between the cell density in the sample versus the desired cell density of McFarland standard 4. It then multiplies this by the suspension sample volume (2 ml). This produces the volume of water-gel required to reconstitute the pellet at a cell density of McFarland standard 4.

$$\text{Volume water-gel for McFarland standard 4 (ml)} = 2 \text{ ml } x\ \frac{\text{Cell density in sample } (x\ 10^8 \text{ CFU/ml})}{\text{Cell density of McFarland standard 4 } (12.4\ x\ 10^8 \text{ CFU/ml})}$$

Equation 2

19. Inside the anaerobic chamber, centrifuge the 2 ml Eppendorf of water-gel suspension at 1500 rcf for 90 seconds, generating a pellet of the bacteria in this sample
20. Inside the anaerobic chamber, reconstitute the preculture pellet at the cell density of McFarland standard 4 by removing the supernatant, adding the calculated volume of water-gel (from Step 8), and gently pipetting up and down to resuspend the pellet.

    Gentle pipetting is important to minimize sheer stress effects on the bacterial cells.

Part B: Prepare 48-well plate and perform growth rate experiment

5. Inoculate 10 µl of the reconstituted pellet onto the solid media in individual wells. Following the new example plate layout (Figure 7), reconstituted pellet is inoculated into Columns 1 – 3 and 5 – 7 of a 48-well plate. The plate, containing two different solid media, was prepared in Basic Protocol 1 (Figure 6).

   Each well is inoculated with the same volume of bacteria at the same concentration. This allows direct comparison between growth rates and analysis of the growth effects of different media ingredients.

   Columns 4 and 8 are used as no isolate controls in this plate layout.

6. Take the time zero timepoint by pipetting 100 µl water-gel onto the zero-hour timepoint replicate wells. In the example plate layout (Figure 7) these wells are Row A, Columns 1 – 3 and 5 – 7. Resuspend bacteria into the water-gel by lightly scraping the plate with a disposable inoculating loop.

7. Pipette the 100 µl water-gel suspension from each of the three replicate zero-hour wells into a 96-well plate for absorbance measurement at 600 nm in an absorbance microplate reader. This absorbance reading can be converted into cell concentration using McFarland standards with Equation 1. In addition, transfer 100 µl of sterile water-gel to another well to serve as a blank reference for the absorbance reading.

   Use of three replicates reduces the influence of any "edge effects" that can cause differences in absorption between wells.

   These zero-hour readings are used for analysis purposes only, not growth rate calculations, as they are outside of the growth period.

8. Calculate at least four further timepoints during growth phase using these methods. Growth rates are measured using a window of at least four timepoints over the growth period, as recommended by Hall, et al., (2013).

   These four timepoints must occur after lag phase, and before stationary phase. These vary among bacteria and must be discovered with preliminary experiments on a chosen isolate. For our fish gut bacteria isolates the growth phase lasted from 3 to 26 hours and timepoints were taken within this range.

Part C: Calculating bacterial growth rate for linear growth

Calculate linear growth rates by increase in colony forming units (CFU) per well over time (hours).

4. Use Equation 1 to convert the absorbance readings of at least four timepoints to a value of approximate cell density in each well in the units of colony forming units per 1 ml (x 10^8 CFU/ml).

5. Take ten percent of these values, this converts the cell density into the approximate cell number in each well (x 10^8 CFU/well), as 100 µl samples are taken of all cells in each well to be measured for absorbance at O.D. 600.

6. Plot CFU/well against time, apply a linear trend line. The gradient of this line is the growth rate of bacteria in units of CFU/hour.

   Comparison of these bacterial growth rates (CFU/hour) on different media allows the development of an optimal media recipe for any anaerobic bacteria of interest. Trialing various nutrient additions allows novel growth factors to be discovered.

Part D: Calculating bacterial growth rate for exponential growth

Calculate exponential growth rates as a first order growth rate constant (α) (minutes^{-1}), or as a doubling time (hours).

3. The first order growth rate constant (α) is expressed in Equation 3 from Hall, et al., (2013), where α is the gradient of a linear trendline applied to a logarithmic graph of OD_{600} versus time. To calculate this growth rate, plot the natural logarithmic value of the mean O.D. values (ln *N*) versus time (*t*) in hours using a graphing program. Equation 3 is generated from the gradient of a straight line drawn between two points (at time *t* and time *0*) on this graph during the exponential growth phase, further explained in Hall, et al., (2013).

$$\ln\frac{N_t}{N_0} = \alpha(t - t_0)$$

Equation 3

Where N is the number of cells at time t and time 0, and α is the first-order growth rate constant. When time (t) is measured in minutes, α is reported in reciprocal minutes (minutes^{-1}) (Hall, et al., 2013).

4. The doubling time (hours) is calculated using the Equation 4 from Hall, et al., (2013) by finding the κ coefficient associated with exponential bacterial growth. The κ coefficient for this data can be found by plotting mean O.D. values against time (hours) and applying an exponential trendline to the graph. The equation associated with this exponential trendline contains the κ coefficient as the exponential index. This is used in Equation 4 to calculate the doubling time.

$$\text{Doubling time (hours)} = \frac{ln_2}{\kappa\, coefficient}$$

Equation 4

Growth rates are estimates and interpreting them requires some judgement, many confounding factors can affect absorbance readings and therefore growth rate calculations (Hall, et al., 2013). Hall, et al., (2013) recommends that if the standard error is >3% of the growth, or if the correlation coefficient is <0.995, then the results can be considered unreliable.

REAGENTS AND SOLUTIONS:

100 ml YCFA media (adapted from Duncan, et al., 2002):

Part 1

Tryptone (Bacto) (BD, cat no. 211705) (0.2 g)

Yeast extract (BBL) (BD, cat no. 211929) (0.05 g)

Agar (Difco) (BD, cat no. 21430) (1.7 g)

K_2HPO_4 at 0.6% w/v in distilled water (3800 µl)

Make 100 ml stock at a time. This stock can be stored in a Schott bottle for up to 30 days at 3 – 5 ^{o}C.

Mineral mix from stock (3800 µl)

Make up stock in advance: KH_2PO_4 phosphate (0.6% w/v), $(NH_4)_2SO_4$ (0.6% w/v), NaCl (1.2% w/v), $MgSO_4.7H_2O$ 0.245% w/v), $CaCl_2.2H_2O$ (0.15% w/v). Autoclave for 15 minutes at 15 psi on a liquid cycle. Make 100 ml stock at a time. This stock can be stored in a Schott bottle for up to 30 days at 3 – 5 ^{o}C

Trace elements from stock (10 µl)

Make up stock in advance: $CuSO_4.4H_2O$ (4 mg), $MnSO_4.4H_2O$ (50 mg), $Ni(II)Cl_2$ (0.2 mg), $FeSO_4$ (10 mg), $Co(II)(NO_3)_2.6H2O$ (0.2 mg), H_2MoO_4 (0.2 mg), and bring to 100 mL with distilled water. Autoclave for 15 minutes at 15 psi on a liquid cycle. Make 100 ml stock at a time. This stock can be stored in a Schott bottle for up to 30 days at 3 – 5 ^{o}C

Resazurin at 0.1% w/v in distilled water (100 µl)

Make 100 ml stock at a time. This stock can be stored in a foil covered schott bottle for up to 30 days at room temperature.

Part 2

$NaHCO_3$ at 4% w/v in distilled water (10000 µl)

Sterilise 4% w/v sodium carbonate by autoclaving in an open flask, this turns to sodium hydroxide during heating. While hot, bubble with CO_2 gas until cool, using a serological pipette attached to the Hungate apparatus, delivering N_2 gas. Solution will has turned into sodium bicarbonate and carbonic acid when white flecks vivible on the end of the serological pipette. Make 100 ml stock at a time. Stopper in 10 ml aliquots under CO_2 gas. This stock can be stored for up to 30 days at 3 – 5 ^{o}C

Cysteine-HCl at 2.5% w/v in distilled water (2000 µl)

Dissolve 1 g Cystine-HCL in 40 ml distilled water. Bring to boil under N_2 atmosphere by gassing stock in Hungate vial with N_2 using the Hungate apparatus. Stopper vial, secure using aluminium wire, and autoclave. Make 100 ml stock at a time. Store in 10 ml aliquots in Hungate tubes under 100% N_2 by gassing tubes using the Hungate technique. This stock can be stored for up to 30 days at 3 – 5 ^{o}C

Vitamin K_3 (0.005% w/v) + Hemin (1% w/v) mix in distilled water (1000 µl)

For Hemin dissolve 10 mg hemin in 0.2 ml 1M NaOH, add 20 ml distilled water and autoclave. For vitamin K_3 add 100 mg sterile vitamin K_1 or vitamin K_3 to 20 ml 95% ethyl alcohol. For mixed stock add 0.2 ml sterile vitamin K_3 to 20 ml hemin. Make 100 ml stock at a time. Store in 10 ml aliquots in Hungate tubes under 100% N_2 by gassing tubes using the Hungate technique. This stock can be stored for up to 30 days at 3 – 5 ^{o}C

Volatile fatty acids mixture from stock (1000 µl)

Make up stock in advance: Acetic acid (17 mL), propionic acid (6 mL), N-valeric acid (1 mL), isovaleric acid (1 mL), and isobutyric acid (1 mL). Make up to ~50 mL with distilled water, neutralize with 5M NaOH to pH 6.5, and make up to 100 mL with water. Autoclave under a N_2 atmosphere by gassing stock in Hungate vial with N_2 using the Hungate apparatus. Stopper vial, secure using aluminium wire, and autoclave. Make 100 ml stock at a time. Store in 10 ml aliquots in Hungate tubes *under 100% N_2 by gassing tubes using the Hungate technique. This stock can be stored for up to 30 days at 3 – 5 °C*

Part 3

Part 3A (GF): Autoclaved and filtered gut fluid supernatant from stock (10000 µl)

Make up stock in advance: Kyphosus sydneyanus hindgut lumen contents from sections IV and V (Figure 4) are filtered through a cheese cloth and the filtrate is centrifuged at 9000 g for 20 min at 4°C. Collect supernatant and autoclave. Centrifuge supernatant at 13500 g for 20 min at 4°C. Make 100 ml stock at a time. Freeze in two 50 ml Falcon tubes at -20°C to store for up to 9 months. Alternatively, store in 10 ml aliquots in Hungate tubes under 100% N_2 using the Hungate technique. These stocks can be stored at 3 – 5 °C for up to 30 days.

Part 3B (M): Mannitol (Sigma, cat no. 63H01621) at 5% w/v (4000 µl)

This stock can be stored for up to 30 days at 3 – 5 °C

YCFA media can be stored up to 90 days at 3 – 5 ^{0}C.

50 ml Water-gel containing 0.1% gelatin (adapted from Casciato, et al., 1974):

0.05 g Gelatin (Gelatin from Porcine Skin, Sigma-Aldrich, cat no. G2625)

50 ml Sterile water (deionized or autoclaved)

6. Add gelatin and water to a Falcon tube, vortex for 30 seconds to mix.
7. Autoclave the solution for 15 minutes at 15 psi on a liquid cycle.

   Twist Falcon lid only halfway on, to allow pressure equilisation inside autoclave.
8. Transfer 10 ml water-gel into each of five Hungate tubes using the Hungate technique.

   A serological pipette can be used to aliquof from the Falcon tube into Hungate tubes in the Hungate apparatus. Hungate tubes should have Hungate needles inserted throught aliquoting. Butyl rubber stoppers are insterted into the Hungate tubes using the Hungate technique (Bryant, 1972; Hungate, 1969). This ensures the headspace is completely free of oxygen.
9. Gas these tubes with anaerobic gas mix of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) using the gassing needles of the Hungate apparatus.

   Gas tubes using the Hungate technique (Bryant, 1972; Hungate, 1969). Secure with a butyl rubber stopper.
10. Leave to become anaerobic overnight inside the anaerobic chamber with bungs loose to allow gas exchange.

    When moving stoppered Hungate tubes into the anaerobic hood, secure stopper into the tube with tape. This prevents gas purging in the airlock from removing the butyl rubber stoppers.

Water-gel can be stored up to 90 days at 3 – 5 ^{0}C with bungs secure.

COMMENTARY:

Background Information:

Why the procedure is performed and how the technique has evolved

The starting point of developing a replicable media for the culture of a novel bacterium is to include the essential elements for growth in sufficient amounts. Some environmentally isolated bacteria grow in simple media with just a single carbon source (Plugge, 2005). Others require addition of macro elements C, H, O, N, P and S, and metals Ca, Cl, Fe, K, Mg and Na, which together make up 95% of average microorganism dry weight (Plugge, 2005). However, many bacteria remain unculturable even when all common essential elements have been added in sufficient amounts (Plugge, 2005). These species require further nutrients to be added, these nutrients are classed as growth factors and are typically one of either amino acids, vitamins, or pyrimidines and purines (Plugge, 2005). For example, many *Bacteroides* strains require the presence of vitamin K_1 and hemin for growth (Gibbons & MacDonald, 1960).

Investigation of anaerobic bacteria in vitro requires specialized media containing all essential nutrients for growth. To improve the accuracy of bacterial function analysis, a medium of reduced complexity with minimal undefined ingredients should be used (Tramonatano, et al., 2018). We present a high-throughput method of developing an optimal medium. This protocol uses solid media because fastidious anaerobic bacteria often do not grow in a liquid media (Speers, et al., 2009). Use of a solid media allows this protocol to be used on a broader range of gut bacteria species.

The central advantages (and disadvantages) of the technique chosen

The key advantage of this technique is the use of a high-throughput set up. Anaerobic bacteria require long incubation times; for routine clinical microbiology anaerobic cultures should be incubated for a minimum of five days (Lagier, et al., 2015; Chapin, et al., 2007). Also, as the gut environment is highly nutritionally complex, bacterial species from the same host often have highly variable nutrient preferences (Egerton, et al., 2018). These complications make developing an optimal medium for previously uncultured bacteria a long process that is nevertheless essential for the successful culture of many species. This high-throughput experimental set up allows three replicates of a potential growth factor over up to six times points on a single 48-well plate. If the anaerobic jar is large enough two plates can be tested at one time. One experimental run using this jar can produce average growth rates of two isolates on two media. This protocol is to our knowledge the quickest way to find the precise nutrient requirements of a species of interest.

Another advantage of this technique is that the novel media recipe developed in these protocols can be exactly replicated in future work, reducing the effect of confounding variables on results. Most human gut bacteria have no growth characterization data available in replicable media (Tramonatano, et al., 2018). These bacteria have only been cultured in media with gut fluid or other complex and undefined nutrient sources. A replicable media recipe has the advantage of allowing metabolomic and proteomic studies to be carried out on bacteria in vitro. Gut bacteria must be cultured in a nutritionally defined media to study the link between host diet, microbiota composition and dynamics, and interspecies interactions (Tramonatano, et al., 2018).

Comparison of Basic and Alternate protocols or comparison with other methods currently in use

Other available protocols for measuring growth rate in a high-throughput fashion are not suitable for strictly anaerobic bacteria. A high-throughput method developed by Hall, et al., (2013) uses microtiter plates to measure absorbance over the growth period aerobically in a plate reader. However, this method is not appropriate for strictly anaerobic species, as an anaerobic environment cannot be maintained in most plate readers.

Another method by Tramontano, et al., (2018) uses a microwell plate inside an anaerobic chamber to measure bacterial growth rates anaerobically. This method was designed specifically for anaerobic bacteria. However, this method requires culture in liquid media (Tramontano, et al., 2018), whereas many fastidious gut bacteria only survive on a solid media (Speers, et al., 2009).

There is a need for our method, the first for high-throughput growth rate analysis of fastidious anaerobic bacteria on solid media. These protocols will enable quick development of novel media recipes for a broad range of fastidious anaerobic bacteria.

Uses and applications of methods

The structure and function of gut microbiome communities and their relation to the host is an area of great research interest. However, few microorganisms isolated from environmental samples survive in vitro culture (Barer & Harwood, 1999; Giovannoni, et al., 1990; Wayne, et al., 1987; Torsvik, et al., 1990; Ward, et al., 1990; Barns, et al., 1994; Kaeberlein, et al., 2002). As culture methods were found to be inadequate for the study of microbial community composition, there was an increased interest in sequence-based genetic techniques that bypassed the need for in vitro culture (Ward, et al., 1990; Barns, et al., 1994). These genetic techniques greatly expanded knowledge of microbial biodiversity and related evolutionary processes (Barns, et al., 1994). While much progress has been made using a metagenomic cultivation-independent approach, the only secure and operational proof of organism viability is culturability (Barer & Harwood, 1999). While genomic 16S and rRNA studies of environmentally isolated bacteria can suggest the presence of metabolic pathways (Béjà, et al., 2000; Béjà, et al., 2002), cultivation is required in order to understand the physiology of novel microbes and the functional processes they engage in (Zengler, et al., 2002; Stewart, et al., 2012). Our protocol can be used to successfully culture anaerobic bacteria from the environment for the first time. This addresses a current challenge for microbiologists of improving in vitro culture methods, leading to greater understanding of the metabolic properties and potentials of these organisms (Zengler, et al., 2002).

These protocols allow the trial of novel nutrient additions to media in a high-throughput fashion. The growth effects of media ingredient removal can also be tested with these protocols. When using a nutrient media, SCFAs and amino acids that act as bacterial growth promotors for some species can inhibit the growth of other species (Tramonatano, et al., 2018). Removal of these inhibitors allows previously unculturable bacteria to be cultured in vitro for the first time. The result of these experiments is the development of an optimal media for the in vitro growth of a chosen anaerobic isolate.

Critical parameters:

There are several critical parameters influencing bacterial growth that can be individually addressed to improve the performance of chosen isolates in these protocols. Maintenance of anaerobic conditions is crucial; hence oxygen must be completely excluded from the system. The Hungate technique must be strictly applied when making media to keep media ingredients anaerobic. The 48-well plates must be stored in an anaerobic jar gassed with standard gas mix of N_2, CO_2, and H_2 (47.5%, 47.5%, 5% v/v/v) at 5 psi at 3 - 5 ^{0}C for at least two days before use. This ensures the solid media is completely reduced. When gassing anaerobic jars, the pressure must not drop below 1 psi at any point. The gas must only move in one direction, away from the gas cylinders, through the jar and out. The seals on the anaerobic jars are verified by minimal loss of pressure over time. Water-gel must be reduced in the anaerobic hood overnight before use. Oxygen levels in the anaerobic hood are monitored with an anaerobic monitor to ensure no oxygen has entered the system.

The amount of trial growth factor added to YCFA media to test growth effects must be decided based on previous work with similar compounds. Basic Protocol 1 explains that the concentration of mannitol in the YCFA-M media used in our study was chosen based on recommendations by Holdeman, et al., (1977), and Browne, et al., (2016), who used a 0.2% (w/v) concentration of sugars in their anaerobic media.

Troubleshooting:

Table 1: Troubleshooting Guide for Measurement of Bacterial Growth Rates on Solid Media

Understanding the results:

As an example of anticipated results, we conducted growth rate experiments on two *Kyphosus sydneyanus* gut section IV (Figure 4) derived bacterial isolates from families Erysipelotrichaceae and Lachnospiraceae. Growth was measured by optical density at 600 nm in two different conditions; YCFA medium with gut fluid (YCFA-GF), and YCFA medium with mannitol (YCFA-M). Standard YCFA medium with gut fluid (Duncan, et al., 2002) is used when culturing previously uncultured gut bacteria in vitro for the first time because it contains many of the nutrients present in the natural environment of these organisms. Mannitol is present at decreasing levels along the fish gut (White, et al. 2010). As this decrease could be caused by fermentation by gut bacteria (White, et al. 2010), this carbohydrate is a possible growth factor for these organisms. In these experiments the effect of mannitol on growth rate was tested on both the Erysipelotrichaceae and Lachnospiraceae isolates. The medium YCFA-M is a defined alternative to YCFA-GF, as all ingredients are industrially sourced. Therefore, YCFA-M is preferable to the complex and nutritionally undefined YCFA-GF for certain metabolic analyses of bacteria in vitro (Tramonatano, et al., 2018).

For representative data, absorbance at wavelength 600 nm was measured at four timepoints during the growth phase of two isolates, BP5G and BP52G (Figure 8). Growth phase for these isolates was found in preliminary work to be between 3 and 27 hours. Three replicates were produced for each of two treatments of YCFA media: addition of gut fluid, or addition of mannitol. Gradients of the linear regression lines applied to the representative data in Figure 8 can be used as approximate growth rates in units of colony forming units per hour (CFU/hour), using the method in Basic Protocol 4, Part C. Alternatively, growth rates of bacteria with exponential growth would be calculated in minutes^{-1}, or hours, as in Basic Protocol 4, Part D. The individual growth rates of the three replicates in this study were averaged to generate Table 2; average approximate growth rates.

For the Lachnospiraceae isolate BP5G, YCFA-GF medium produced an average growth a rate of 1.1×10^5 (± 0.44) CFU/hour (Table 2). This result is used as the desired growth rate against which the alternative media recipe of YCFA-M can be compared. The average growth rate on YCFA-M was found to be 1.3×10^5 (± 0.45) CFU/hour (Table 2). For a linear regression model of OD_{600} against time for this isolate, growth on both YCFA-GF ($R^2 = 0.5574$, $p = 0.001386$)

and YCFA-M were significant ($R^2 = 0.9302$, $p = 6.82 \times 10^{-9}$) compared to no growth. Also the interaction effect (Hour:Media) is significant ($p = 0.0302842$), indicating a significant difference in the growth rate of BP5G on YCFA-GF and YCFA-M. These results show that, for Lachnospiraceae isolate BP5G, YCFA-M produces a significantly faster growth rate than YCFA-GF. Based on these results, mannitol can replace fish gut fluid in YCFA medium for culture of Lachnospiraceae isolate BP5G as it is a more effective growth factor. This creates YCFA-M, a defined and replicable alternative to YCFA-GF for fish gut bacteria isolate BP5G.

For the Erysipelotrichaceae isolate BP52G, YCFA-GF medium produced an average growth rate of 0.4×10^5 (± 0.10) CFU/ml (Table 2). This result is used as the desired growth rate against which the alternative media recipe of YCFA-M can be compared. The average growth rate on YCFA-M was found to be 0.05×10^5 (± 0.01) CFU/ml (Table 2). For a linear regression model of OD_{600} against time for this isolate, growth on YCFA-GF was significant compared to no growth ($R^2 = 0.3678$, $p = 0.01653$), whereas YCFA-M was not significant ($p > 0.05$). Although there was some growth of BP52G on YCFA-M (Supplementary material) this growth was poor and not statistically significant. Based on these results, mannitol cannot replace fish gut fluid in YCFA media for culture of Erysipelotrichaceae isolate BP52G as it produces significantly inferior growth.

These results demonstrate the unique nutritional requirements of isolates from different families isolated from the same fish gut environment, even from the same gut section (IV) (Figure 4). These results demonstrate that within the fish gut microbiota there is family level variation in nutrient requirements. These same protocols can be used to search for other essential growth factors present in nutritionally complex media additions sourced from the natural environment. By evaluating the growth rate of anaerobic bacteria on different media, these protocols can be used to discover novel defined alternatives to YCFA-GF for in vitro gut bacteria culture.

Time considerations:

The preparation of multi-well anaerobic plates (Basic Protocol 1) takes three days. The initial isolation of fish gut bacteria from an environmental sample takes approximately 4 days (Basic Protocol 2). Though this step, along with all other steps involving bacteria culture, may vary depending on the growth profile of the isolate used. Time taken to reach saturation phase should be identified for an isolate in preliminary work before it is used in this protocol. The saturation phase should be reached by the end of incubation for all steps in Basic Protocol 2. Identification of isolates takes 1 day of laboratory work, further time depends on the sequencing facility used. Preculture preparation (Basic Protocol 4) takes 4 days. Performing growth rate experiments (Basic Protocol 4) takes at least 2 days. This may take more time if isolates take more than 48 hours to reach saturation phase.

Conflict of Interest Statement:

The authors declare no conflicts of interest.

Data Availability Statement:

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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INTERNET RESOURCES:

geneious.com

Geneious Prime 2019.2.1 bioinformatics software (Kearse, et al., 2012).

Software to place a 16S sequence within a phylogenetic tree of sequences in the NBCI database. These trees of phylogeny can be used to compare relatedness of isolates and understand the genetic relationship between all isolates under investigation.

Geneious Prime 2019.2.1. was used in this study.

nih.gov

NCBI BLAST: Basic Local Alignment Search Tool) (Altschul, et al., 1990)

Algorithm and program to compare 16S sequence data with sequences in the NBCI database.

FIGURE LEGENDS:

Figure 1: *Basic protocols for high throughput assessment of growth effects of different media ingredients on fish gut bacteria. Culture plates of 48 wells are prepared, containing two trial growth media recipes with controls (Basic Protocol 1). Bacteria is recovered from the fish gut and streaked to single isolates (Basic Protocol 2). The 16S rRNA marker gene is amplified from the genomic DNA of each isolate, then sequenced and genetically identified (Basic Protocol 3). The chosen isolate is streaked across a whole plate to create a preculture, the culture is adjusted to McFarland Standard 4 in water-gel and inoculated onto the culture plates. Timepoints taken by absorbance in a microplate reader, timepoint samples are culture taken from a well by washing it in water-gel (Basic Protocol 4).*

Figure 2: *Anaerobic jars custom built by Mason Tool and Engineering Limited (Auckland, New Zealand). Has the option of agar plate vertical mode of operation as well as microwell plate horizontal mode of operation. Input and output valves allow gassing cylinder input through the Hungate gassing apparatus. Pressure gauge enables maintenance of a specific pressure during incubation.*

Figure 3*: Copper tube attachment to Hungate apparatus allowing anaerobic delivery of* N_2, CO_2, *and* H_2 *(47.5%, 47.5%, 5% v/v/v) gas from cylinders, through the Hungate apparatus, to the anaerobic jar.*

Figure 4: *Anatomy and gut section I, II, III, IV, and V allocation of K. sydneyanus.*

Figure 5: *Basic Protocol 1, generating solid anaerobic media for the culture of obligately anaerobic gut bacteria. Includes steps from Basic Protocol 1 Part A, generating petri dishes containing a version of YCFA (Yeast extract-casein hydrolysate-volatile fatty acids) media. Als including steps from Basic Protocol 1 Part B, generating 48-well plates containing anaerobic media, for use in growth rate measurement experiments (Basic Protocol 4).*

Figure 6: *Example of media layout in a 48-well plate for comparison of bacterial growth on two trial media. In our experiment Medium 1 is YCFA-GF (Yeast extract-casein hydrolysate-volatile fatty acids with gut fluid), and Medium 2 is YCFA-M (Yeast extract-casein hydrolysate-volatile fatty acids with mannitol).*

Figure 7: *Example plate layout for growth rate experiment in the 48-well plate prepared in Basic Protocol 1. Six timepoints are possible (rows A - F), on two trial media (YCFA-GF and YCFA-M). One timepoint at zero hours, four timepoints during the growth period, and a timepoint at stationary phase. Reconstituted pellet at McFarland Standard 4 pipetted onto plate in columns 1 - 3, and 5 – 7 for three replicates of each condition at each timepoint. No isolate controls are placed in columns 4 and 8.*

Figure 8: *Representative data of bacterial growth by absorbance at* OD_{600} *on two different media.* ***Top:*** *Growth comparison of strain BP5G in YCFA + GF and YCFA + M media.* ***Bottom:*** *Growth comparison of strain BP52G in YCFA + GF and YCFA + M media. Four timepoints taken over the growth period for three replicates in each treatment (timepoints: 3, 6, 9 and 22 hours). Each replicate fitted to a linear regression line. YCFA+GF, Yeast extract-casein hydrolysate-volatile fatty acids media with gut fluid. YCFA+M, Yeast extract-casein hydrolysate-volatile fatty acids media with mannitol. Analysis was conducted in R (R Core Team, 2014) producing figures using the ggplot2 package (v3.3.3; Wickham, 2016).*

TABLES:

Table 1: Troubleshooting Guide for Measurement of Bacterial Growth Rates on Solid Media

Problem	Possible causes	Potential solutions
Not enough bacteria CFU collected from one petri dish	Fastidious or slow growing isolate	Create two petri dishes for this isolate, transfer bacteria suspended in water-gel from the first petri dish to the second to collect bacteria from both
Bacteria adhere tightly to solid media and cannot be removed by rubbing with disposable inoculating loop	Isolate strongly adheres to nutrient source	Incubate at 20 ^{0}C for a shortened time period, to harvest before strong adherence occurs
Unsuccessful growth of inoculum on 48-well plate	Bacteria was dead before inoculation	Needs a shorter incubation time on petri dishes
		Less spin time in centrifuge required to reduce sheer stress on cells. A range of 60 to 120 seconds recommended at 1500 rcf
		Pipette gently to reduce sheer stress
		Warm plates in hood for at least 20 minutes before streaking to reduce temperature shock effects on bacteria
	Bacteria did not pellet	More spin time in centrifuge required to pellet cells. A range of 60 to 120 seconds recommended at 1500 rcf
	Bacteria growth was interrupted	Maintain strict anaerobic conditions at all points during growth
		Store anaerobic jars containing plates at 3 – 5 °C to prevent media drying
		Ensure palladium and charcoal is freshly baked before use inside the

		anaerobic jar. Activate by heating at 150^{0}C for 1 hour.
		Increase gaps between timepoints for longer uninterrupted incubation time
		Increase the length of the growth period, as some bacteria have long lag phases

Table 2: *Average approximate growth rate in CFU/hour for fish gut bacteria isolate BP52G in the Erysipelotrichaceae family and isolate BP5G in the Lachnospiraceae family, cultured on two different media: YCFA-GF, and YCFA-M. Average calculated from three replicates of each condition, with standard deviation. (Expanded table with all replicates in appendix).*

Isolate	**Media**	**Average approximate growth rate from three replicates (± standard deviation) (x 10^5 CFU/hour)**
BP5G (Lachnospiraceae)	YCFA-GF	1.10 (± 0.44)
	YCFA-M	1.30 (± 0.45)
BP52G (Erysipelotrichaceae)	YCFA-GF	0.40 (± 0.10)
	YCFA-M	0.05 (± 0.01)