Isolation and characterization of prebiotic oligosaccharides from algal extracts and their effect on gut microflora

Submitted in partial fulfillment for the Degree of Master of Applied Sciences in Biotechnology in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

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PROMOTER/ SUPERVISOR: **Prof. B Odhav**

REFERENCE DECLARATION

I, Miss N. Hadebe (Student no: 20515187) and Prof. Bharti Odhav do hereby declare that in respect of the following dissertation titled:

Isolation and characterization of prebiotic oligosaccharides from algal extracts and their effect on gut microflora

1. As far as we ascertain:

a) no other similar dissertation exists;

2. All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.

Signature of student	**Date**
Signature of promoter/ supervisor	**Date**

AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Bharti Odhav**.

_____ _____

Student's signature **Date**

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ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation and heartfelt gratitude to:

God Almighty, through Him everything was possible. I give thanks to him the beneficent and gracious God who in spite of everything, He consistently gave me hope, direction, wisdom, and the strength to continually press on. In him I move, I live and I have my being. If it weren't for the Lord I wouldn't have made it this far;

Professor Bharti Odhav, my supervisor**,** within the Department of Biotechnology and Food Technology, Durban University of Technology, for her remarkable insight, constructive criticism, expert guidance and support, time and energy, patience, invaluable assistance during the course of my degree, and for her motivation;

Priscilla Phillips, Sohana Rangals and John Mellem, Department of Biotechnology and Food Technology, Durban University of Technology staff members for their assistance, tolerance and support;

Kabange Kasumbwe, Department of Biotechnology and Food Technology, Durban University of Technology, a post-graduate student, for being God sent, being my eyes where I cannot see and reaching where I cannot reach, his contribution was major in this project;

Dr Zakhele Khumalo, a dear friend to me, for always believing in me throughout and constantly motivating me;

Simangele and Sibusiso Hadebe, my parents for their love, encouragement, support, tolerance and guidance throughout this period in my life;

Sizakele Mdluli, my sister for her love, assistance, guidance, friendship and tolerance

Sabelo Hadebe, my little brother for his love and assistance.

ABBREVIATIONS

AF6:	Artificial Fresh Water
ASW:	Artificial Salt Water
CFU:	Colony Forming Units
CNS:	Central nervous system
CSIR:	Council for Scientific and Industrial Research
Dha:	Deoxy-lyxo-2-heptulosaric acid
DP:	Degree of polymerization
DUT:	Durban University of Technology
ESIMS:	Electrospray ionization mass spectrometry
EPS:	Exocellular polysaccharides
FDR:	False discovery rate
FOS:	Fructooligosaccharides
GalA:	Galactopyranuronic acid
GALT:	Gut-associated lymphoid tissue
GIT:	Gastrointestinal tract
GLC:	Gas liquid chromatography
GLP:	Glucagon-like peptide
GOS:	Glucooligosaccharides
GRAS:	Generally Regarded As Safe
HPLC:	High performance liquid chromatogram
HPAEC:	High performance anion exchange chromatography
IBD:	Inflammatory bowel disease
IBS:	Irritable bowel syndrome
IMO:	Isomaltooligosaccharides

Kdo:	Keto-sugar acids 3-deoxy-manno-2-octulosonic
MALDI-TOF MS:	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MeKdo:	Methyl-manno-2-octulosonic acid
Ml:	Millilitre
Min:	Minute
Mg:	Milligram
NDF:	Non-digestible fibre
NDOs:	Non-digestible oligosaccharides
OD:	Optical density
OF:	Oligofructose
RI:	Refractive index
RS:	Resistant starch
Rpm:	Revolution per min
RPS:	Released polysaccharides
SCCs:	Short-chain carbohydrates
SCFA:	Short chain fatty acids
SERS:	Surfaced-enhanced Raman scattering
Spp:	Species
Subsp:	Subspecies
TLC:	Thin layer of chromatography
TFA:	Trifluoroacetic acid
TLR:	Toll-like receptors
TOS:	Transgalactooligosaccharides
Wt:	Weight
XOS:	Xylooligosaccharides

PUBLICATION UNDER REVIEW

HADEBE, N., ODHAV, B. 2016. Isolation and characterization of prebiotic oligosaccharides from algal extracts and their effect on gut microflora. *Journal of Functional Foods*.

INTERNATIONAL CONFERENCE PRESENTATIONS

HADEBE, N., ODHAV, B. 2009. Isolation and characterization of oligosaccharides from *Spirulina platensis*. Poster presented at the Human Evolution Symposium, University of the Western Cape, Bellville, South Africa, 12 October 2009.

HADEBE, N., ODHAV, B. 2016. Isolation and characterization of prebiotic oligosaccharides from algal extracts and their effect on gut microflora. Poster presented at the South African Society of Microbiology, Coastlands Umhlanga, Durban, South Africa, 18 January 2016.

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ABSTRACT

Prebiotics are defined as non-digestible oligosaccharides (NDOs) or polysaccharides (NDPs), which promote the growth of beneficial lactic acid bacteria in the colon. Algae are rich in polysaccharides and can be exploited as prebiotics for functional food ingredients to improve human and animal health. Currently, inulin is the most widely used ingredient in the prebiotics market, which is produced from live plants and requires expensive production processing. There is a vast repository of marine life with algae as a major source of nutrients. Therefore, this study provides an alternative source for prebiotic production and examines marine and freshwater algae that promote the growth of two strains of *Lactobacillus delbrueckii* subs. *(Lactobacillus lactis* and *Lactobacillus bulgaricus*) and one strain of *Bifidobacterium* spp. (*Bifidobacterium longum*). Monosaccharides of the oligosaccharide fraction of marine and freshwater algal extracts were investigated with the use of thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) after acidic hydrolysis of cell matrix polysaccharides.

A total of fifty-five marine and freshwater aqueous algal extracts were assessed for their effect on the growth of *L. lactis, B. longum* and *L. bulgaricus* over a 96 hour period. Relative to the negative control, 34.5% algal extracts showed improved growth on one or more probiotic bacteria. The optimum time for maximum bacterial growth was noted at 48 h for all the tested aqueous algal extracts. Five marine and freshwater algal cultures (*Spirulina platensis*, *Chlorococcum* spp.*, Dunaliella salina, Scenedesmus magnus, Chlorella* spp. and algal extract no. 48*)* from various aquatic environments in Kwa-Zulu Natal showed the best growth dynamics and demonstrated the greatest potential as sources of biomass for prebiotic production. These algal extracts were able to significantly increase the growth of at least one of the three probiotic bacteria ($p < 0.05$). Aqueous algal extract from *S. platensis* was regarded as the best algal source for prebiotics as it demonstrated a greater stimulatory effect on the growth of all three probiotic bacteria (*L.*

lactis, *B. longum* and *L. bulgaricus*) compared to tested aqueous algal extracts and the inulin used as a positive control. The results obtained by HPLC for characterization confirmed TLC data, as xylose and galactose were detected by both chromatograms. These data indicated that xylose and galactose were present in aqueous algal extracts from *S. magnus* and S*. platensis* and galactose in aqueous algal extract no. 48. Xylose was most abundant in aqueous algal extracts from *S. platensis* (3mg/ml) and *S. magnus* (2.3mg/ml). In conclusion aqueous algal extracts from *S. platensis*, *Chlorococcum, D. salina, S. magnus, Chlorella* and algal extract no. 48 are potential sources for prebiotic production. *Spirulina platensis* extract was regarded as the best algal source. Xyose and galactose characterized by HPLC in algal extracts make up oligosaccharides that function as prebiotic compounds for stimulation of probiotic bacteria. There is a great scope for successful production of prebiotics from algal sources in South Africa.

Key words: Algae, Polysaccharides, Non-digestible oligosaccharides, Prebiotics, Probiotics

1. INTRODUCTION

Microalgae have been identified as a potentially viable feedstock for the biological production of transportation biofuels (Brennan et al., 2010; Greenwell et al., 2010; Wijffels and Barbosa, 2010). Although lipids are considered the most valuable components of algal biomass in the context of a biofuels process (Griffiths and Harrison, 2009; Laurens et al., 2012; McNichol et al., 2012), other biomass components such as proteins and carbohydrates also make up a large fraction of the biomass. Characterization of microalgal carbohydrates is currently one of the major barriers to the detailed compositional analysis of algae (Laurens et al., 2012). Algal carbohydrates exist as oligosaccharides or polysaccharides in algal biomass and require a hydrolysis (acid or alkaline) procedure to break up the polymers into their monomeric constituents. The term 'carbohydrates' refers to both monomers and polymers of sugars and sugar derivatives such as uronic acids and amino sugars. Polymers can have widely varying molecular weights depending on the degree of polymerization (Sluiter et al., 2010).

The marine environment offers a tremendous biodiversity and original polysaccharides that have been discovered presenting a great chemical diversity that is largely species specific. Marine polysaccharides present an enormous variety of structures which are still exploited and should therefore be considered as an extraordinary source of prebiotics (Senni et al., 2011). Polysaccharides of algal origin such as alginates, laminarins, fucans and their derivatives have prebiotic potential that have been used for decades to enhance animal and human health (O'Sullivan et al., 2010). Prebiotic oligosaccharides are defined as non-digestible oligosaccharides (NDOs) and polysaccharides (NDPs), which promote the growth of beneficial lactic acid bacteria in the colon (Roberfroid, 2007). There exists an array of prebiotics from various origins with different chemical properties that have been reviewed and classified based on a set of common criteria i.e., (i) resistance to

gastric acidity; (ii) hydrolysis by mammalian enzymes; and (iii) selective stimulation of growth, and/or activity of intestinal bacteria (Sridevi et al., 2014).

The ultimate aim of supplementation of the human diet with prebiotics is beneficial management of the gut microbiota (Kolida et al., 2002). The probiotic- and prebiotic-containing product market is a fast growing industry worldwide and the list of available products increases on a daily basis (Stanton, et al, 2001). In South Africa, the probiotic and prebiotic market is dominated by dairy products, although fortified cereals, especially baby cereals, and supplements also seem to be growing markets (Brink et al., 2005). The range of products on the South African market includes probiotic and prebiotic supplements (capsules) and fortified food items and as well as fermented foods containing probiotics. Inulin and fructooligosaccharides (FOS) are manufactured by ORAFTI in Belgium and distributed in South Africa by SAVANNAH Fine Chemicals. The range includes Raftiline®GR (inulin), Raftilose®L95 (Oligofructose) and Raftilose®Synergy1 (combination of inulin and oligofructose) (Brink et al., 2005).

The production of prebiotics at industrial scale faces several challenges, including the use of novel techniques and economical sources, and the low-cost production. Most oligosaccharides with prebiotic status are normally obtained by enzymatic treatment of cheap raw materials such as sucrose, lactose and plant derivatives (Figueroa-González et al., 2011). The amount and nature of oligosaccharides formed depend upon several factors such as the enzyme source, the concentration and nature of the substrate and the reaction conditions. Nevertheless, the current processes used to obtain oligosaccharides have very low yields, thus increasing the production cost (Panesar et al., 2006). Therefore, this study provides an alternative source for prebiotics from marine and freshwater algae that promote the growth of *Lactobacillus* and *Bifidobacterium* spp. which are the widely studied probiotic strains of lactic acid bacteria. Bifidobacteria and

Lactobacilli have been attributed to beneficial aspects such as modification of the intestinal microbiota and stimulation of the immune response, thereby promoting non-specific host resistance to microbial pathogens (Rubel et al., 2014). Methods for isolation of algal polysaccharides are rather labour intensive and time consuming (Girod et al., 2002; Maksimova et al., 2004 and Pugh et al., 2001). This work also includes conditions for extracting polysaccharides from algal biomass to improve polysaccharide yield and shorten procedure time, providing a feasible method for polysaccharide extraction.

1.1. Aim and objectives

1.1.1. Aim

The aim of this study was to investigate prebiotic oligosaccharides from marine and freshwater microalgae that may offer potential for use as prebiotics.

1.1.2. Objectives

1) To obtain aqueous extracts from marine and freshwater algal species
2) To screen marine and freshwater algal aqueous extracts for prebiotic activity at different time intervals
3) To evaluate the prebiotic effect of selected aqueous algal extracts based on the ability to improve growth of *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Bifidobacterium longum*
4) To characterize the selected aqueous algal extracts for oligosaccharides using TLC and HPLC

2. LITERATURE REVIEW

The concept of probiotics and prebiotics continues to expand. Current global research efforts have significantly contributed to the understanding of the role of gastrointestinal tract (GIT) commensal organisms on its extraordinary symbiotic relationship with humans. Continued studies into the microbiota will no doubt help lead to an improved insight into the impact of probiotics and prebiotics on human health. Probiotics are designed to provide added functions that can compensate for, substitute for, or add to the gut microbiota, and therefore impact the host directly or indirectly through “cross-talk” with the gut microbiota and/or the host. While prebiotics are designed to improve the intrinsic microbiota by selectively stimulating those groups that are thought important for eubiosis (Al-Sheraji et al., 2013, Hemarajata and Versalovic, 2012). Eubiosis is a state where the host and microflora live together in symbiosis, meaning, with mutual benefit. Accordingly the host provides good living conditions and in exchange, the intestinal microflora, when in the state of eubiosis, supports the host with essential activities (Mohnl, 2015).

Research over past decades has demonstrated potential health benefits of dietary probiotics and prebiotics and contributed to our understanding of the mechanisms by which these benefits derived. The most commonly reported impact of probiotics and prebiotics is on intestinal function, including transit time, antibiotic-associated diarrhea and infectious diarrhea. Evidence continues to emerge that probiotics and prebiotics have an influence on the immune system that may thereby, enhance resistance to infections. This is particularly within the GIT or respiratory tract where they help to mitigate allergies, predominantly in infants and young children. (Isolauri et al., 2001, Gerritsen et al., 2011).

2.1. Prebiotic oligosaccharides

Prebiotics are short-chain carbohydrates (SCCs) that are non-digestible by digestive enzymes in humans referred to as resistant SCCs. They are also referred to as non-

digestible oligosaccharides with a degree of polymerization (DP) of two or more, which are soluble in 80% ethanol and are not susceptible to digestion by pancreatic and brush border enzymes (Al-Sheraji et al., 2013). They beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon called probiotics, usually Bifidobacteria and Lactobacilli. In the intestine, prebiotics are fermented by beneficial bacteria to produce short-chain fatty acids such acetic acid, propionic acid and butyric acid, which are used by the host organism as an energy source and that improve health (Roberfroid, 2007). Most non-digestible oligosaccharides (NDOs) contain three to ten sugar moieties (Van Laere, 2000), although the DP could go up to sixty for some NDOs, like chicory inulin or down to two like lactulose (Tanabe et al., 2014). Various health benefits conferred by the activity of prebiotics in the large intestine include reduction of cancer risk and increase calcium and magnesium absorption. They are found in several vegetables and fruits, and are being used in the food industry as functional food ingredients (de Sousa et al., 2011). Some of the applications of prebiotics in food and health industry are illustrated in Figure 1.

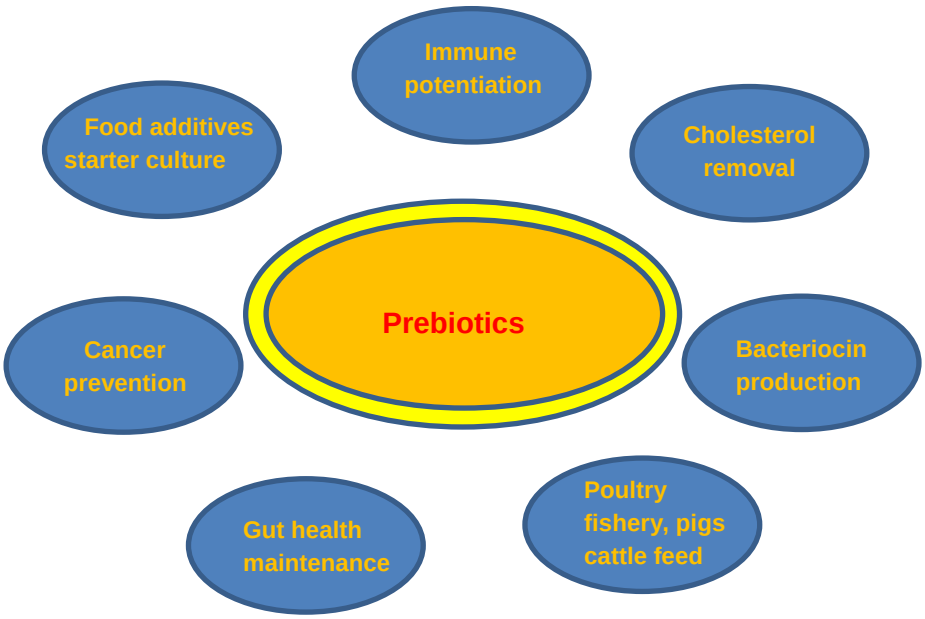


Figure 1: Prebiotic applications in food and health industry (Patel and Goyal, 2012)

Prebiotics can be manufactured using three different methods. They are obtained either by extraction from plants, for example, inulin (extracted from chicory), by enzymatic hydrolysis of plant polysaccharides, for example, XOS (produced by enzymatic hydrolysis of xylans from cereal grains), and by transgalactosylation reactions catalysed by an enzyme, using either a mono-saccharide or a di-saccharide as the substrate, for example, GOS (produced from lactose using *β*-galactosidase as the biocatalyst) (Charalampopoulos and Rastall, 2012).

Only non-digested carbohydrate molecules, a range of di-oligo- and polysaccharides, resistant starches and sugar polyols have been claimed to have prebiotic properties (Cummings et al., 2001). According to Sridevi et al. (2014), a non-digestible carbohydrate can be considered as a prebiotic if the following criteria are met; i) It must be capable of passing through the small intestine without being absorbed or digested in the upper part of the GIT, ii) It must be capable of being digested in the large intestine by beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* and able to enhance the growth of these beneficial bacteria; iii) The digestion and utilization of this compound by the gut microflora should have a positive effect on human health by improving the absorption of Ca, Mg, and Fe; (iv) It must able to prevent cancer and destroy any pathogens in the large intestines.

Prebiotic oligosaccharides used for the promotion of growth of beneficial bacteria are called bifidogenic oligosaccharides. These include fructooligosaccharides (FOS), inulin, glucooligosaccharides and other long chain oligosaccharides polymers of fructose and/or glucose, trisaccharide and raffinose (Mussatto and Mancilha, 2007). In the GIT, they pass through the small intestine to the lower gut and become accessible to probiotic bacteria without being utilized by other intestinal bacteria and they also increase the level of nutrient supplementation and enhance nutrient solubility. The bifidogenic oligosaccharides

are metabolized exclusively by the indigenous Bifidobacteria and Lactobacillus and not by detrimental microorganisms such as *Clostridia, Staphylococcus*, *Salmonella* and *Escherichia coli* (Thantsha, 2007). The use of bifidogenic oligosaccharides together with lactic acid bacteria allows beneficial, probiotic bacteria to grow and out-compete any undesirable, pathogenic microorganisms within the GIT (Sullivan and Nord, 2002).

2.2. Properties of prebiotic oligosaccharides

Carbohydrates are classified according to their molecular size or degree of polymerization (number of monosaccharide units combined), into monosaccharides, oligosaccharides and polysaccharides (Foschia et al., 2013). The prebiotic properties of carbohydrates are likely to be influenced by monosaccharide composition, glycosidic linkage which is a crucial factor in determining both selectivity of fermentation and digestibility in the small intestine and lastly, molecular weight (Gibson, 2004). Currently, all known and suspected prebiotics are carbohydrate compounds, primarily oligosaccharides, known to resist digestion in the small intestine that reach the colon where they are fermented by the gut microflora (Slavin, 2013). Studies have provided evidence that inulin and oligofructose (OF), lactulose, and resistant starch (RS) meet all aspects of the definition, including the stimulation of *Bifidobacterium*, a beneficial bacterial genus. Other isolated carbohydrates and carbohydrate containing foods, including galactooligosaccharides (GOS), transgalactooligosaccharides (TOS), polydextrose, wheat dextrin, acacia gum, psyllium, banana, whole grain wheat, and whole grain corn also exhibit prebiotic effects (Slavin, 2013).

2.3. Fermentability and non-digestibility of prebiotic oligosaccharides

The concept of non-digestible oligosaccharide originates from the observation that the anomeric carbon atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharide has a configuration that makes their glycosidic bonds non-digestible

during hydrolytic activity of human digestive enzymes (Boler and Fahey Jr, 2012). The indigestibility of NDOs either results from the configuration of the glycosidic bond between monomeric sugar units or the substrate selectivity of gastrointestinal digestive enzymes. Most NDOs have a *β*-configuration and cannot be degraded by human gastrointestinal digestive enzymes, which are specific for *α*-glycosidic bonds. The *β*-galactosidase enzyme, localized at the brush border membrane of the small intestine, has the potential to degrade *β*-galactooligosaccharides. However, degradation of NDOs with *α*-glycosidic bonds or *β*-galactooligosaccharides by the above mentioned gastrointestinal digestive enzymes is low because the enzymes usually have weak activities and/or show other substrate selectivity (Mussatto and Mancilha, 2007).

Fermentation involves a variety of reactions and metabolic processes involved in the anaerobic microbial breakdown of organic matter, yielding metabolizable energy for microbial growth and maintenance as well as use of other metabolic end products by the host (Topping and Clifton, 2001). Non-digestible oligosaccharides, which are water soluble, are highly likely to be fermented in the colon. Their utilization is mediated by hydrolytic enzymes of colon bacteria (Voragen, 1998). Any carbohydrate that reaches the cecum is a potential substrate for fermentation by the probiotic bacteria, producing short chain fatty acids (Al-Sheraji et al., 2013). Short chain fatty acids (SCFAs) are organic fatty acids with 1 to 6 carbon atoms and are the principal anions, which arise from bacterial fermentation of polysaccharides, oligosaccharides, proteins, peptide, and glycoprotein precursors in the colon (Ganapathy et al., 2013). There is data from both *in vitro* and *in vivo* experiments on fermentation of carbohydrates by bacteria of the large intestine. *In vitro* experimental studies indicate that several SCFAs enhance bacterial survival and activity (Al-Sheraji et al., 2013).

Cummings et al. (2001) found that the fermentation of oligosaccharides by different strains of *Bifidobacterium, Clostridium* and *Lactobacillus* is based on the structure of the oligosaccharide that could either be linear or branched. Linear oligosaccharides breakdown to a larger extent than those with branched structures and *Bifidobacteria* use low degrees of polymerised carbohydrates first whereas, Bacteroides utilise carbohydrates with a high degree of polymerization.

2.4. Direct blocking of pathogenic intestinal microbes

The ability of NDOs to inhibit pathogen binding to the surface of epithelial cells has attracted considerable interest for more than 20 years (Quintero-Villegas et al., 2013). Pathogens such as *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Escherichia coli*, *Vibrio cholera* and *Clostridium perfringens* are bacteria that are capable of causing diseases, colonise and grow within the GIT, invade the host tissues and excrete toxins. These toxins disrupt the function of the intestinal mucosa, causing nausea, vomiting and diarrhoea (Cummings and Macfarlane, 2002; Jain et al., 2014; Wittke et al., 2014). Bacterial adherence to host cell surfaces is the first, and in many cases, the most critical step in bacterial pathogenesis (Boyle and Finlay, 2003). Many short chain oligosaccharides can exert a direct anti-microbial effect, as they act as blocking factors by dislodging the adherent pathogen by adhering to the binding sites of the bacteria on the enterocyte surface (Gourbeyre, 2011).

Adherence is the mechanism by which bacteria can avoid the host's natural displacement mechanisms (peristalsis, acid excretion, flux) (Quintero-Villegas et al., 2013). In a study curried out by Hopkins and Macfarlane (2003), GOS and FOS were able to stimulate bifidobacterial growth, with concomitant reduction in *C. difficile* population.

2.5. Types and sources of prebiotic oligosaccharides

2.5.1. Prebiotic oligosaccharides from plants

A literature search from 2013 to date reveals the presence of prebiotic oligosaccharides in some food crops with vegetable, root and tuber crops being the predominant sources. Natural sources of oligosaccharides exist, e.g. galactooligosaccharides in breast milk, fructans in onion (*Alliumcepa*), leeks (*Allium porrum*), garlic (*Allium sativum*) and stachyose (soybean). Table 1 provides a list of several types of prebiotics including oligomers with their sources that are best recognised (e.g. FOS, inulin, and GOS from plants) and the emerging prebiotics (IMO, XOS, and lactitol) (Al-Sheraji et al., 2013). In addition, the raffinose family of oligosaccharides and resistant starch (the type that is not absorbed in the GIT) have also been recognized as prebiotic carbohydrates because these are not absorbed in the intestine but promote the growth of beneficial bacteria in the gut (Van den Ende, 2013). A review of these prebiotics listed is provided subsequently in this chapter.

Table 1: Several types of prebiotics and their sources

Prebiotic	Sources	References
Fructooligosaccharides	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato and rye	(Sridevi et al., 2014)
Xylooligosaccharides	Bamboo shoots, fruits, vegetables, milk, honey and wheat bran	(Panesar et al., 2013)
Galactooligosaccharides	Human's milk and cow's milk	(Torres et al., 2010)
Cyclodextrins	Water-soluble glucans	(Al-Sheraji et al., 2013)
Raffinose oligosaccharides	Seeds of legumes, lentils, peas, beans, chickpeas, mallow composite, and mustard	(ElSayed et al., 2014)
Lactulose	Lactose (Milk)	(Awad et al., 2014)
Palatinose	Sucrose	(Al-Sheraji et al., 2013)
Isomaltooligosaccharides	Honey, sugarcane juice	(Barea-Alvarez et al., 2014)

Table 1 continues

Lactosucrose	Lactose	(Zhou et al., 2014)
Maltooligosaccharides	Starch	(Beeren and Hindsgaul, 2014)
Soybean oligosaccharide	Soybean	(Fei et al., 2014)
Enzyme-resistant	Potato starch	(Lim et al., 2014)
Arabinoxylooligosaccharides	Wheat bran	(François et al., 2014)

2.5.1.1. Inulin

Inulin (Figure 2) is a linear fructan, which is a plant reserve polysaccharide constituted by fructose molecules linked by β(2→1) bonds, with a terminal glucose unit linked by an α (1→2) bond with a DP typically ranges from 2 to 60 units (Rubel et al., 2014). Since inulin is a fructan type of prebiotics, it is resistant to digestion and absorption in the human small intestine, and reaches the colon, where it is selectively fermented by beneficial bacteria, such as *Lactobacillus* and *Bifidobacteria* (Peshev and Van den Ende, 2014). The inability of the human digestive system to hydrolyze fructans is due to our lack of effective hydrolytic enzymes that can break β linkages. However, the microflora in the colon can degrade these bindings. When fructans reach the colon, they are more or less intact, and become a substrate for bacterial enzymes belonging to the glycoside hydrolase family, GH32 (Van den Ende et al., 2011).

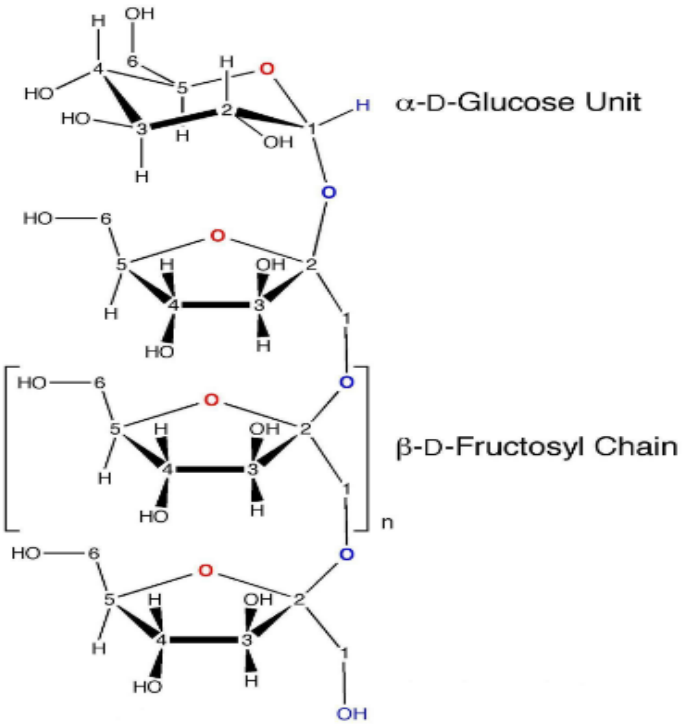


Figure 2: Inulin Polymer (Petrovsky, 2010)

2.5.1.2. Fructooligosaccharides

Fructoologosaccharides (FOS) are fructose oligosaccharides joined by β-(2→1) or β-(2→6) linkages and are terminated with a glucose molecule linked to fructose by an α-(1→2) bond (Figure 3) (Fernández et al., 2013). They have been classified as prebiotic oligosaccharides because, they modulate the composition and metabolic activity of the intestinal microbiota, promoting the growth of bifidogenic bacteria, reducing the risk of colon cancer (Charalampopoulos and Rastall, 2012). This prebiotic has demonstrated to have beneficial properties such as reducing blood pressure, cholesterol and blood glucose levels and better absorption of calcium and magnesium. Fructooligosaccharides promote intestinal health by stimulating the growth and activity of beneficial bacteria as well as enhance immune response. The Bifidobacteria in the colon secrete ß- fructosidase enzyme responsible for FOS hydrolysis (Sridevi et al., 2014).

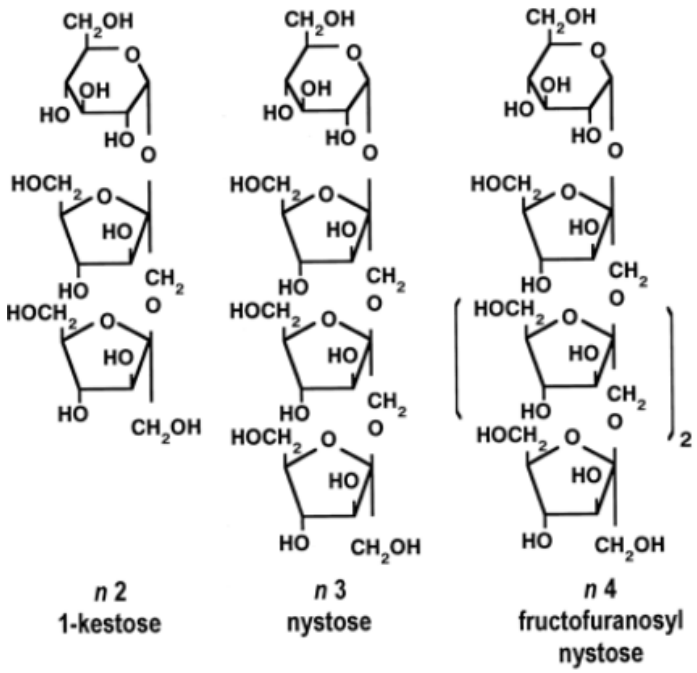


Figure 3: Chemical structures of some fructooligosaccharides (FOS) (Ohta et al., 1998)

2.5.1.3. Galactooligosaccharides

Galactooligosaccharides (GOS) are produced by β- D- galactodases derived from *Bacillus circulans* by transferring galactosyl residues to lactose molecules. They have a DP between 2 and 8 and are composed of galactose oligomers in β1-3/4/6 linkages with a terminal glucose residue (Figure 4) (Sen et al., 2014, Torres et al., 2010). These substrates have been extensively studied for their prebiotic status, promoting the growth of beneficial microorganisms such as *Bifidobacteria* and *Lactobacilli*, therefore providing putative health benefits (Garrido et al., 2013).

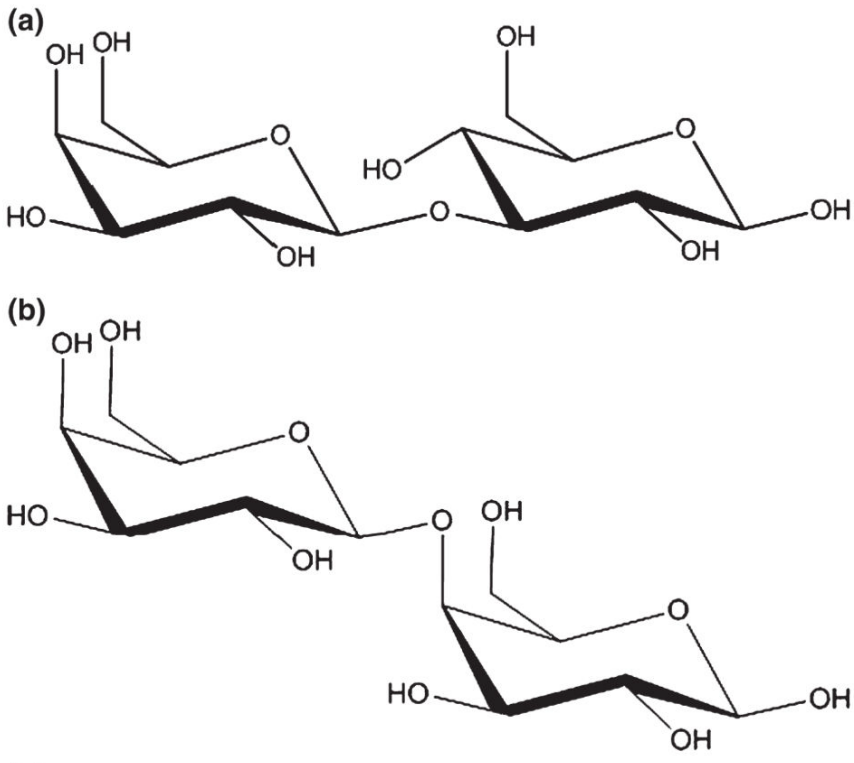


Figure 4: Structures of some GOS: (A) β-D-Galp-(1→ 3)-D-Glc ; (B) β-D-Galp-(1→4)-D-Gal (Intanon et al., 204)

2.5.1.4. Xylooligosaccharides

Xylooligosaccharides (XOS) are mixtures of oligosaccharides formed by xylose residues linked through β- (1→4) linkages (Figure 5). The number of xylose residuals known as xylobiose or xylotriose involved in the formation of XOS vary from 1 to 10 (Kumar et al., 2012). Xylooligosaccharides are extensively used by several species of Bifidobacteria, hence a study done by Childs et al. (2014) discovered that when prebiotics were compared, *Bifidobacterium* spp. favored XOS over hexoses (such as glucose) during *in vitro* growth experiments. Rycroft et al. (2001) also evaluated the fermentative properties of some prebiotics *in vitro* and found that XOS and lactulose produced the highest concentration of Bifidobacteria.

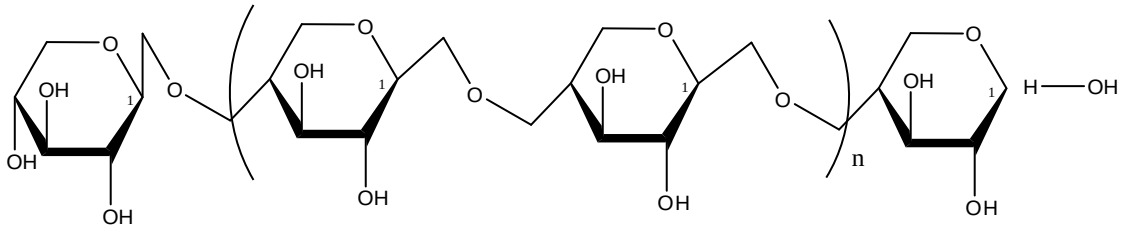


Figure 5: Chemical structure of xylooligosaccharide (Doerr et al., 2002)

2.5.2. Prebiotic oligosaccharides from algae

Macroalgae are regarded as a rich source of sulfated polysaccharides, and the particular types of polysaccharides varied depending on the taxonomic group. The key function of these relatively high molecular weight polysaccharides is that they are rich in hydroxyl (OH) groups, making them hydrophilic. They are known to establish intra-chain H-bond networks, making them stiff, rigid and suitable as thickeners. The regularity of their structures also promotes their interaction with external ions and inter-chain H-bonding (e.g. gelation) (O'Sullivan et al., 2010). Different carbohydrates including ulvans, carrageenan, or alginates are extracted from macroalgae and are widely used in the food and pharmaceutical industries as functional ingredients. Key polysaccharides in macroalgae such as Chlorophyta, Phaeophyta and Rhodophyta and their prebiotic effect on the intestinal microflora are summarized in Table 2 (Warrand, 2006, O'Sullivan et al., 2010).

Table 2: Prebiotics from marine macroalgae

Prebiotic	Sources	Prebiotic effect
Alginate-(1-4) linked a-L-guluronic acid and b-D-mannuronic acid and pyranose residues	Brown seaweed species (Phaeophyta) but also bacterial sources (*Azotobacter*)	Alginate can alter the colonic microflora and the quantities of short chain fatty acids produced. Faecal Bifidobacteria levels increased while the number of some potentially pathogenic bacteria decreased during alginate consumption.
Carrageenan- 1,3-linked b-D-galactose and 1,4-linked a-D-galactose	Red seaweed species *(*Rhodophyta*)*	Degraded carrageenans may cause ulcerations in the GIT and gastrointestinal cancer
Ulvan- uronic acids, rhamnose (sulphated or not), xylose and the disaccharide composed of b -D-glucuronic acid-(1-4)-a-L-rhamnose	Green seaweed species (*Ulva)* (Chlorophyta)	Fermentated by Bifidobacteria and serve as growth promotors that are involved in the intestinal epithelial growth and wound repair.

2.6. Production of non-digestible oligosaccharides

Over twenty different types of NDOs are on the world market. Industrial production methods have been employed to produce NDOs from natural sources by hydrolyzing polysaccharides, and by enzymatic and chemical synthesis from disaccharides (Saad et al., 2013). Production of NDOs using various food sources is summarized in Figure 6, meanwhile a detailed description of processes involved in producing these NDOs is provided subsequently in this proceeding section in this chapter.

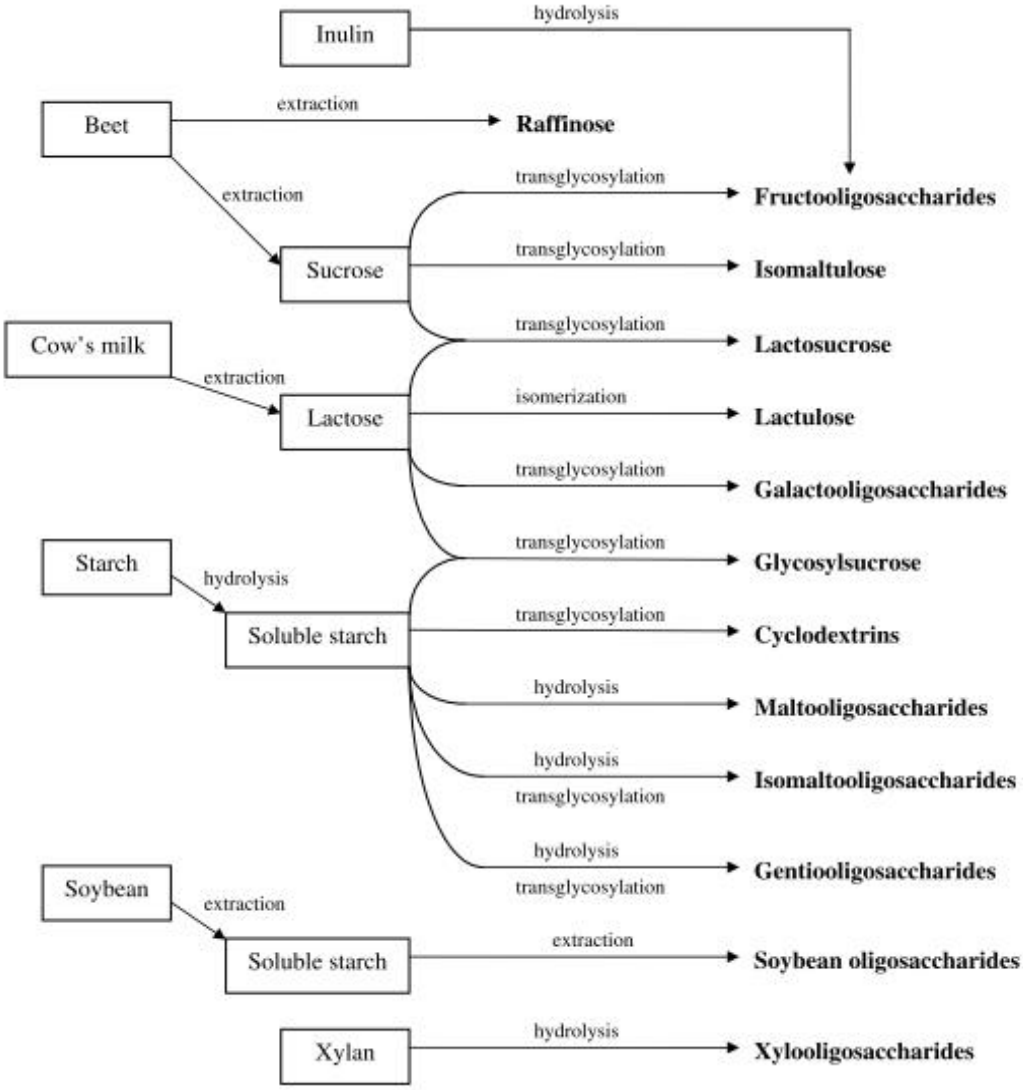


Figure 6: Production of non-digestible oligosaccharides (Mussatto and Mancilha, 2007)

2.6.1. Extraction from natural sources by hydrolysis processing

The naturally occurring NDOs in plants such as chicory roots, artichoke jerusalem and asparagus root can be obtained by direct extraction (Whelan, 2014). Raffinose oligosaccharides can be directly extracted from plant materials using water, aqueous methanol or ethanol solutions (Johansen et al., 1996). Soybean oligosaccharide are also extracted from soybean whey (Fei et al., 2014).

2.6.2. Enzyme processing

Most polysaccharides such as inulin, xylan and starches, are treated with specific enzymes to produce oligosaccharides. In enzymatic glycosylation strategies, a high number of stereoselective glycosyltransferase and glycosylsynthetase enzymes are commonly used for enzymatic synthesis of NDOs to produce lactose and sucrose. The glycosyltransferases families are classified according to the sugar transferred from donor to acceptor and by the acceptor specificity. This process has allowed for the synthesis of bioactive oligosaccharides such as fructooligosaccharides from sucrose using fructosyltransferase or the formation of trans-galactosylated oligosaccharides or galactooligosaccharides from lactose (Figure 6) (Saad et al., 2013).

2.6.3. Chemical production

Several polysaccharide degradation mechanisms have been developed and they include acid hydrolysis, thermomechanical degradation and oxidative degradation. Ultrasonication is most effective at depolymerizing polysaccharides (Hosseini et al., 2013). More environmentally friendly technologies, such as autohydrolysis (also referred to as hydrothermolysis), have gained considerable attention. In addition, the use of water as the only solvent has several advantages and these include limited problems derived from equipment corrosion owing to the mild pH of reaction media, and reduced operational costs (Morimoto et al., 2014).

2.7. Identification and characterization of non-digestible oligosaccharides

2.7.1. Chromatographic, spectrometric and spectroscopic techniques

Previously, the most common techniques used to analyse oligosaccharides was gas liquid chromatography (GLC) and high performance liquid chromatography (HPLC) (Knudsen

and Li, 1991). The first HPLC methods were based on the separation of NDOs up to a DP of 15 by low-pressure ion exchange or permeation chromatography Alternative methods were developed because these methods were time consuming (Swennen et al., 2006). One method in particular is the use of high performance anion exchange chromatography (HPAEC), that is more efficient than HPLC in terms of separation and detection (Hell et al., 2014). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization mass spectrometry (ESIMS) are powerful techniques for NDO analysis (Selvakumar et al., 1996). NMR-spectroscopy is also powerful for identification of NDOs (Soininen et al., 2014). Surface-enhanced Raman scattering (SERS) spectroscopy has also recently been used for NDO analysis. Surfaced-enhanced Raman scattering is a vibrational spectroscopic technique in which a monochromatic laser light is non-elastically scattered from a sample and shifted in frequency by the energy of its characteristic molecular vibrations Due to the high sensitivity to structural differences between equal-mass isomers, SERS can be effective in studying oligosaccharide structures. Furthermore, it has lower detection limits than NMR-spectroscopy, is more sensitive than normal Raman spectroscopy, and better suited for the analysis of aqueous solutions (Mary et al., 2014).

2.8. Prebiotic oligosaccharides and the gut system

The mucosal surface of the human GIT is about 200–300 m^2 and is colonized by $10^{13–14}$ bacteria of 400 different species and subspecies (Hao et al., 2004). The colon is densely populated with beneficial resident bacteria (e.g., *Bifidobacterium* and *Lactobacillus* spp.) and or harmful resident bacteria (e.g., *Clostridium* spp., *Shigella* spp. and *Veillonella* spp) (Figure 7) (Liu et al., 2014). These organisms that constitute the normal microflora.perform several functions, such as metabolic digestion, assist in food digestion, and protection from harmful bacteria (Serban, 2014).

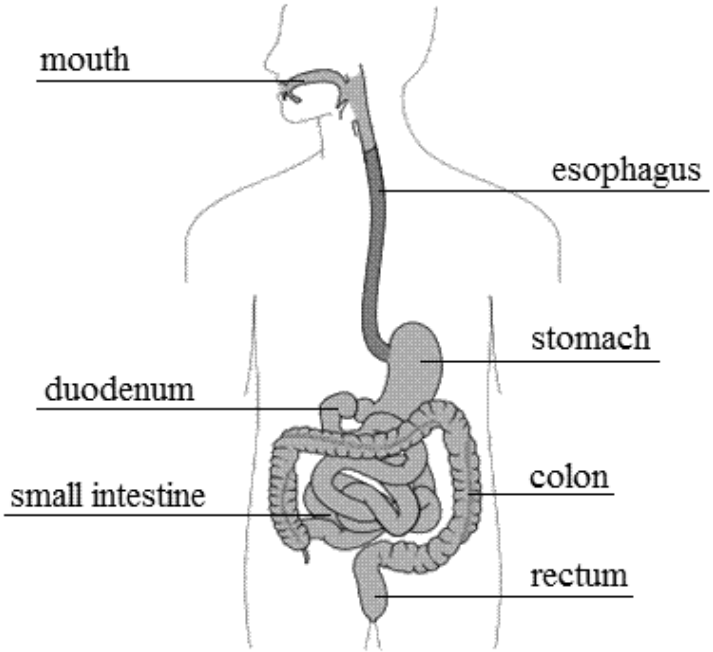


Figure 7: Human gastrointestinal tract (Lewandowska, 2010)

Due to the aggressive intestinal fluids (e.g., bile, pancreatic juices) and the short trait time, the duodenum also represents a hostile environment that contains relatively low numbers of transit microbes and each bacteria has its spectrum of metabolic activities (Quigley, 2010). Types and numbers of microorganisms in the small and large intestine vary from person to person. In a healthy individual, most of these species are advantageous or benign to the host, but some are potentially pathogenic provided their numbers are allowed to increase to high levels (Crittenden and Playne, 1996). Disturbances to the ecological balance in the intestinal microflora caused by, for example, changes in diet, stress or antibiotic treatment can lead to the overgrowth of deleterious bacteria, which may lead to gastrointestinal disorders. These disorders may be as minor as intestinal discomfort or increased flatulence, or relatively serious health problems. These disorders can be severe diarrhoea, irritable bowel syndrome and colitis. Undesirable bacteria in the colon have been implicated in the development of colon cancer (Thammarutwasik et al., 2009).

In a diet, part of the food is digested and absorbed as nutrients in the small intestine. The parts that are not digested in the small intestine will pass into the large intestine and be utilised by beneficial bacteria called probiotics. During the process, the food (prebiotics) is fermented by probiotics and a large amount of lactic acid is produced causing a reduction of pH value, which results in the inhibition of the growth of pathogens. At the same time, short chain fatty acids, e.g., acetic acid, butyric acid and propionic acid, and other useful compounds such as vitamin B are produced and absorbed by the host during probiotic fermentation. Butyric acid can assist the intestinal cell wall to fight against progression of cancer (Hijova and Chmelarova, 2007, Duncan and Flint, 2013). Types of prebiotics affect their usefulness in the human body, therefore those prebiotics that can be fermented more quicker produces large amount of gases, which cause discomfort to the host, and therefore, of lesser benefit to the host than those that get fermented much slower (Thammarutwasik et al., 2009).

The different mechanisms of prebiotic action are summarized with a clear presentation of most of the physiological effects occurring in the caeco-colon following the ingestion of FOS, and their putative consequences outside the gastrointestinal tract is displayed (Figure 8). After ingestion with oligosaccharides, a prebiotic effect occurs by allowing a (re)equilibrium of the colonic microbiota. The production of short-chain fatty acids by the probiotics in the large intestines allows oligosaccharides to play a role of proliferation in normal or altered colonic cells (butyrate), lowering pH, with consequences on cation absorption, reaching the liver and playing a role in lipid and glucose homeostasis. Bacterial colonic changes may also contribute in lowering carcinogen activation in the colon, modulate mucin production and stimulate the immune system, with consequences on host resistance to infection and other immune system-dependent processes (perhaps cancer). Other interesting effects are exhibited such as an increase in faecal N excretion, and a promotion of the production of intestinal hormones (for example, glucagon-like

peptide-1; GLP-1). CNS, central nervous system; GALT, gut-associated lymphoid tissue (Delzenne, 2003).

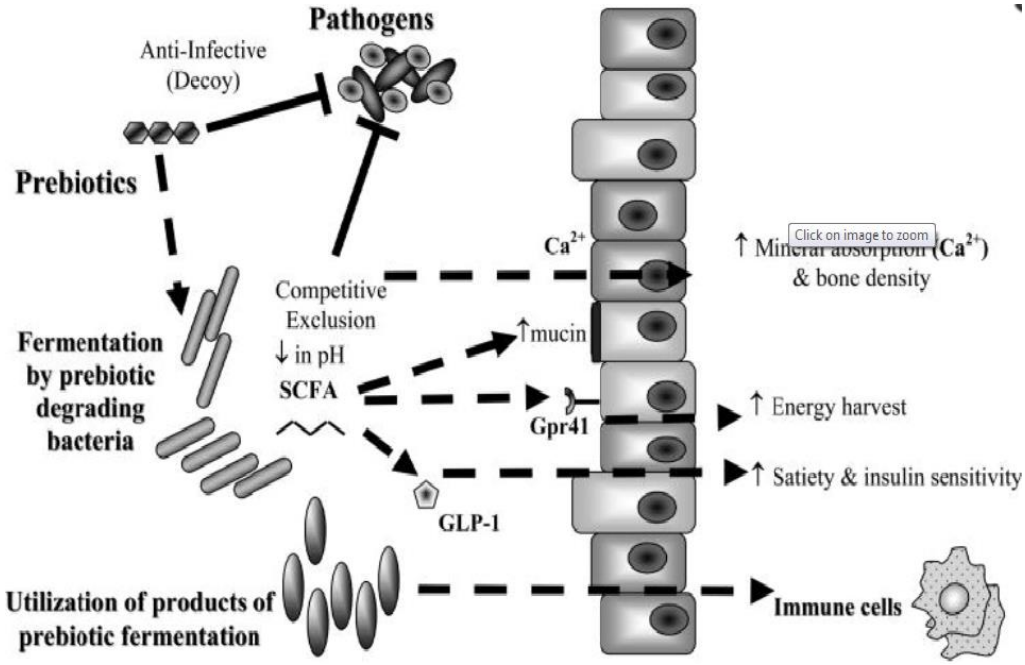


Figure 8: Depiction of the beneficial roles of prebiotics in the mammalian GIT and their systemic effects (Saulnier et al., 2009)

2.9. Probiotics

Probiotics are live microbial feed and food supplements that beneficially affect the humans and animals by improving gut microbial balance (Chandok et al., 2014). They are composed of large numbers or one or more strains of a single species or a mixture of several microbial species that are common components of the normal GIT. The most studied probiotics belong to the genera Lactobacilli and Bifidobacteria that reside in the human GIT, capable of lactic acid fermentation. These genera have a considerable safety record both within the fermented food industry, where they have been widely used for many years in probiotic foods (He et al., 2006). As evidence accumulates for their beneficial effects on human health, these bacteria are increasingly being included as functional ingredients, particularly in dairy products such as yoghurts and other fermented milk products (Shah, 2014). *Lactobacillus* and *Bifidobacterium* species are also dominant

inhabitants in the human intestine (*Lactobacillus* in the small intestine and *Bifidobacterium* in the large intestine). However, species belonging to the genera Lactococcus, Enterococcus, Saccharomyces and Propionibacterium yeasts (e.g. *Saccharomyces cerevisiae* and *Saccharomyces boulardii*) and filamentous fungi (e.g. *Aspergillus oryzae*) are also used as probiotics due to their health promoting effects (Pyar et al., 2013, Tripathi and Giri, 2014). Probiotic products on the market are available in the form of tablets or capsules, dried powder, liquid suspension and sprays. Most preparations destined for human consumption are fermented in milk or given as powder or tablets. They can contain one or several species of bacteria or fungi (Reyed, 2007). Probiotics have been widely reported to alleviate lactose intolerance, suppress diarrhea, reduce irritable bowel symptoms and prevent inflammatory bowel diseases (Baquerizo Nole et al., 2014). Some of these health benefits are indicated in Figure 9.

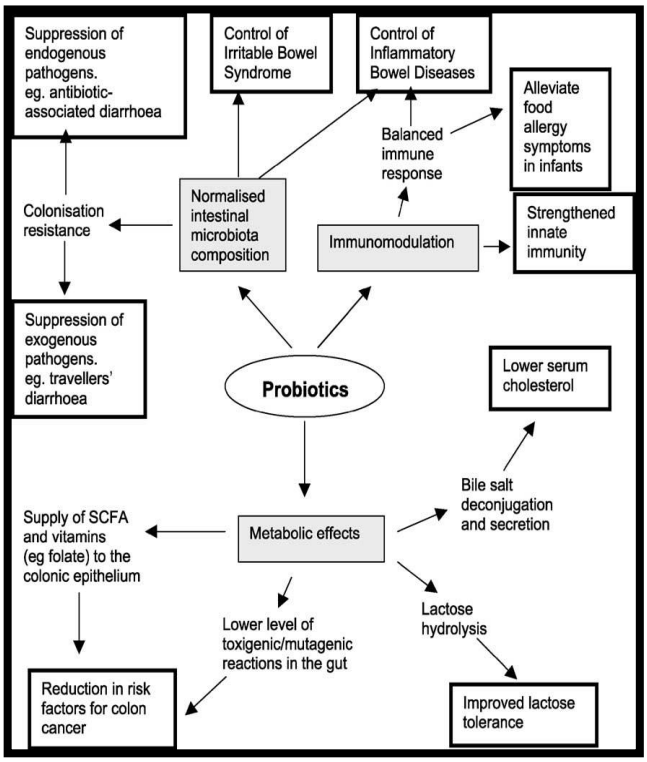


Figure 9: Proposed health benefits stemming from probiotic consumption (Saarela et al., 2002)

Normalisation of the indigenous microbiota by specific strains of the healthy gut microbiota forms the basis of probiotic therapy. Oral introduction of probiotics may decrease abnormally high intestinal permeability and alter gut microecology, affecting the intestinal immunological barrier functions and alleviating the intestinal inflammatory response. The targets for probiotic therapy are thus, identified as clinical conditions with impaired mucosal barrier function, mainly manifested by infectious and inflammatory disease. The presence of these bacteria in the human intestine has been considered as one of the most important aspects of a healthy intestinal microflora. Therefore, many attempts have been made to increase their numbers in the intestine by the administration of certain probiotic or prebiotic strains including oligo- and polysaccharides that stimulate the growth and activity of probiotic bacteria (Liu et al., 2014, Reyed, 2007).

Probiotics have the following characteristics:

a) Able to withstand bile salts, since the liver secretes bile salts into small intestine to digest fatty foods at a concentration of 0.15-0.30% (Erkkilä and Petäjä, 2000).
b) Able to withstand stomach acid. Stomach excretes HCl to help digest food, causing the pH in the stomach to be as low as 1-3, therefore the probiotic must be able to withstand these pH levels to be able to survive (Kontula et al., 1998).
c) Able to adhere to human cell (Gismondo et al., 1999). Must be able to colonize the intestinal wall to prevent colonization by pathogens, and resist the peristaltic movement of the food in the intestine, making absorption and digestion to be as normal as possible (Gourbeyre, 2011), and adherence to human intestinal cells is the first step in the mechanism of probiotic action. In fact, microorganisms of probiotics are tested for their ability to colonize intestinal epithelia. The capability to colonize epithelial cells is very important because, bacteria ingested as probiotics must be able to multiply, and colonize the gut (Reyed, 2007). Lactobacilli, Bifidobacteria and other bacterial strains probiotics.

2.9.1. Lactobacilli, Bifidobacteria and Bacillus as probiotics

The indigenous bacteria may be grouped either as potentially pathogenic or as health promoting. The strains with beneficial properties include among others Bifidobacteria and Lactobacilli, which are also among the predominant microbes in healthy infants (Liu et al., 2014). These are widely studied probiotic strains of lactic acid bacteria have been shown to exert several health benefits. Bifidobacteria and Lactobacilli have been attributed with beneficial aspects such as modification of the intestinal microbiota and stimulation of immune responses thereby promoting non-specific host resistance to microbial pathogens (Mumcu and Temiz, 2014). Bifidobacteria and Lactobacilli belong to different taxonomic groups with varying phenotypic and genotypic properties and are natural residents of the human intestine. A diverse ecological distribution and quantity in the intestinal microflora exists between these bacteria but they possess many similarities in their health-promoting effects, for instance, shortening and preventing diarrhea caused by various types of pathogenic agents, and stimulation of human immune responses (He et al., 2006).

2.9.1.1. Lactobacilli

Lactobacilli are Gram-positive, non-spore forming rods, catalase negative, usually non-motile and do not reduce nitrates. As glucose fermenters, they can be divided into different biochemical subgroups on the basis of the metabolic route by which glucose is metabolised, either homofermentatively or heterofermentatively. The *Lactobacillus* population of the human gastrointestinal system consists of various species, subspecies and biotypes within the same genera, with the most frequently isolated Lactobacilli belonging to six species, i.e., *L. acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum* and *L. brevis*. Lactobacilli have GRAS status (He et al., 2006). Lactic acid is the major metabolic end-product of Lactobacilli during glucose fermentation. The presence of lactic acid and hydrogen peroxide, and other byproducts of *Lactobacillus* metabolism have also been shown to be beneficial in controlling overgrowth of other

potentially pathogenic bacteria (Slover and Danziger, 2008). Acetic acid and succinic acid are also produced, but only in small amounts. Lactobacilli are required to maintain a healthy GIT and are not usually considered to be pathogens in the healthy host except when associated with dental caries (Aguirre and Collins, 1993). They are considered protective organisms and are thought to inhibit the growth of pathogenic organisms via the production of lactic acid and other metabolites. All of these characteristics make Lactobacilli essential bacteria in the human microflora for keeping other “more pathogenic” bacteria in check and not allowing them to cause infection (Slover and Danziger, 2008).

2.9.1.2. Bifidobacteria

Bifidobacteria are Gram-positive, non-spore forming rods, with distinct cellular bifurcating or club-shaped morphologies. Since Bifidobacteria constitute up to 25% of the gut microflora in some adult individuals, they make a significant contribution to carbohydrate fermentation in the colon. Hexoses are fermented by the fructose-6-phosphate, or ‘bifidus’ shunt, which is characterized by the presence of the key enzyme, fructose-6-phosphate phosphoketolase (He et al., 2006). The main species present in humans are *Bifidobacterium adolescentis, B. infantis, B. breve* and *B. longum* in the colon and *B. dentium* in the oral cavity. Five more species have been isolated from humans which include *B. catenulatum,* and *B. pseudocatenulatum.* It is claimed that a high number of Bifidobacteria in the colon improves human health. A high number of Bifidobacteria may prevent colonization of pathogens, and may have positive effects on intestinal peristalsis, the immune system, cancer prevention, cholesterol metabolism and carbohydrate metabolism in the colon. This has led to the recognition of Bifidobacteria as probiotics, especially in the dairy industry (Gibson, 2004).

2.9.1.3. *Bacillus* species

Bacillus species have been used as probiotics in at least 50 medicinal supplements. There is scientific interest in *Bacillus* species as probiotics, though it has only occurred in the last 15 years (Cutting, 2011). The species that have most extensively been examined are *B. subtilis*, *B. clausii*, *B. cereus*, *B. coagulans* and *B. licheniformis*. Spores being heat-stable have some advantages over other non-spore formers such as *Lactobacillus* spp. For example, the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. The second advantage is that the spore is capable of surviving the low pH of the gastric acid (Barbosa et al., 2005), which is not the case for all species of *Lactobacillus* (Tuohy et al., 2007), so in principle, a specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria will reach the small intestine intact.

2.10. Immunomodulation by probiotics

The most complex of the postulated mechanisms by which probiotics and stimulated endogenous microbes may act is their interaction with the GIT immune cells and lymphoid tissues to modulate immune and inflammatory responses of the host. Figure 10 depicts three levels of probiotic action in the GIT. Probiotic bacteria can interfere with the growth or survival of pathogenic microorganisms in the gut lumen (Level 1); probiotic bacteria can improve the mucosal barrier function and mucosal immune system (Level 2); and, beyond the gut, have an effect on the systemic immune system, as well as other cell and organ systems such as liver and brain (level 3) (Binns, 2013).

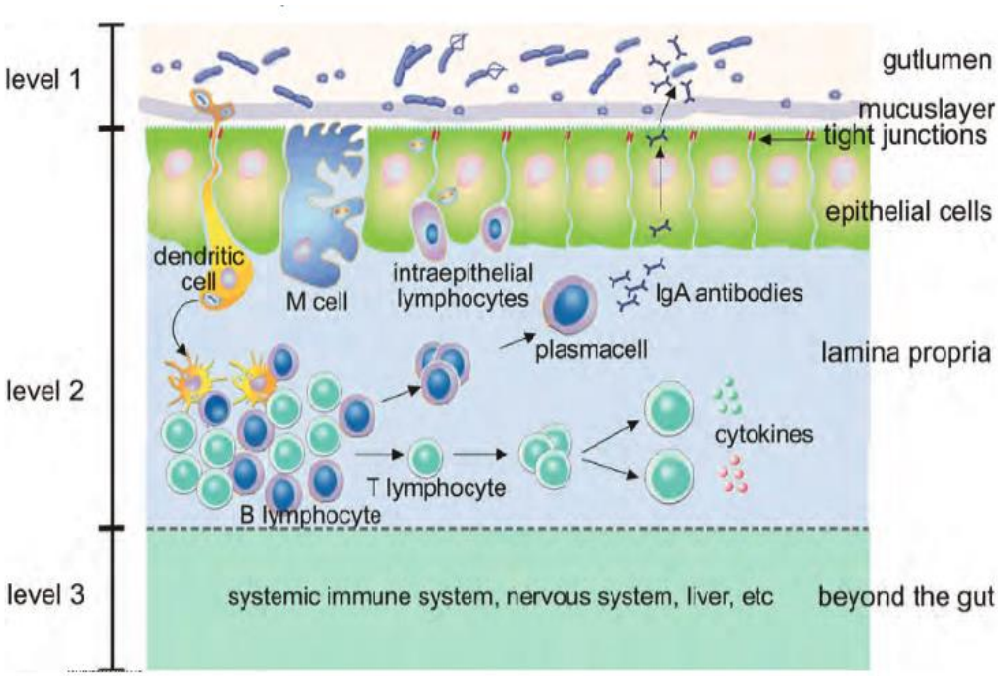


Figure 10: Depiction of the three levels of action of a probiotic in the GIT (Binns, 2013)

Ingested and endogenous microbes can impact both on the innate and adaptive responses of the host immune system. The interaction between microbial cells (commensal, probiotic or pathogen) and host cells is mediated by their interaction with specific receptors such as Toll-like receptors (TLR) that are associated with cells lining the mammalian GIT. The activation of these receptors initiates a cascade of concerted immune signals leading to different responses. Probiotics from different strains can stimulate immunity by increasing mucosal antibody production, boosting pro-inflammatory cytokine expression and enhancing host defense production. Suppressive effects are manifested by decreasing cytokine expression, systemic inflammation, cellular proliferation and increasing apoptosis. Surface proteins have been implicated as key factors involved in immunomodulation (Saulnier et al., 2009). For example, aggregation-competent, *L. crispatus* modulates the expression of innate immune receptors TLR-2 and TLR-4 on the surfaces of epithelial cells in the colonic mucosa of mice (Voltan et al., 2007). Also, mutant strains of *L. casei* deficient in cell wall-associated polysaccharides are unable to exert immunosuppressive effects on macrophage cytokine production as seen

by the wild-type strains (Yasuda et al., 2008). Probiotic bacteria are also known to secrete factors responsible for modulating immune responses. For instance, secreted factors from *L. reuteri* decrease nuclear factor-κB dependent gene expression, resulting in diminished cell proliferation and enhanced mitogen-activated protein kinase activities important for inducing apoptosis (Iyer et al., 2008). As fermented milk drinks are popular sources of probiotics, it is important to note that *L. helveticus* is capable of producing factors during milk fermentation responsible for increasing calcineurin expression, resulting in an augmented population of mast and goblet cells in the mouse GIT (Vinderola et al., 2007).

2.11. Macroalgae and microalgae

Algae are photosynthetic organisms possessing reproductive simple structures. These organisms constitute a total number of 25 to 30,000 species, with a great diversity of forms and sizes as shown in Figure 11. They exist from unicellular microscopic (microalgae) to multicellular organisms (macroalgae or aquatic plants) (Swennen et al., 2006). They live in complex habitats and can continue to survive in extreme conditions, for example; changes of salinity, temperature, nutrients and ultraviolet irradiation. Therefore, they adapt rapidly to new environmental conditions to survive, producing a great variety of secondary (biologically active) metabolites, which cannot be found in other organisms (Carlucci et al., 1999). Considering their great taxonomic diversity, investigations relating to search of new biologically active compounds from algae can be seen as an almost unlimited field. Besides its natural character, other important aspects relating to the algae are their ease of cultivation, their rapid growth comparison with many other species and the control of the production of some bioactive compounds by manipulating the cultivation conditions (Plaza et al., 2008, De Morais et al., 2015). Algae can be classified into two major groups according to their size i.e, macroalgae and microalgae, which are discussed below.

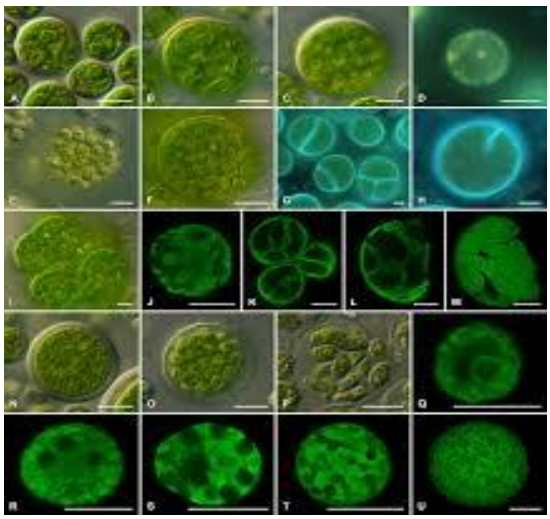


Figure 11: Diversity of algae (Škaloud et al., 2013)

2.11.1. Macroalgae

Macroalgae, commonly known as seaweeds, are multi-celled organisms that live in sea water and are between 2 to 30 mm in size. Several characteristics are used to classify them including the nature of their chlorophyll, their cell wall chemistry, and the presence or absence of flagella. The common feature that is usually employed in algal classification to its various divisions is the presence of specific pigments such as the carotenoid fucoxanthin in brown algae (Phaeophyceae), chlorophyll a and b in green algae (Chlorophyceae) and phycoerythrin and phycocyanin in red algae (Rhodophyceae) (O'Sullivan et al., 2010). The presence of these different phytopigments in algae is related to their sea habitat because, not all macroalgae need the same light intensity to perform photosynthesis. Thus, green macroalgae, which are able to absorb large amounts of light energy, abound in coastal waters, while red and brown macroalgae prevail at greater depths where penetration of sunlight is limited (Bocanegra et al., 2009). Macroalgae are a source of biologically active phytochemicals, which include carotenoids, phycobilins, fatty acids, polysaccharides, vitamins, sterols, to copherol and phycocyanins. Many of these compounds are known to possess biological activity and hence have potential beneficial use in healthcare (Kadam and Prabhasankar, 2010).

2.11.2. Microalgae

Microalgae are the most primitive and simply organised members of the plant kingdom, with a majority existing as small cells of about 3–20 μm. These algae are ubiquitous in nature and have been isolated from areas ranging from hot springs to glacial ice flows. Microalgae are found in both benthic and littoral habitats and also throughout the ocean waters as phytoplankton. Phytoplankton comprises organisms such as diatoms (bacillariophyta), dinoflagellates (dinophyta), green and yellow-brown flagellates (Chlorophyta; prasinophyta; prymnesiophyta, Cryptophyta, Chrysophyta and Rhaphidiophyta) and blue-green algae (Cyanophyta). As photosynthetic organisms, they plays a critical role in the productivity of oceans and constitute the basis of the marine food chain (Lordan et al., 2011).

Compounds isolated from marine microalgae have demonstrated various biological activities, such as anti-oxidant (Yuan and Walsh, 2006), anti-inflammatory (Kang et al., 2008), anti-coagulant (Wijesekara et al., 2011), anti-bacterial (Kellam and Walker, 2007), and anti-viral activity (Talyshinsky et al., 2002). As a result, compounds derived from algae have significant applications in a range of products in the food, pharmaceutical and cosmetic industries (d'Ayala et al., 2008), with some are illustrated in Figure 12. Further more, algae are a rich source of dietary fiber (25–75% dry weight), of which water-soluble fiber constitutes approximately 50–85% (O'Sullivan et al., 2010).

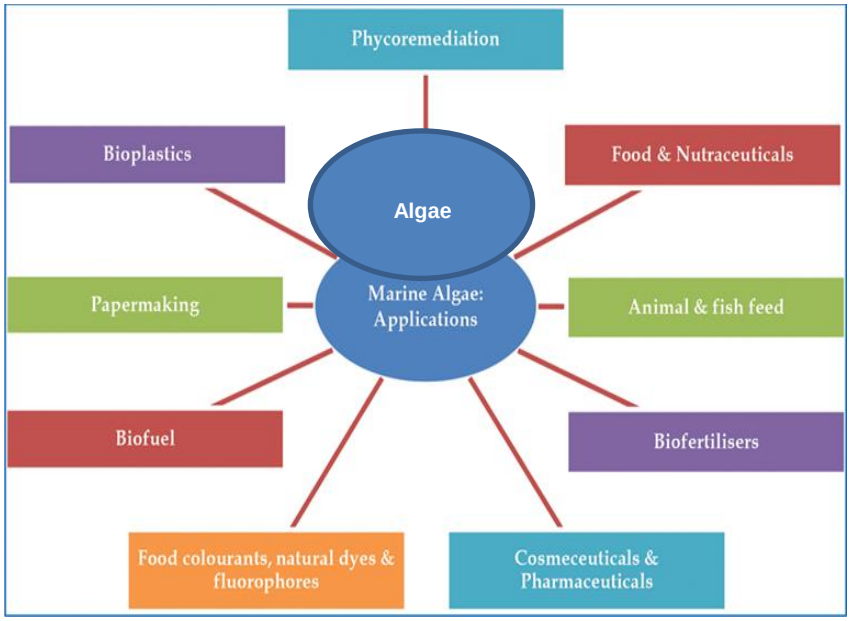


Figure 12: Applications of algae (Beetul et al., 2016)

2.11.3. Structural characteristics of oligo/polysaccharides produced by macroalgae and microalgae

The chemical structure of polysaccharides produced by macroalgae and microalgae is significantly determined by their properties, namely physico-chemical and biochemical, and reflect their physical behavior and biological activities. The characteristics of the various polysaccharides produced by microalgae, including their composition and structure are discussed further on in this section. Table 3 shows the main classes of polysaccharides that are commonly found in higher plants, algae and fungi (Harborne, 1973).

Table 3: Main classes of Polysaccharides in higher Plants and Algae

Class name	Sugar unit	Linkage	Distribution
	HIGHER PLANTS		
Cellulose	Glucose	β1→4	Universal as cell wall material
Starch-amylose	Glucose	α1→4	Universal as storage material in artichoke, chicory, etc.
Starch-amylopectin	Glucose	α1→4, α1→6	
Fructan	Fructose/Glucose	β2→1	

Table 3 continued

Class name	Sugar unit	Linkage	Distribution
Xylan	Xylose/Arabinose/Uronic acid	β1→4	Widespread, e.g. grasses
Glucomannan	Glucose/Mannose	β1→4	Widespread, especially in coniferous wood
Arabinogalactan	Arabinose/Galactose	1→3, 1→6	
Pectin	Galacturonic acid	α1→4	Widespread
Galactomannan	Mannose/Galactose	β1→4,α1→6	Seed mucilages in *Acacia* and *Prunus* species
	ALGAE (Seaweeds)		
Laminaran	Glucose	β1→4	Phaeophyceae (brown algae)
Polysaccharide Sulphate	Fucose (& others)	-	
Alginic Acid	Mannuronic & Guluronic acid	-	
Amylopectin	Glucose	α1→4, 1→6	Rhodophyceae (red algae)
Galactan	Galactose	1→3, 1→4	
Starch	Glucose	α1→4, α1→6	
Polysaccharide Sulphate	Xylose, Rhamnose, Glucuronic acid	-	Chlorophyceae (green algae)

2.11.3.1. Macroalgae

Macroalgae contain large to least amounts of polysaccharides, such as alginates, laminarins, fucans and cellulose in Phaeophyta (brown seaweeds); carrageenans and agar in Rhodophyta, (red seaweeds); and ulvans in Chlorophyta (green seaweeds). However, most of these polysaccharides are not digestible with the human GIT and therefore, can be regarded as non-digestible fibre (NDF). Moreover, they can be regarded as prebiotics as they promote growth of intestinal microflora and probiotics (Lordan et al., 2011, Varfolomeev and Wasserman, 2011). Fucoidans are sulphated polysaccharides, which are found in the cell walls of brown algae that have been reported to display

numerous physiological and biological properties such as anti-tumour, anti-viral, anti-coagulant benefits (Cumashi et al., 2007). Another sulphated polysaccharide, porphyran makes up the main components of the red macroalgae (*Porphyra)*. This polysaccharide has been reported to be used as a gelling agent, a nutritional supplement and as an anti-oxidant (Jiménez-Escrig and Sánchez-Muniz, 2000).

Numerous oligosaccharides from algae with immune stimulating activities have been characterized as having anti-hypertensive, anti-microbial, anti-oxidant and anti-tumour properties (Patel and Goyal, 2011). Moreover, oligosaccharides, for example, can benefit a person's health when they are added to the diet as they enhance the growth of prebiotic bacteria (Courtois, 2009).

2.11.3.2. Microalgae and Cyanobacteria

The polysaccharides released by Cyanobacteria are complex heteropolymers that, in about 80% of the cases, are composed of six to ten different monosaccharides. These ten monosaccharides are the hexoses (glucose, galactose and mannose); the pentoses (ribose, arabinose and xylose); the deoxyhexosesfucose, rhamnose and the acidic hexoses (glucuronic and galacturonic acid) (De Philippis et al., 2001).

A significant variation in the ratio among the monosaccharides has also been found, glucose being, in 60% of the cases, the most abundant. Another important feature, common to most cyanobacterial polysaccharides, is their anionic character (Shah et al., 2000), with about 90% polymers being characterized by the presence of uronic acids that, in polysaccharides of half of the strains studied, exceeding 20% of the released polysaccharides (RPS) dry weight (Wt) (De Philippis et al., 2001). In addition, significant levels of other charged substituents (sulphate and ketal-linked pyruvyl groups) have been found in many cyanobacterial RPS (De Philippis et al., 2000), contributing to the global charge of the macromolecules that acquire a rather high anion density (De Philippis and Vincenzini, 1998). The presence of hydrophobic groups has also been reported for a

rather large number of cyanobacterial polysaccharides, ester-linked acetyl groups, up to 12% of dry Wt, together with peptidic moieties, and the deoxysugarsfucose with rhamnose contributing to a significant hydrophobic behaviour of these otherwise hydrophilic macromolecules (Shepherd et al., 1995).

2.11.4. Algal species with prebiotic potential

There are a number of algal species that have been used in the food and pharmacological industries. These species alongside those with potentials for future use are reviewed in the proceeding section of this chapter. The microscopic descriptions of these algal species are presented along with prebiotic properties.

2.11.4.1. *Spirulina platensis*

Spirulina platensis (*S. platensis*) is a planktonic, filamentous Cyanobacterium or blue–green alga found in many freshwater environments including ponds, lakes, and rivers (Babadzhanov et al., 2004). *Spirulina platensis* contains about 13.6% carbohydrates; some of which are glucose, rhamnose, mannose, xylose and galactose (Sánchez et al., 2003). *Spirulina platensis* biomass is known to stimulate bacterial growth, and studies performed indicated that the algae consumes nitrogen from the growth medium and releases extracellular carbohydrates and other growth substances which, may be responsible for stimulating growth of Lactobacilli (and other lactic acid-producing strains) (Parada et al., 1998).

Carbohydrate moieties from *Spirulina platensis* exist in the form of lipopolysaccharide and lipoglucan as observed in Figure 13. The major oligosaccharide fraction of the lipopolysaccharide comprises the O-antigen (with 40 repeating sugar units) and the core polysaccharide. These carbohydrate segments consist of rhamnose, mannose, galactose, 2-keto-3-deoxymanno-octanic acid and glucosamine. Galactose, mannose and xylose

sugar units generally exist in very low quantities as part of the lipopolysaccharide structure and 2, 3-di-O-methylpentose and 2-O-methyl-6-deoxyhexose also occur in trace amounts. The sugars which comprise the majority of the O-antigen and core polysaccharide structure are rhamnose and glucose residues, the former sugars linked by 1,2- and 1,3-bonds, and the latter by 1,4- bonds (Mikheiskaya et al., 1983).

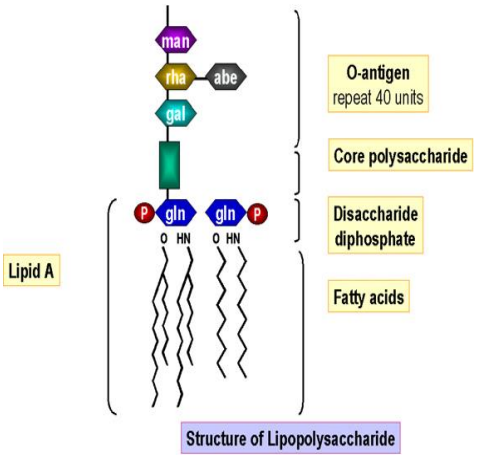


Figure 13: The basic molecular structure of a lipopolysaccharide (Gemma et al., 2016)

2.11.4.2. *Chlorella* species

Chlorella spp. are unicellular green algae of Chlorophyta found in both fresh and marine water (Suárez et al., 2005). Polysaccharide complexes from *Chlorella pyrenoidosa* and possibly, *Chlorella ellipsoidea*, contain glucose and any of the combination of galactose, rhamnose, mannose, arabinose, N-acetylglucosamide and N-acetylgalactosamine (Lordan et al., 2011). An acidic polysaccharide was isolated from *Chlorella pyrenoidosa* containing mostly rhamnose (52%) with both arabinose and galactose in about equal amounts (12 and 13%, respectively). These complexes are believed to have immunostimulating properties, inhibiting the proliferation of *Listeria monocytogenes* and *Candida albicans* (Mata et al., 2010, Suárez et al., 2005). *Chlorella vulgaris* contains about 0.09% carbohydrates; some of which are rhamnose, mannose, xylose, galactose, 2-O-methyl rhamnose, 3-O-methyl rhamnose (in molar ratios of 25:2.3:1.7:1.0:3.9:2.1:1.2) (Sui et al., 2012). Yalcin et al. (1994) reported the isolation of another extracellular

polysaccharide from *Chlorella* spp. containing glucuronic acid and arabinose as major components (38.3 and 32.5%, respectively).

2.11.4.3. *Tetraselmis* species

Tetraselmis is a sizeable genus (more than 50 species) of green flagellates. Most species are known from inshore marine environments, tide pools in particular, but a few freshwater species are also known (Mohammadi et al., 2015).The cell walls (theca) of *Tetraselmis* spp. consist mainly of acidic polysaccharides (82% of dry weight) (Becker et al., 1998). Major monosaccharide constituents are the unusual 2-keto-sugar acids 3-deoxy-manno-2-octulosonic (Kdo, 54–60 mol% of total carbohydrate), 3-deoxy-5-O-methyl-manno-2-octulosonic acid (5OMeKdo, 4%), and 3-deoxy-lyxo-2-heptulosaric acid (Dha, 6–8%) (Becker et al., 1991). In addition, galacturonic acid (18–21%) and small amounts of galactose (7%), gulose (3–4%), and arabinose (1%) are present (Becker et al., 1991). However, not much is known about the structural elements of the theca. Two Kdo and galactopyranuronic acid (GalA) containing trisaccharides obtained by partial hydrolysis of the theca of *Tetraselmis striata* were isolated by Becker et al. (1995).

2.11.4.4. *Dunaliella salina*

Dunaliella salina (*D. salina*) is a unique unicellular species of Chlorophyta with no cell wall found in saline environments (Abu-Rezq et al., 2010). This alga has been reported to produce extracellular polysaccharides that are known to have immunostimulant, anti-viral and anti-tumor activities (Mishra and Jha, 2009). The first reported polysaccharide isolated from *D. salina* residue after extraction of carotene and its monosaccharide composition was identified as glucose, galactose, xylose, mannose, and rhamnose by paper chromatography (Dai et al., 2010). Xue et al. (2003) and Xie et al. (2005) obtained three polysaccharide fractions from *D. salina* by basic hot water extraction and purification with DEAE-32 ion-exchange column and Sephadex G-100 gel filtration column. The three fractions were determined as a glucan, a sulfated proteoglycan and a sulfated

heteropolysaccharide mainly containing glucose by GC, IR and barium sulfate turbidimetry.

2.11.4.5. *Scenedesmus* species

Scenedesmus spp. are ubiquitous organisms and are frequently dominant in freshwater lakes and rivers. (Kim et al., 2007). They are well-known for their nutritional value and considered as potential food additives (Kumar, 2015). *Scenedesmus* spp. has also been used for producing lipid and carbohydrate suitable for making liquid biofuels (Ho et al., 2010). Colony formation in algae is linked to polysaccharide production, however polysaccharide from this algae is limited (Liu et al., 2010).

2.11.4.6. *Chlorococcum* species

The marine green alga *Chlorococcum* is a unicellular spherical strain with cells of diameter about 10 μm belonging to Chlorococcaceae family (Berberoglu et al., 2009). It is of interest due its tolerance to high CO_2 concentrations and the fact that it can grow to high cell density (Hu et al., 1998). Just like all other microalgae, it can produce value-added by-products that can enable their processes more economical. Some of the examples include their use in medicinal and pharmaceutical industry as well as health drinks for their immunostimulatory, antioxidant, antiviral, and anticancer activities (Hu et al., 1998, Skjånes et al., 2007).

2.11.4.7. *Cylindrospermum* species

Cylindrospermum usually inhabits soft, acidic freshwater lakes and is one of the filamentous, heterocystous and non-branched cyanobacteria, classified traditionally in Nostocaceae family. The filaments form fine or compact benthic mats or colonies and may be epiphytic or metaphytic (Hijova and Chmelarova, 2007). *Cylindrospermum* spp are employed in agriculture as biofertilizers and soil conditioners. They are capable of fixing atmospheric nitrogen and are effectively used as biofertilizers its application is useful for

the reclamation of soils (Harborne, 1973). In previsions studies 31% carbohydrate and 21% protein content was recorded in the imolecular composition of *Cylindrospermum* (Kellam and Walker, 2007). Figure 14 illustrates microscopic structures of some fresh and marine microalgae with prebiotic potential.

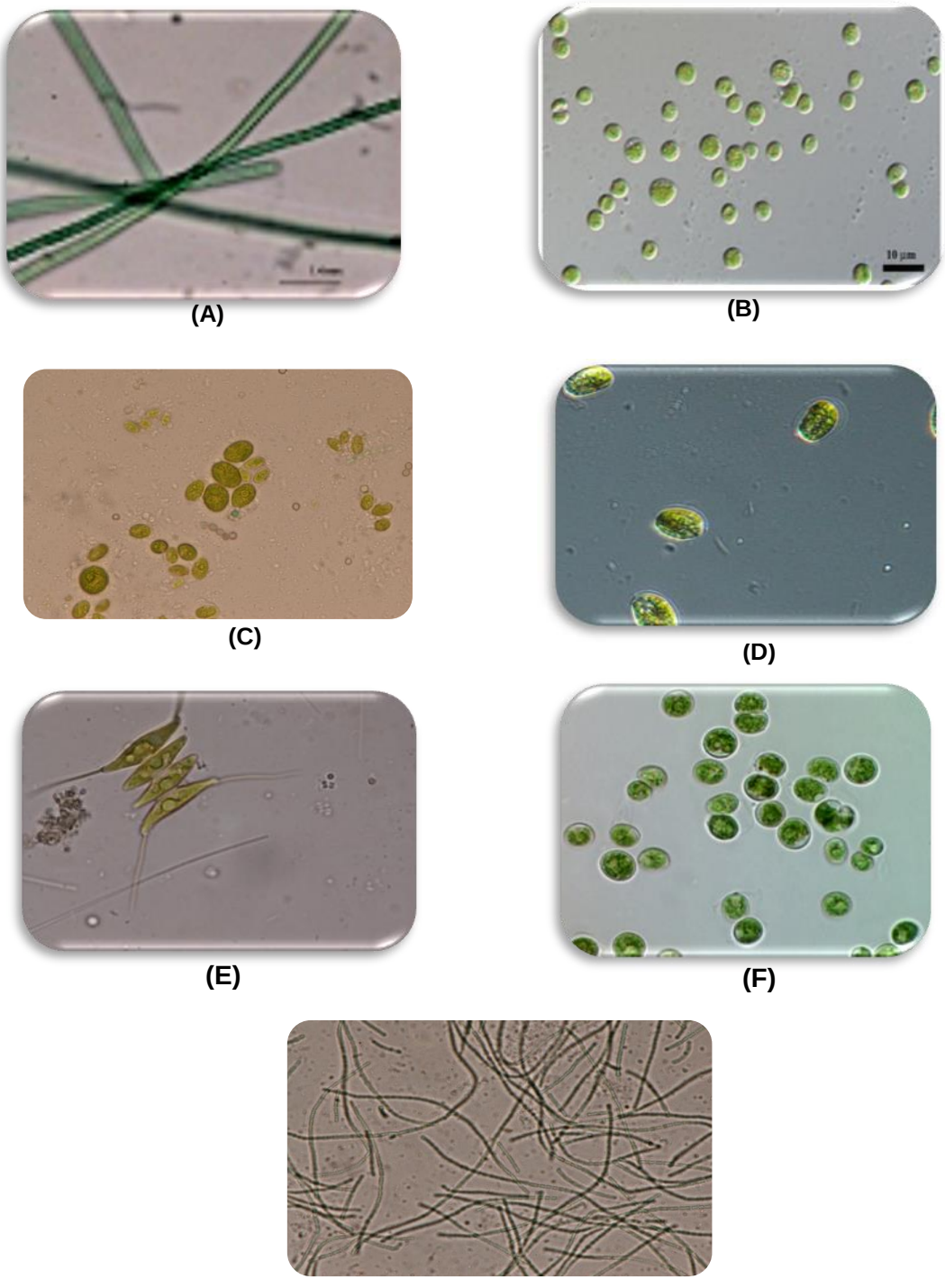


Figure 14: Examples of microscopic structures of some fresh and marine microalgae with prebiotic potentials. (A) *Spirulina platensis*; (B) *Chlorella vulgaris*; (C) *Tetraselmis* spp.; (D) *Dunaliella salina*; (E) *Scenedesmus* spp.; (F) *Chlorococcum* spp.; (G) *Cylindrospermus* spp.

2.12. Synbiotic effect of algal oligosaccharides

A combination of probiotic(s) and prebiotic(s) constitutes a synbiotic, which can stimulate and increase the survival of probiotic and autochthonous-specific strains in the intestinal tract (Gourbeyre, 2011). The combination of a prebiotic of plant material for example; inulin and a probiotic in one product has shown to confer benefits beyond those of either prebiotic or probiotic (Holzapfel and Schillinger, 2002). Probiotic and prebiotic treatment has been shown to be a promising therapy to maintain and repair the intestinal environment. Consumption of healthy live microorganisms (lactic acid bacteria) with prebiotics such as inulin, galactooligosaccharide and oligofructose to mention a few, may enhance healthy colonic microbiota composition. This combination might improve the survival of the bacteria crossing the upper part of the GIT, thereby boosting their effects in the large bowel (Jain et al., 2014).

However, relative success of the symbiotic of algal material is dependent on the changes elicited during the metabolism of the bacterial populations present, their ecological interactions and their population composition (Buddington, 2009). Although various clinical trials have thus far led to the belief that symbiotic systems for colonic treatment might be the most efficient, too few studies have been conducted to draw conclusive results.

Algal oligo- and polysaccharides show effects on health similar to and sometimes more effective than other oligosaccharides from different sources. Their chemical structures include oligosaccharides that are not degraded by enzymes in the upper part of the GIT. Therefore, algal polysaccharides present a great potential for emergent prebiotics to be used directly, in the case of microalgae, or as dried biomass or nutraceuticals, after extraction from the biomass or from the culture medium (De Jesus Raposo et al., 2016).

3. METHODOLOGY

3.1. Introduction

Aqueous extraction of the algal oligosaccharide from marine and freshwater algae was performed, utilizing sonication and centrifugation followed by a period of storage. Probiotic bacteria were isolated, enumerated on Man, Rogosa Sharpe agar (MRS) and later characterized. The prebiotic properties of algal extracts were assessed in three phases and in each case inulin, a known prebiotic, served as the positive control, meanwhile bacterium culture without algal extract served as the negative control. The effect of algal extracts from a library of fifty-five algal species were screened for improvement of bacterial growth on three probiotic bacteria (*L. lactis, L. bulgaricus* and *B*. *longum*) at 0, 24, 48, 72 and 96 hours by spectrophotometric measurements to establish a optimum time at which the growth of probiotic bacteria is significantly enhanced. Nineteen algal extracts were selected based on their ability to significantly increase the growth of at least one probiotic bacterium in comparison to the positive (inulin) or negative control. Subsequently these nineteen algal extracts were evaluated at 48 hour period on the growth of all three probiotic bacteria. Evaluation of the growth of probiotic bacteria was investigated using a spread plate technique and colony forming units (CFU). The best algal extracts were selected for prebiotic identification and characterization. Acid hydrolysis was performed on algal extracts to obtain a complete decomposition into monosaccharides. Thin layer chromatography (TLC) was performed to identify the monosaccharide sugars comprising the oligosaccharide isolated from these algae. The hydrolysed monosaccharides were characterized by high performance liquid chromatography (HPLC) using appropriate standards.

3.2. Culture collection, maintenance and confirmation of cultures

3.2.1. Algal cultures

Seven marine and 43 freshwater species were obtained from the Council for Scientific and Industrial Research (CSIR), Modderfontein, South Africa. These microalgae were previously isolated from various regions in South Africa, ranging from the Drakensburg to the Cape. Five algal species namely; *Chlorella* spp., *Scenedesmus magnus*, *Chlorococcum* spp., *Dunaliella salina* and *Spirulina platensis* were obtained from Durban University of Technology (DUT), Waste Water Research Institute, South Africa, which were previously isolated from various aquatic environments in KwaZulu-Natal, South Africa. Table 4 shows the source number generated from the initial sampling point, laboratory generated numbers in a sequence, location area of sampling and the respective media for each microalgae.

Algal species from CSIR were received on slants and were aseptically transferred onto a respective agar plate. Marine algal isolates were cultured on Artificial Salt Water (ASW) media, freshwater algal isolates were grown on Artificial Fresh Water (AF6) media as per CSIR protocols (Appendix Table A_{1-8}). and *S. platensis* was cultured on Zarrouks media according to the protocol described by Parada et al. (1998) (Appendix Table A_9). Algal cells were cultured in their respective growth media at 26°C in a shaker incubator at 120 rpm for 14 days with an artificial light (using a 60 watts light bulb) provided.

Table 4: Freshwater and marine algal species from various regions in SA, ranging from the Drakensberg to the Cape

Source number	Lab generated number	Location	Culture media used
79	1	Drakensberg	AF6
82	2	Drakensberg	AF6
84	3	Drakensberg	AF6
85	4	Drakensberg	AF6
92	5	Drakensberg	AF6
95	6	Drakensberg	AF6
97	7	Drakensberg	AF6
98	8	Drakensberg	AF6
100	9	Drakensberg	AF6
101	10	Drakensberg	AF6
105	11	Drakensberg	AF6
106	12	Drakensberg	AF6
29	13	ST. Lucia Estuary	ASW
27	14	Milley's Caltex	AF6
23	15	Milley's Caltex	AF6
34	16	Milley's Caltex	AF6
35	17	Milley's Caltex	AF6
41	18	Milley's Caltex	AF6
45	19	Kiepersdol	AF6
111	20	Kiepersdol	AF6
112	21	Kiepersdol	AF6
118	22	Kiepersdol	AF6
119	23	Kiepersdol	AF6
119	24	Kiepersdol	AF6
121	25	Kiepersdol	AF6
129	26	Kiepersdol	AF6
15	27	Bergrivier	AF6
12	28	Bergrivier	AF6
19	29	Bergrivier	AF6
20	30	Lake 3	AF6
22	31	Lake 3	AF6
25	32	Lake 3	AF6
30	33	Lake 3	AF6
36	34	Lake 3	AF6
62	35	Sedgevield Lagoon	AF6
67	36	Sedgevield Lagoon	AF6
1	37	Milley's Caltex	AF6
7	38	Milley's Caltex	AF6
72	39	Cape Recife Dam	AF6

Table 4 continued

Source number	Lab generated number	Location	Culture media used
75	40	Cape Recife Dam	AF6
78	41	Cape Recife Dam	AF6
70	42	Cape Recife Dam	AF6
80	43	Cape Recife Dam	AF6
87	44	ST. Lucia Estuary	ASW
91	45	ST. Lucia Estuary	ASW
93	46	ST. Lucia Estuary	ASW
99	47	ST. Lucia Estuary	ASW
108	48	ST. Lucia Estuary	ASW
103	49	ST. Lucia Estuary	ASW
104	50	Cape Recife Dam	AF6
***Chlorella* spp.**	51	DUT	AF6
Scenedesmus magnus	52	DUT	AF6
***Chlorococcum* spp.**	53	DUT	ASW
Dunaliella salina	54	DUT	ASW
Spirulina platensis	55	DUT	Zarroucks

Algal species from CSIR (1 to 50) and DUT (51 to 55)

3.2.2. Probiotic strains of Lactobacilli and Bifidobacteria

Lactobacillus delbrueckii subsp. *lactis* (*L. lactis*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) were obtained from the Durban University of Technology Culture Collection, South Africa, whereas *Bifidobacterium longum* (*B. longum*) was isolated from breast milk. *Lactobacillus* subsp. *lactis*, *L. bulgaricus* and *B. longum* were cultured on Man, Rogosa and Sharpe (MRS) agar, incubated and grown under anaerobic conditions in a jar (Anaerocult® A, Merck, Germany) at 30°C for 24-48 hours. The cultures were stored in micro banks at -70°C until further use.

3.3. Confirmation of probiotic and algal cultures

Lactobacillus lactis, *L. bulgaricus* and *B. longum* were confirmed based on their colonial and microscopic morphology and also on their Gram Reaction. The marine and freshwater algal cultures from CSIR and DUT were viewed with the naked eye under the light microscope (Nikon, Japan) to confirm and determine the purity of cultures. The probiotic bacteria and algal cultures were studied for their shape, size, colour and arrangement

using a light microscope (Nikon, Japan). Algal keys were used for the identification of some unknown algal species for CSIR, South Africa according to John et al. (2002).

3.4. Aqueous extraction of oligosaccharides from algal species

To isolate oligosaccharides, the algae were grown for 14 days and collected by centrifugation (Eppendorf Centrifuge 580R) at 11,000 *g* for 20 minute (min) at 4°C. The supernatant was discarded, and the pellets from replicated cultures pooled, and an equal volume of sterile distilled water was added. The mixture was sonicated using a ViriSon 100 Sonicator 100W for 5 min at an interval of 1 min to ensure algal cell disruption. After sonication, the mixture was centrifuged for 20 min at 11 000 rpm (Revolutions per minute). The pellets were discarded, the supernatant was retained and filter sterilized using a 0.22 µm filter (Millipore filter). The filtrate was kept in a bio-freezer (-20°C) for two days and thereafter dried using a freeze dryer (Vertis) for three days. The dried extracts were used for further analysis.

3.5. Prebiotic effect of aqueous algal extracts on the growth of probiotic bacteria

3.5.1. Screening of marine and freshwater algal aqueous extracts for prebiotic activity

The batch culture containing aqueous algal extracts and probiotic bacteria was carried out according to the procedure outlined by López-Molina et al. (2005), with slight modifications (Figure 15). Two-hundred ml each of Man, Rogosa broth and Sharpe (MRS) broth (Biolab; Merck, Gauteng) was prepared according to the manufacturer's protocol. Fifty ml of MRS broth was transferred into three 100 ml flasks, inoculated with 5 loops full of *L. lactis*, *L. bulgaricus* and *B. longum* and incubated in anaerobic chambers overnight. The cultures were standardised at A_{620} using a Varian-Carey UV-Vis spectrophotometer by diluting each culture with sterile MRS broth to match the turbidity of 2 X 10^3 CFU/ml.

The algal samples were prepared by dissolving 1 mg each of *S. magnus, D. salina, S. platensis*, *Chlorella* and *Chlorococcum* algal extracts from DUT, South Africa, and 50 unknown algal species from CSIR, South Africa into one ml of MRS broth and mixed thoroughly. A positive control was prepared by dissolving 1 mg Chicory inulin (Sigma; Germany) into 1 ml of MRS broth. A quantity of 50 µl of the positive control was added to sterile tubes each containing 50 µl of the standardised bacterial suspension with 5 ml sterile MRS broth. The test sample was prepared by adding 50 µl of the algal extract to sterile tubes, each containing 50 µl of the standardised bacterial suspension with 5 ml sterile MRS broth. The negative control contained 5 ml sterile MRS broth with 50 µl the standardised bacterial suspension. The tubes were incubated at 30°C anaerobically using an anaerobic jar (Anaerocult® A, Merck, Germany) at 0, 24, 48, 72 and 96 hours by taking spectrophotometric measurements (A_{620}) to establish the optimum time at which the growth of probiotic bacteria is significantly enhanced.

3.5.2. Evaluation of prebiotic effect of the selected aqueous algal extracts

Aqueous algal extracts that showed an increase in probiotic bacterial growth over a period of 96 h were selected. The effect of aqueous algal extracts was tested as described in section 3.5.1. The tubes were incubated at 30°C anaerobically using an anaerobic jar (Anaerocult® A, Merck, Germany) on the time at which the optimal bacterial growth was observed. Triplicate serial dilutions up to 10^{-6} of the samples were prepared and plated on MRS agar. Incubation of plates was carried out at 30°C anaerobically using an anaerobic jar (Anaerocult® A, Merck, Germany) for 48 hours. The growth of probiotic bacteria was enumerated using a spread plate technique and CFU were calculated using the formula below

$$\text{CFU} = \frac{\text{No of colonies x dilution factor}}{\text{volume}}$$

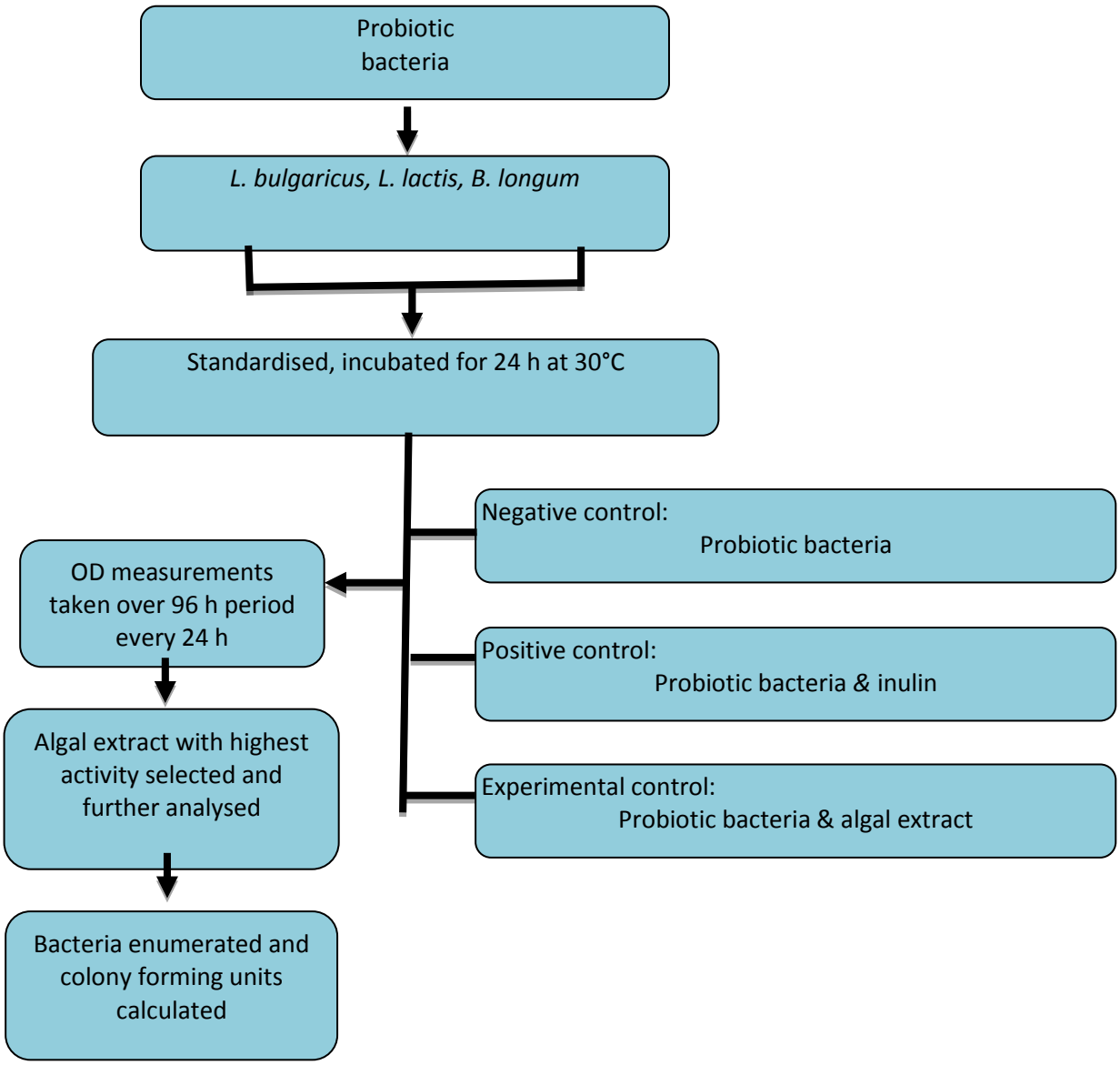


Figure 15: Diagram depicting experimental procedure used for determining the effect of aqueous algal extracts on the growth of probiotic bacteria

3.6. Acid hydrolysis of aqueous algal extracts

A mild acid hydrolysis with 2 M trifluoroacetic acid (TFA) of the algal extracts was performed to decompose oligosaccharides into monosaccharides. Aqueous algal extracts (5 mg each) were hydrolyzed using 0.5 ml of 2 M TFA acid in boiling water for 2 h at 100°C. After the hydrolysis, the reaction medium was neutralized to pH of 7 by adding

$Ba(OH)_2$, and the aliquot of hydrolysate was filtered through a 0.22 µm millipore filter (Emaga et al., 2012).

3.7. Characterization of oligosaccharides present in algal extracts

3.7.1. Identification of monosaccharide subunits of oligosaccharide by Thin Layer Chromatography

The monosaccharide subunits of the algal oligosaccharide present in the algal extracts were identified by TLC as illustrated in figure 16. This was done to separate the molecular size and compare with known monosaccharide standards namely; glucose, xylose, glucuronic acid, fructose and galactose (Merck, Gauteng) used as certified reference material (CRM). One to four µl aliquots taken from the algal extracts and standards were spotted on 1 cm from the bottom and between the samples on the silica gel plate (Merck, Gauteng). The mobile phase used for this experiment was composed of ethyl acetate: acetic acid: 2-propanol: formic acid: water (25:10:5:1:15, v/v/v/v/v). When the mobile phase reached 1 cm from the top of the plate, the plate was removed and dried at room temperature. The plate was sprayed using a solution of orcinol reagent (Appendix B), heated at 100°C in oven for 5 -7 min and viewed under a UV detector set at wavelength 360nm (Naidoo, 2010).

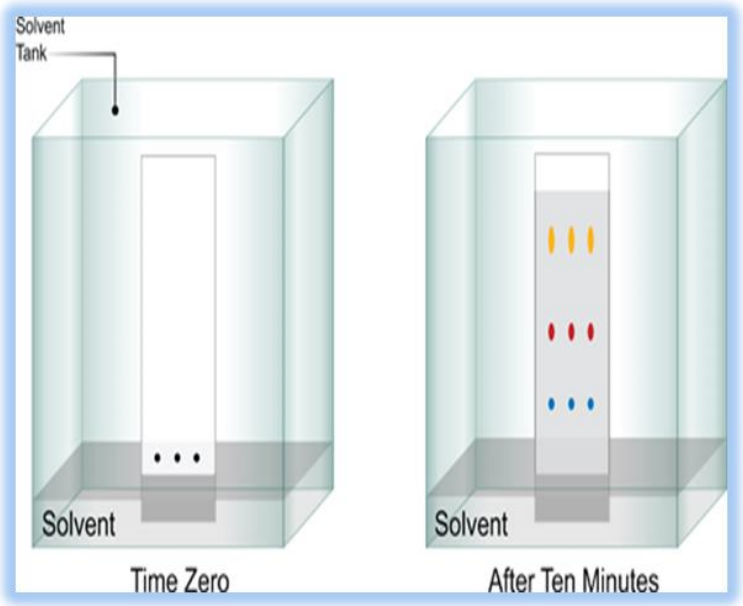


Figure 16: Diagram illustrating migration of compounds during TLC

3.7.2. Identification and quantification of algal oligosaccharide by High Performance Liquid Chromatography

Chromatographic separation of carbohydrates in the algal extracts was performed on a Hi-Plex H (Merck, Gauteng) column. Mobile phase used was 1 mM H_2SO_4 with 0.7 ml/min as flow rate and 10 µl injection volume using a refractive index (RI) detector. Monosaccharide standard solutions of glucose, xylose, glucuronic acid, fructose and galactose were prepared at different concentrations (2 to 10 mg). All the samples were filtered through a 0.22 µm millipore filter before injection.

The monosaccharides in aqueous algal extracts were quantified by comparison with standard curves (Appendix Figure B_1 and $_2$) of monosaccharide standard sugars and using a formula below (Behera et al., 2010).

$$y=mx+c$$

y = peak area

m = slope

x = concentration (mg/ml)

c = intercept

3.8. Statistical analysis

The results of algal extracts that showed improved growth against any single bacterial isolate were taken to the second phase of the experiment. The raw growth data was transformed to log base 10 value (log_{10}) to make highly skewed distributions less skewed and patterns in the data more interpretable The results were expressed as mean and standard deviations of triplicates. One-way analysis of variance (ANOVA) at 95% confidence intervals using GraphPad Prism software (version 6.05, GraphPad, Inc, CA, USA) was used followed by a student t-test for multiple comparisons by Benjamini-Hochberg false discover rate (FDR) procedure in order to compare the log_{10} growth of each bacteria to the negative control. Aqueous algal extracts with $p < 0.05$ were considered statistically significant and regarded as potential prebiotic sources.

4. RESULTS

4.1. Confirmation of algal and probiotic cultures

The algal and probiotic cultures used in this study were all viewed under a light microscope for confirmation. The probiotic cultures were confirmed to be *L. lactis*, *L. bulgaricus* and *B. longum*. Using taxonomic keys, two algal species form CSIR were identified as members of *Tetraselmis* spp. (marine isolates 46, 47 and 48) and a member of *Cylindrospermum* spp. (freshwater isolate 12). Algal species from DUT were confirmed to be *S. platensis*, *D. salina*, *Chlorococcum* spp, *S. magmus* and *Chlorella* spp. The remainder of the algal species was also viewed using the light microscope but were not further investigated in this study due to their minimal effect on growth.

4.2. Prebiotic effect of aqueous algal extracts on the growth of probiotic bacteria

4.2.1. Screening of marine and freshwater algal aqueous extracts for prebiotic activity

The results presented in Figure 17 shows the effect of aqueous algal extracts on probiotic growth over 96 hours. A total of fifty-five aqueous algal extracts were assessed for their effect on the growth of *L. lactis, B. longum* and *L. bulgaricus* over a 96 hour period. Relative to the negative control, 34.5% algal extracts showed improved growth on one or more probiotic bacteria and their effect on each probiotic bacteria is reported. The time exposure for maximum bacterial growth was noted at 48 h for all tested aqueous algal extracts.

4.2.1.1. *Bifidobacterium longum*

Twenty-two percent aqueous algal extracts showed an effect on the growth of the *B. longum* as indicated in Figure 17A. An increased stimulation of *B. longum* was observed

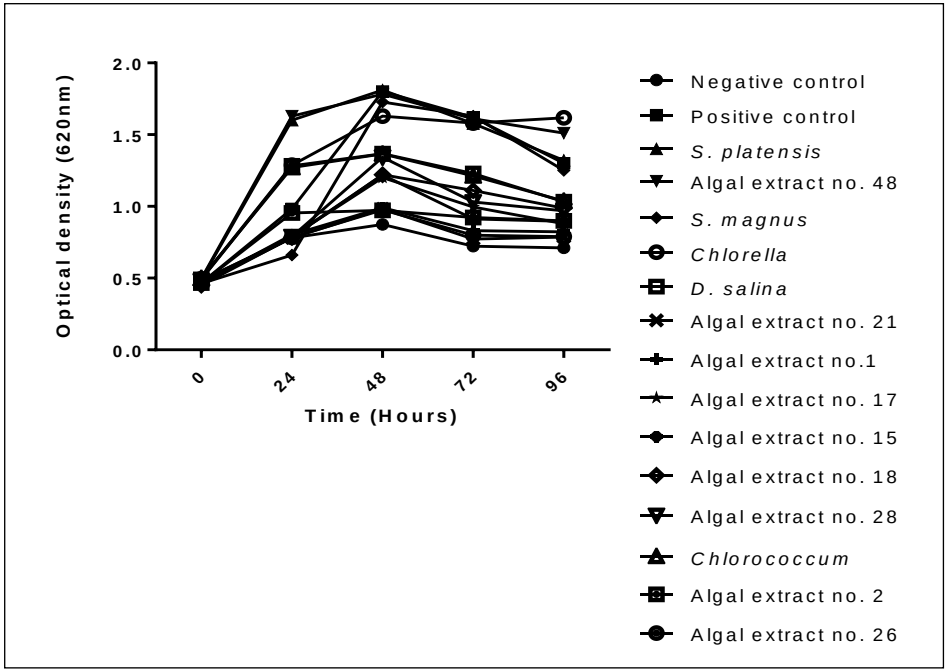
to be much more evident after the 48 hour period. Aqueous algal extracts from *Spirulina platensis* had the highest growth stimulation as compared to the other aqueous algal extracts. Seventy-eight percent aqueous algal extracts showed no significant improvement on the growth of the *B. longum* (Appendix Table B_2). *Spirulina platensis*, *Chlorococcum* and *D. salina* aqueous algal extracts had the highest stimulatory effect on the growth of *B. longum* when compared to the positive control inulin during the 96 hour period.

4.2.1.2. *Lactobacillus delbrueckii* subsp. *lactis*

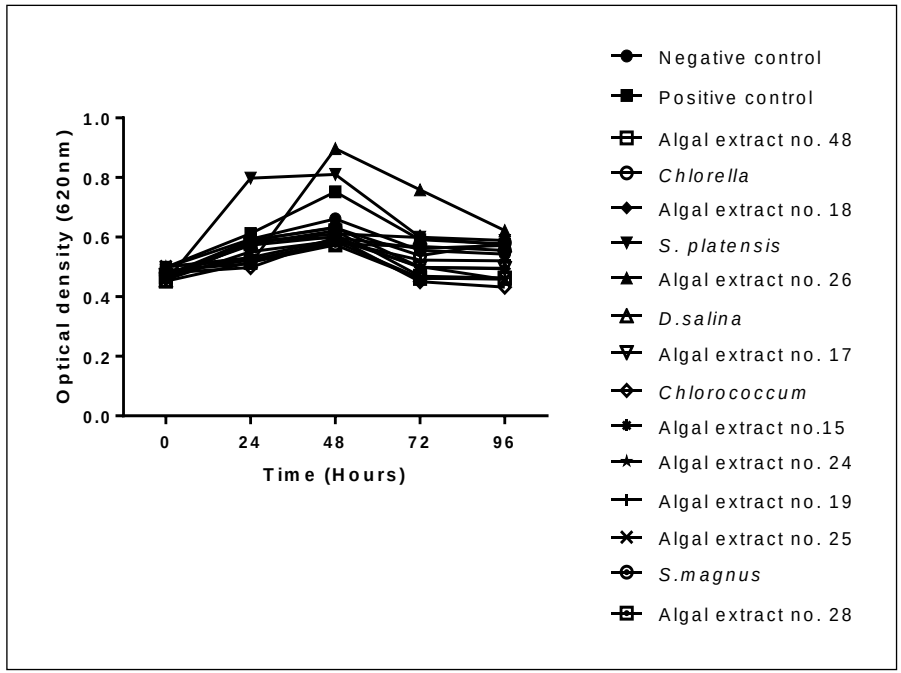
Twenty-five percent aqueous algal extracts namely: aqueous algal extracts from *S. platensis*, *Chlorococcum*, *Chlorella*, *S. magnus*, *D. salina* exerted a stimulatory effect on the growth of *L. lactis* as compared to the negative control as indicated in Figure 17B. Seventy-five percent aqueous algal extracts exerted a lower stimulatory effect on *L. lactis* growth, as the growth was either similar or lower than that of the negative control (Appendix Table B_1). Aqueous algal extracts from *S. platensis, Chlorococcum, S. magnus*, and *D. salina* demonstrated high stimulatory effect on the growth of *L. lactis* when compared to the positive control inulin. This can be deduced from the improvement on *L. lactis* growth over 96 hour period.

4.2.1.3. *Lactobacillus delbrueckii* subsp. *bulgaricus*

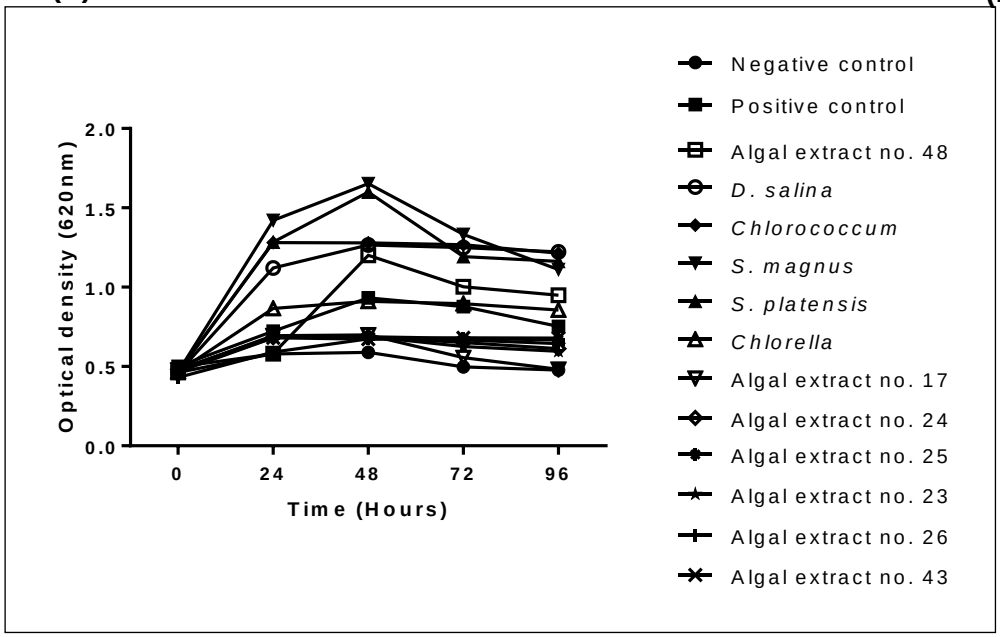
Lactobacillus bulgaricus growth was increased by 22% of tested aqueous algal extracts whereas 78% showed no significant growth improvement (Figure 17C). Aqueous algal extract no. 48, 43, 26, 25, 24, 23, 19, 18, 17 as well as *S. platensis*, *Chlorococcum*, *Chlorella*, *S. magnus* and *D. salina* aqueous algal extracts showed *a* greater stimulatory effect on the growth of *L. bulgaricus* when compared to the negative control. Aqueous algal extracts from *S. platensis, Chlorococcum, S. magnus*, and *D. salina* had a greater stimulatory effect on the growth of *L. bulgaricus* when compared to the prebiotic inulin.



(A)



(B)



(C)

Figure 17: Screening of algal extracts for prebiotic activity over 96 hours.
(A) Growth of *B. longum*; (B) Growth of *L. lactis*; (C) Growth of *L. bulgaricus*

4.2.2. Evaluation prebiotic effect of aqueous algal extracts at 48 hour period

4.2.2.1. *Bifidobacterium longum*

The effect of aqueous algal extract no. 48, 28, 18, 17, 15, 1 as well as *S. platensis, Chlorococcum*, *D. salina*, *S. magnus* and *chlorella* aqueous algal extracts significantly increased the growth of *B. longum* when compared to the negative control ($p < 0.05$) (Appendix Table B_4) as observed in Figure 18. Inulin, used as a positive control, stimulated a high number of *B. longum* cells when compared to all aqueous algal extracts, but *S. plantesis* aqueous algal extract. The initial viable cell numbers of *B. longum* were 3.30 Log CFU/ml and after 48 h incubation with the aqueous algal extract from *S. platensis* and inulin, the growth percentage of *B. longum* increased by 170.3% (8.92±0.004 Log CFU/ml) and 196% (8.89±0.01Log CFU/ml) respectively (Appendix Table B_4). Both aqueous algal extract from *S. magmus* and algal extract no. 48 increased the same number of cells (8.76 Log CFU/ml); indicating that they both had the same effect on *B. longum*.

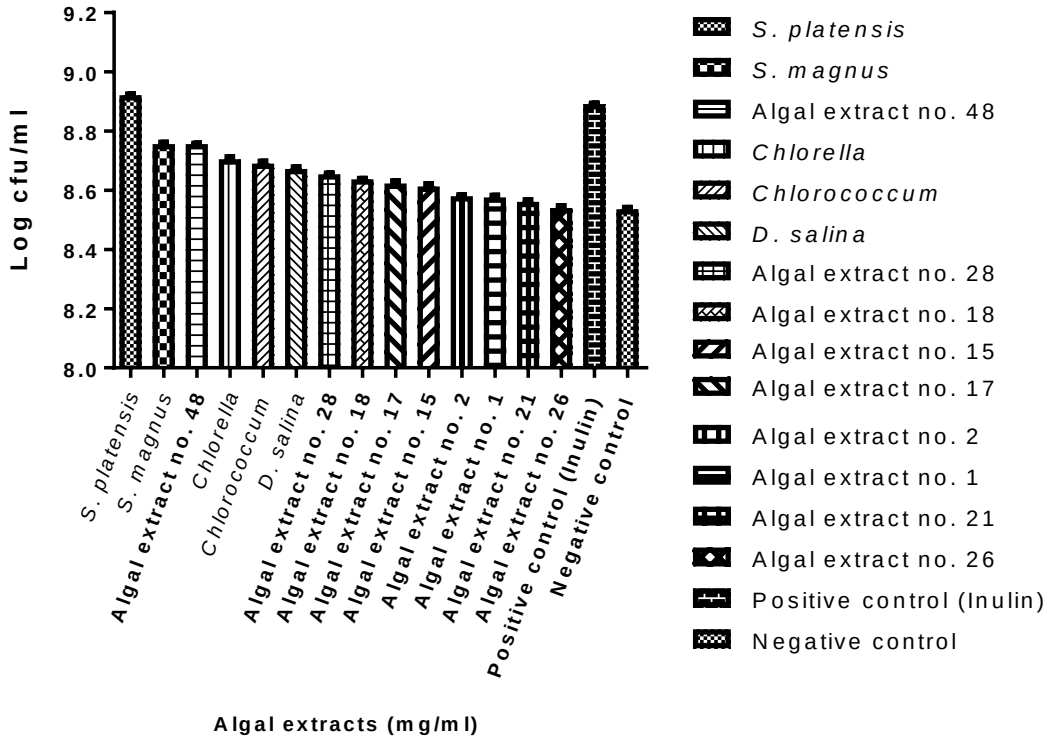


Figure 18: Effect of aqueous algal extracts on *B. longum* after 48 h treatment

Data are given as mean ± S.D for triplicates (Appendix Table B_4)

4.2.2.2. *Lactobacillus delbrueckii* subsp. *lactis*

The effect of aqueous algal extracts from *S. platensis, Chlorococcum*, *D. salina*, *Chlorella, S. magnus* and aqueous algal extract no. 15, 25, 28 and 48 significantly increased the growth of *L. lactis* ($p < 0.05$) (Table B_5) when compared to the negative control as indicated in Figure 19. *Spirulina platensis, Chlorococcum*, *D. salina*, *S. magnus* and *chlorella* aqueous algal extracts had a greater stimulation on probiotic bacterial cells than inulin. *S. platensis* aqueous algal extract stimulated a high number of *L. lactis* cells when compared to all aqueous algal extracts, negative control and inulin. The initial viable cell count of *L. lactis* was 3.30 Log CFU/ml and after 48 h incubation with algal extracts, an increase in growth percentage was observed. The aqueous algal extract from *S. platensis* stimulated *L. lactis* by 172% (8.99±0.004 Log CFU/ml), whereas inulin, used as a positive control stimulated the growth of the same bacterium by 156.7% (8.47±0.03 CFU/ml) (Appendix Table B_5).

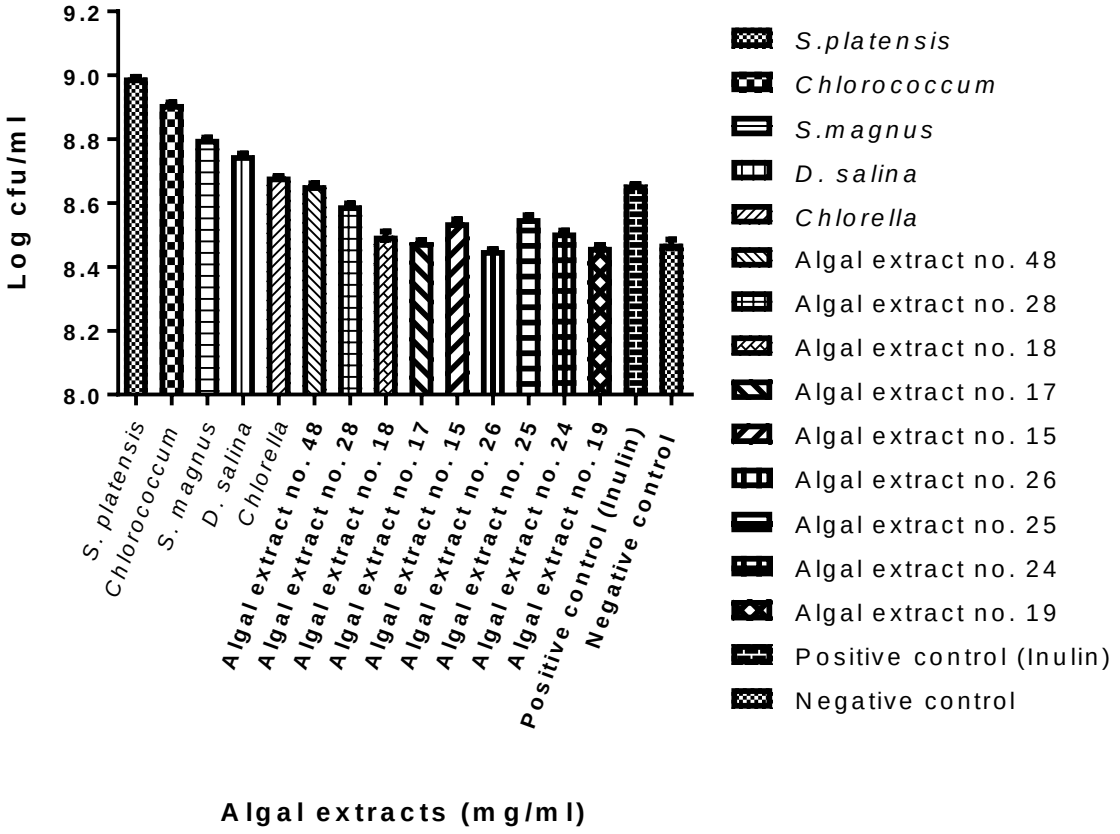


Figure 19: Effect of aqueous algal extracts on *L. lactis* after 48 h treatment

Data are given as mean ± S.D for triplicates (Appendix Table B_5)

4.2.2.3. *Lactobacillus delbrueckii* subsp*. bulgaricus*

Aqueous algal extracts from *S. platensis, Chlorococcum*, *D. salina*, *S. magnus* and *a*queous algal extract no. 48 significantly increased the growth of *L. bulgaricus* ($p < 0.05$) (Appendix Table B_6) when compared to the negative control as shown in Figure 20. Aqueous algal extracts from *Spirulina platensis, S. magnus, D. salina*, *Chlorococcum* and algal extract no. 48 had a greater stimulation of *L. bulgaricus* bacterial cells than the positive control inulin. The aqueous algal extract from *D. salina* stimulated a high number of *L. bulgaricus* cells when compared to all aqueous algal extracts including the positive control inulin and the negative control. The initial viable cell count of *L. bulgaricus* was 3.30 Log CFU/ml and after 48 h incubation with the algal extract from *S. magnus*, growth percentage increased by 168% (8.83±0.01 Log CFU/ml), whereas inulin, used as a positive control stimulated the growth of the same bacterium by 162% (8.65±0.05 CFU/ml). Five marine and freshwater algal cultures (*S. platensis*, *Chlorococcum, D. salina, S. magnus, Chlorella*, algal extract no. 48*)* isolated from various aquatic environments in Kwa-Zulu Natal (CSIR) showed the best growth dynamics and demonstrated the greatest potential as sources of biomass for prebiotic production as they were able to significantly increase the growth of at least one of the three probiotic bacteria.

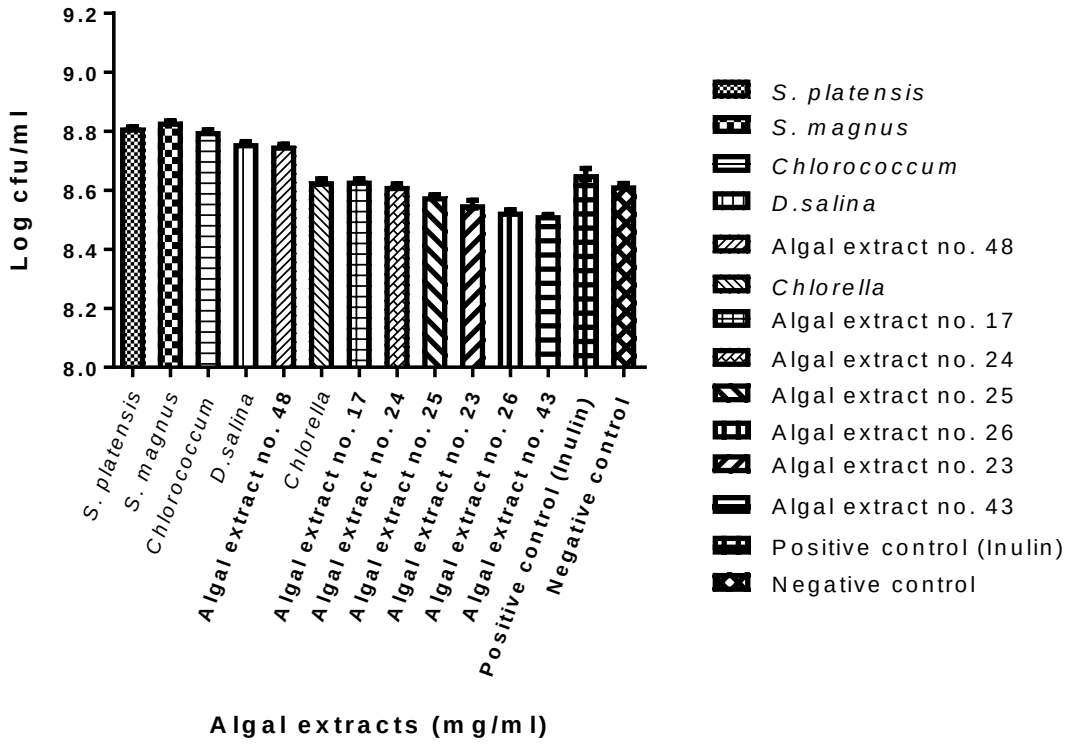


Figure 20: Effect of aqueous algal extracts on L. bulgaricus after 48 h treatment

Data are given as mean ± S.D for triplicates (Appendix Table B_6)

4.3. Characterization of oligosaccharides present in algal extract

4.3.1. Identification of monosaccharide subunits of oligosaccharide by thin layer chromatography

Thin layer chromatography (TLC) profile of hydrolysed aqueous algal extracts shown is shown in Figure 21. Five monosaccharide standards (glucose, xylose, glucuronic acid, fructose and galactose) formed clear banding patterns after derivitization with orcinol reagent and oven treatment. Aqueous algal extracts indicated carbohydrate fractions that comprised the oligosaccharide. These results showed that *S. magnus* (spot 7) and *S. platensis* (spot 8) had xylose and galactose, whereas galactose was detected in aqueous algal extract no. 48 (spot 6) after orcinol treatment. The chromatography did not show separation of glucose, fructose and glucuronic acid. None of the monosaccharide standards used were detected on *Chlorococcum, D. salina* and *Chlorella* algal extracts by TLC.

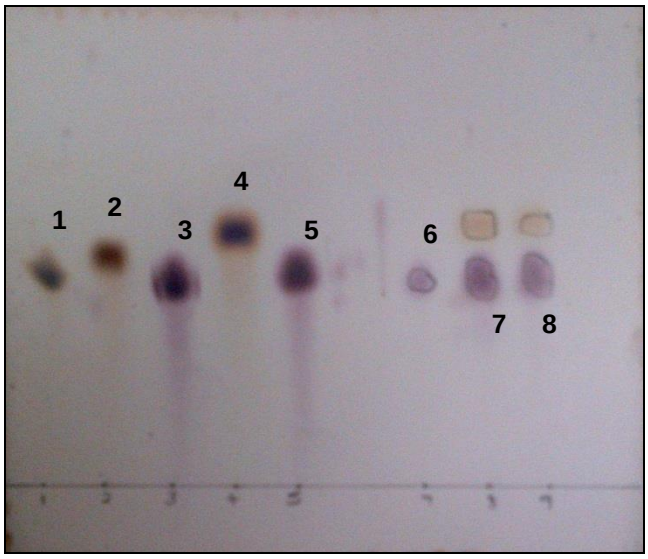


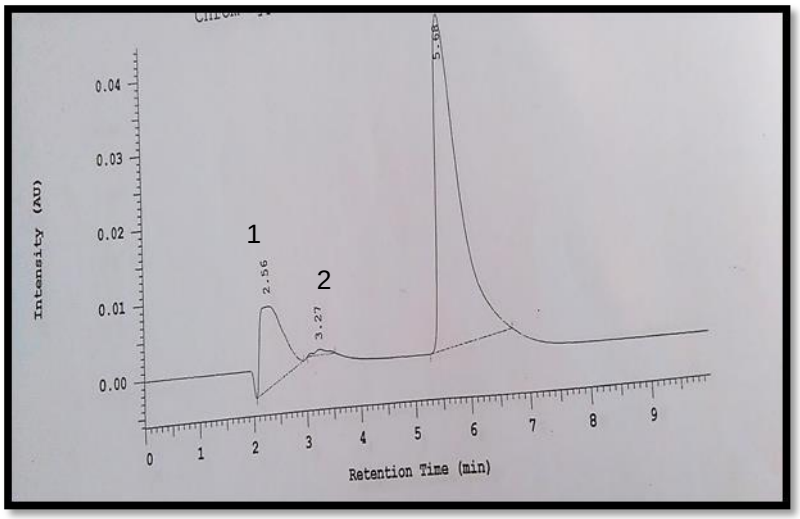
Figure 21: TLC plate of hydrolysed algal extracts compared to five reference carbohydrate standards: 1 - Glucuronic acid; 2 – Fructose; 3 – Galactose; 4 - Xylose; 5- Glucose; 6 – Algal extract 48; 7 - *S. magnus;* 8 - *S. Platensis*

4.3.2. Identification and quantification of algal oligosaccharide by High Performance Liquid Chromatography

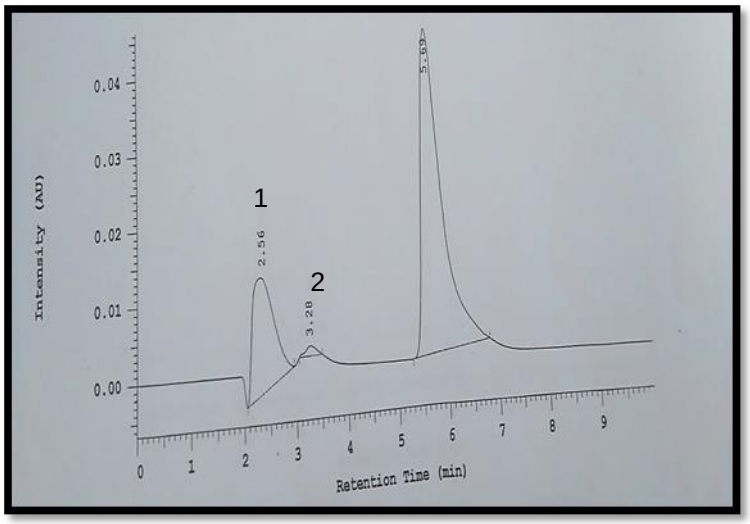
The separation profile of monosaccharides in aqueous algal extracts on the Hi-Plex H column is shown in Figure 22A, B and C. Three peaks were observed and compared with the monosaccharide standards (Appendix Figure B_1) to confirm the monosaccharide identity of peaks. Aqueous algal extracts from *S. magnus* and S. *platensis* contained similar compounds at retention times of 3.28 and 2.56 min. These data indicated that xylose and galactose were present in aqueous algal extracts from *S. magnus* and S. *platensis* and galactose in aqueous algal extract no. 48. Xylose was most abundant in aqueous algal extracts from *S. platensis* (3mg/ml) and *S. magnus* (2.3mg/ml) (Table 5). The chromatography did not show separation of glucose, fructose and glucuronic acid. None of the monosaccharide standards used were detected on *Chlorococcum, D. salina* and *Chlorella* algal extracts by HPLC.

Table 5: Concentration and retention times of hydrolysed oligosaccharides present in algal extracts

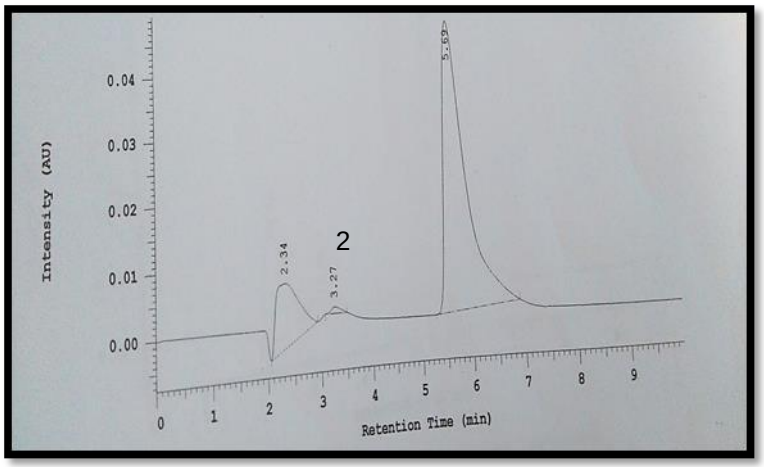
Algal extracts	Concentration (mg/ml)		Retention times (min)	
	Xylose	Galactose	Xylose	Galactose
Spirulina platensis	3.0187	0.1660	2.56	3.28
Scenedesmus magnus	2.2300	0.1020	2.56	3.27
Algal extract no. 48	-	0.0001	-	3.27



(A)



(B)



(C)

Figure 22: Chromatograms of a Hi-Plex H column with RI detection, separation of monosaccharides in aqueous algal extracts. (A) *S. magnus* aqueous algal extract; (B) *S. platensis* aqueous algal extract; (C) Unknown aqueous algal extract No. 48 (Peaks: 1. xylose; 2. galactose)

5. DISCUSSION

5.1. Prebiotic effect of aqueous algal extracts on the growth of probiotic bacteria

In order to demonstrate that lactic acid bacteria can effectively digest prebiotic compounds from algal sources or able to demonstrate bifidogenic effect, this study was carried out by using a batch culture technique containing aqueous algal extracts and probiotic bacteria.

Growth enhancement of tested aqueous algal extracts on each bacterium was established by measuring cell density over 92 h (Figure 17A, B and C), and the maximum growth for each strain was obtained at 48 h. During the initial phase of bacterial growth, termed the lag phase, bacteria undergo intracellular alterations in response to the new environmental or cultural conditions they find themselves exposed to (Yates and Smotzer, 2007). The length of the lag phase is thus related to the time taken for and the manner with which the probiotic bacteria adapt to a new environment. Many factors influence the duration of lag time, including inoculum size, the physiological history of the cells, and the precise physiochemical environment of both the original and the new growth medium (Rolfe et al., 2012; Swinnen et al., 2004). Such a theoretical model assisted in providing an insight into the stimulatory effect on probiotic bacteria due the presence of aqueous algal extracts. Due to various intracellular changes and adaptations, probiotic bacteria were still adjusting to their metabolism to enable degradation of complex oligosaccharides present in each aqueous algal extract during the lag phase. However some probiotic bacteria displayed a shorter lag phase as compared to other strains as a result of oligosaccharides present within algal cell structure. Aqueous algal extract comprising the oligosaccharide with longer degree of polymerization and slower fermentation rate enable byproducts produced to create an environment that is more favorable for the probiotic bacteria to utilize the oligosaccharide in the algal extract, thus enhancing bacterial growth. Aqueous algal extracts with longer lag phase than others indicate that the aqueous algal extract took

longer to adapt to the environment. Van De Wiee et al. (2007) reported that oligosaccharides with slower fermentation rate and longer degree of polymerization have higher prebiotic potency and beneficially influence the microbial community at both the proximal and distal regions of the colon.

During the exponential growth phase (20 – 50 hrs), aqueous algal extracts displayed a clear prebiotic effect on probiotic bacterial growth (Figure 17A, B and C). Sierra et al. (2014) define the prebiotic effect as ‘‘stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health”. During this phase, the probiotic bacteria undergo rapid cell division, utilizing growth substances and nutrients in the growth medium for the purposes of metabolism (Yates and Smotzer, 2007). Algal species contains carbohydrates (De Philippis et al. 2001), therefore the carbohydrates from algal extracts provided an additional source of nutrients for conversion to energy required for the probiotic growth and metabolism, resulting in stimulation of probiotic bacterial growth (Behera and Varma, 2016).

During the death phase of probiotic bacterial growth (51 – 96 hrs), the growth of probiotic bacteria declines in the presence of either aqueous algal extracts or inulin (Figure 17A, B and C). This was a result of high nutrients in aqueous algal extracts being consumed by the probiotic bacteria during the exponential phase, with little or no nutrient material left for subsequent growth stages. In some instances in the latter phase of the probiotic bacterial growth, after a decline of bacterial cells was observed, a slight increase in cell numbers for very few hours occurred but then declined thereafter. This can be explained by the fact that these probiotic bacteria tried to recover and begin to multiply, but failed to do so because of nutrient depletion, accumulation of inhibitory end products and lack of biological space (Madigan et al., 2003).

5.1.1. Evaluation of prebiotic effect of aqueous algal extracts at 48 hour period

Aqueous algal extracts that exerted a greater stimulatory effect, i.e., by significantly increasing the growth of tested probiotic bacteria at 48 h were *S. platensis* on all three probiotic bacteria; *D. salina*, *S. magus, Chlorococcum* and *Chorella* on *L. lactis; D. salina*, *S. magus, Chlorococcum* and algal extract no. 48 on *L. bulgaricus* ($p < 0.05$) (Figure 20). These aqueous algal extracts significantly increased the growth of probiotic bacteria when compared to inulin, and were regarded as exhibiting prebiotic activity. A key and surprising observation in this study is the poor utilisation of inulin as a carbon source by probiotic bacteria given the widespread use of inulin as a prebiotic in commercial preparations. By contrast *S. platensis* algal extract was more utilised by probiotics both *Lactobacillus* and *Bifidobacteria* (Figure 18, 19 and 20). A study conducted by Adebola et al. (2014), also indicated that inulin is poorly utilised by *Lactobacilli* spp. as a carbon source yielding less growth of probiotic bacteria.

Aqueous algal extracts from *S. platensis, S.magnus, Chlorococcum, D.salina* and algal extract no. 48 had significant effect on the growth of *L. lactis, B. longum* and *L. bulgaricus* when compared to the negative control (Figure 18, 19 and 20). Optimum growth was only achieved with specific combinations of probiotics and prebiotics as not all aqueous algal extracts tested increased the growth of probiotic bacteria. Aqueous algal extract from *S. platensis* was recognized as the best prebiotic source as it demonstrated a greater stimulatory effect on the growth *L. lactis* and *L. bulgaricus* in comparison to the positive control inulin (Figure 19 and 20). Bhowmik et al. (2007) also found that the growth of lactic acid bacteria was enhanced in the presence of *S. platensis*. *Spirulina platensis* biomass consumes nitrogen from the growth medium and releases extracellular carbohydrates and other growth substances which, may be responsible for stimulating growth of Lactobacilli

(and other lactic acid-producing strains) (Parada et al., 1998). Therefore *S. platensis* can potentially replace inulin (from chicory) as a prebiotic.

Twenty-one percent aqueous algal extracts showed stimulatory effect on the growth of *B. longum*, *L. lactis* and *L. bulgaricus* as probiotic bacterial growth was enhanced; indicating that the algal extracts provided an additional source of nutrients for conversion to energy required for probiotic bacterial growth and metabolism. Inulin appears to have a greater stimulatory effect on the growth of probiotic when compared to most aqueous extracts prepared from algal species obtained from CSIR, especially on the growth of *B. longum* where all aqueous algal extracts except for *S. platensis* had a lower stimulatory effect (Figure 20). This highly defined prebiotic effect is consistent with growth results obtained by Pompei et al. (2008) wherein, the effect of inulin-type oligofructans on stimulating growth of Bifidobacteria was seen. In the same study, inulin and other highly soluble oligofructans significantly enhanced the growth of Bifidobacterial spp., including *B. longum, B. infants, B. adolescentis* and *B. breve* in the presence of inulin. Due to production of algal toxins and probiotic antimicrobial substances, 12.9% aqueous algal extracts had no stimulatory effect the growth of probiotic bacteria during the 48 h period. This resulted in cell death or at best inhibited of probiotic bacterial growth such as aqueous algal extract no. 26 on the growth of all three probiotic bacteria (Figure 18, 19 and 20). The results obtained showed that inulin was the best prebiotic compound for stimulation of *B. longum,* whereas *S. platensis* aqueous algal extract was found to be the best prebiotic algal source for the stimulation *L. lactis*, *B. longum* and *L. bulgaricus.*

5.2. Characterization of oligosaccharides in algal extracts

5.2.1. Identification and quantification of monosaccharide subunits of oligosaccharide by TLC and HPLC

Monosaccharides of the oligosaccharide fraction from marine and freshwater algal extracts were investigated using TLC and HPLC after acidic hydrolysis of cell matrix polysaccharides. De Philippis et al. (2001) reported that polysaccharides that are commonly found in algae are consisting of glucose, rhamnose, galactose, xylose, glucuronic acid. However galactose and xylose were the only monosaccharides detected in the algal extracts.

The presence of both xylose and galactose were observed in spots identified in *S. platensis* and *S. magnus* extracts, whereas for algal extract no. 48, only galactose was identified (Figure 21). The results obtained by HPLC for characterization confirmed TLC data, as xylose and galactose were detected by both chromatograms (Figure 21 and 22). The method used allowed for the detection of xylose and galactose, however glucose, fructose and glucuronic acid were not detected. The poor separation makes this method unsuitable for algal biomass derived monosaccharide quantification, also the poor resolution can cause misidentification of the monosaccharides found in microalgae.

The low concentration of xylose and galactose in the *S. platensis* extract supports the report by Mikheiskaya et al. (1983) that galactose, mannose and xylose sugar units generally exist in very low quantities as part of the lipopolysaccharide. However it was anticipated that the concentration of these monosaccharides is much higher than what was detected in all three algal extracts (Table 5), but was insufficiently hydrolyzed with TFA hydrolysis conditions used. Since polysaccharides released by algae are complex and requires thorough hydrolysis for more efficient separation, a harsher HCl hydrolysis procedure would aid with this and subsequent quantification. A parametric study of

different hydrolysis conditions and acids is ongoing and will be reported in a future study. The use of a sonication procedure was found to be effective in polysaccharide extraction for its shorter time and higher yields and may be potentially useful for mass production of algal polysaccharides.

The monosaccharides identified in these three algal extracts, i.e., *S. platensis* and *D. salina* and algal extract no 48 from various equatic environments in Kwa-Zulu Natal are a result of enhanced bacterial growth of probiotic bacteria. These algal extracts demonstrated the greatest potential as sources of biomass for prebiotic production. Therefore algal polysaccharides present a great potential for emergent prebiotics to be used directly, in the case of microalgae, or as dried biomass or nutraceuticals, after extraction from the biomass or from the culture medium. They may be included in food and/or feed, or administered as pills, for example. This enables us to tune these polysaccharides and produce novel prebiotics.

6. CONCLUSION

Polysaccharides are regarded as key ingredients for the production of bio-based materials in life sciences (e.g., medical devices, pharmaceutics, food, cosmetics). There are an enormous variety of polysaccharides that can be synthetized and/or released by marine macroalgae and microalgae. Both these organisms are excellent sources of polysaccharides. The characteristics of some algal polysaccharides described above and the experimental results indicate that marine and freshwater algae-derived oligosaccharides can be considered an exciting and promising source of prebiotic production, but further investigations are imperative. In these *in vitro* anaerobic experiments there is clear evidence that not all symbiotic combinations may result in an increased probiotic survival and growth. It was concluded that, each prebiotic and probiotic is strain-specific or oligosaccharide specific.

Our results showed that HPLC was able detect and accurately quantify 2 out of 5 sugars (glucuronic acid was not derivatized or detected under the reaction conditions tested). The inability to detect glucuronic acid limits complete carbohydrate quantification, as algal cells can contain large quantities of glucuronic acids. These chromatographic tools have allowed characterization of monosaccharides released after TFA hydrolysis of *S. platensis*, *S. magnus* and algal extract no. 48. Further work in structural carbohydrate release, either by chemical or enzymatic hydrolysis, will share light not only on the total carbohydrate content in these tested algae, but also on the structural understanding of algal polymeric carbohydrates. This knowledge can be applied to conversion and fermentation experimental work as well as guide research into cell wall degradation procedures to aid the release of soluble sugars.

Thus, aqueous algal extracts from *S. platensis*, *Chlorococcum, D. salina, S. magnus, Chlorella* and algal extract no. 48 are potential sources for prebiotic production. *Spirulina*

platensis extract was regarded as the best algal source for prebiotic as it had a greater stimulatory effect on the growth of all three probiotic bacteria (*L. lactis*, *B. longum* and *L. bulgaricus*). Xylose and galactose characterized by HPLC in algal extracts make up oligosaccharides that function as prebiotic compounds for stimulation of probiotic bacteria. There is a great scope for successful production of prebiotics from algal sources in South Africa.

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8. APPENDICES

APPENDIX A: METHODOLODY

AF6 media preparation

All the ingredients except the vitamin stock solution were added to distilled water (dH_2O) to make 100 ml. The medium was autoclaved at 121°C for 15 min and allowed to cool. Prior to being added into the medium, the vitamin stock was filter sterilized.

Table A_1: AF6 media composition

Compound	Quality used (g/L)
Fe-citrate	0.002
Citric Acid	0.002
$NaNo_3$	0.14
NH_4NO_3	0.022
$MgSO_4.7H_2O$	0.03
KH_2PO_4	0.01
KH_2PO_4	0.005
$CaCl_2$	0.01
Bacteriological Agar	15
Trace Metal Solution	1 ml
Vitamin Solution (pH 6.6)	1 ml
Distilled water	Up to 1000ml

Table A_2: Trace metal stock solution

Compound	Quality Used (g/L
$FeCl_3.6H_2O$	0.98
$ZnSO_4.7H_2O$	0.11
$CoCl_2.6H_2O$	0.02
$MnCl_2.4H_2O$	0.18
$Na_2MoO_4.2H_2O$	0.01
$Na_2EDTA.2H_2O$	5

Table A_3: Vitamin stock solution

Compound	Quality used (mL)
B-12 (0.0114 g/10 ml)	1
Biotin (0.0104 g/100 ml)	1
Thiamine-HCl	200

ASW media preparation

Each of anhydrous and hydrous salts were dissolved separately in 300 ml of dH_20 and these two solutions were then be combined (dissolved while continuously mixing using a magnetic stirrer).

To this solution each nutrient was added and 0.5 ml of trace metal stock solutions and 0.5 ml of Fe/EDTA stock was added. This was autoclaved at 121°C for 15min. Thereafter the vitamin solution was added after sterilization to avoid degradation of the vitamins.

Table A_4: Hydrous and anhydrous salt solutions

Compound anhydrous salts	**Unit**	**50%ASW (ASP)**	**Concentration in final medium (M)**
Nacl	g/l	0.98	
$ZnSO_4.7H_2O$	g/l	0.11	
$CoCl_2.6H_2O$	g/l	0.02	
$MnCl_2.4H_2O$	g/l	0.18	
$Na_2MoO_4.2H_2O$	g/l	0.01	
$Na_2EDTA.2H_2O$	g/l	5	
Compound	**Unit**	**50%ASW (ASP)**	**Concentration in final medium (M)**
Anhydrous salts			
NaCl	g/l	13.98	2.39×10^{-1}
KCl	g/l	0.39	9.97×10^{-3}
$NaHCO_3$	g/l	0.2	2.38×10^{-3}
H_3BO_3	g/l	0.6	9.71×10^{-4}
Hydrous salts			
$MgCl_2.6H_2O$	g/l	2.6	1.28×10^{-2}
MgSO4.$7H_2O$	g/l	3.56	1.45×10^{-2}
$CaCl_2.H_2O$	g/l	0.77	5.24×10^{-3}

Table A_5: Major nutrient stock preparation

Compound	**Stock solution**	**Quantity to be used**	**50%ASW (ASP)**	**Concentration in final Medium (M)**
	(g/l dH_2O)		(g/l)	
$Na2SiO_3.9H_2O$	60.82	5 ml	0.3	1.05 x 10-3
$NaNO_3$	149.6	1 ml	0.14	1.65 x 10-3
$NaH2PO_4$	8.8	0.5 ml	0.04	3.33 x 10-4

Table A_6: Trace metal stock preparation

Compound	g/200 ml
$CuSO4.5H_2O$	3.92
$ZnSO4.7H_2O$	8.8
$CoCl2.6H_2O$	4
$MnCl2.4H_2O$	72
$Na2MoO4.2H_2O$	2.52
$Na2EDTA.2H_2O$	0.41

Individual 200 ml stocks of the first components were prepared; thereafter 250 microlitres of each of stocks was added to 200 ml water. 0.41g of Na_2EDTA was added and this solution was boiled for 3 min and thereafter cooled at room temperature. The volume was adjusted to 205 ml. Half a ml of this solution was added to all media formulated. EDTA was stored away from light at 4°C

Table $A_{7:}$ EDTA stock preparation

Compound	g/L
$FeC6H5O7$	5.71
$Na2EDTA.2H_2O$	17.35

Ingredients will be dissolved in 800 ml of dH_2O and neutralized using 5 N NaOH. The stock will be boiled to dissolve compounds, cooled, and then adjust to a volume of 1L. 0.5 ml of this solution will be added to all media formulated.

Table $A_{8:}$ Preparation of vitamin stock

Compound	Quantity (mg/L)
Thiamine	200
B-12	1

Primary stocks of B-12 (11.4 mg/ml) and biotin (10.4 mg/100 ml) were initially prepared. The biotin mixture required the pH to be adjusted to 10 in order to dissolve. All stocks were adjusted to pH 4.5 – 5.0 using 5 N sodium hydroxide (NaOH) and made up to final volume of 1L and stored at 4°C. The vitamin stock was prepared by adding: 1 ml of biotin

stock; 0.1 ml B-12 primary stock, and 20 mg thiamine HCl in 100 ml water. 0.5ml of this solution was added to all media formulated.

Table A_9: Zarrouks media preparation (Parada et al., 1998)

Compound	**Quantity (g/L)**
$NaHCO_3$	16.8
KH_2PO_4	0.5
$NaNO_3$	2.5
K_2SO_4	1
$MgSO_4.7H_2O$	0.2
NaCl	1
$CaCl_2$	0.04
$FeSO_4.7H_2O$	0.01
$EDTA.Na_2$	0.08
Trace metals	4 ml
Trace metals	g/l
H_3BO_3	2.86
$MnCl2.4H_2O$	1.13
$ZnSO_4.7H_2O$	0.222
$CuSO_4.5H_2O$	0.079
$NaMoO_4.5H_2O$	0.39
$CO(NO_3)_2.6H_2O$	0.049

Preparation of MRS broth

MRS broth was prepared by suspending 50 g of MRS broth in one litre of distilled water and allowed to stand for 15 min to allow the media to dissolve. After the media had dissolved, it was sterilized by autoclaving at 121°C for 15 min. After the media had cooled sufficiently, it was used immediately.

Preparation of MRS agar

MRS Agar was prepared by suspending 74.5 g of MRS Agar in one litre of distilled water and boiled until the media had completely dissolved; it was sterilized by autoclaving at 121°C for 15 min. After the media had cooled, it was poured into Petri dishes and allowed to set. The plates were then stored at 4°C.

Preparation of orcinol reagent

Eighty mg of Orcine-Monohydrate (Sigma) was dissolved in 160 ml acetone and 8 ml of concentrated sulphuric acid was added.

APPENDIX B: RESULTS

Table B_1: Spectrophotometric measurements of the algal extracts on *L. lactis.*

Time (Hours)	0	24	48	72	96
Negative control	0.498	0.593	0.661	0.555	0.543
Positive control	0.499	0.612	0.752	0.591	0.577
S. platensis	0.452	0.798	0.81	0.6	0.589
Chlorella	0.472	0.59	0.631	0.538	0.581
D. salina	0.455	0.583	0.611	0.599	0.573
Chlorococcum	0.4800	0.498	0.599	0.45	0.432
S. magnus	0.483	0.523	0.577	0.569	0.555
Algal extract no.1	0.488	0.61	0.619	0.594	0.584
Algal extract no.2	0.479	0.601	0.675	0.597	0.588
Algal extract no.3	0.466	0.593	0.694	0.523	0.493
Algal extract no.4	0.485	0.617	0.703	0.578	0.568
Algal extract no.5	0.461	0.611	0.600	0.547	0.533
Algal extract no.6	0.473	0.598	0.822	0.573	0.567
Algal extract no.7	0.457	0.493	0.593	0.472	0.47
Algal extract no.8	0.456	0.501	0.582	0.463	0.462
Algal extract no.9	0.455	0.522	0.578	0.465	0.463
Algal extract no.10	0.456	0.562	0.577	0.511	0.506
Algal extract no.11	0.459	0.580	0.590	0.500	0.597
Algal extract no.12	0.451	0.593	0.611	0.475	0.470
Algal extract no.13	0.467	0.577	0.621	0.469	0.463
Algal extract no.14	0.478	0.567	0.584	0.467	0.462
Algal extract no.15	0.466	0.589	0.599	0.562	0.572
Algal extract no.16	0.457	0.587	0.623	0.584	0.580
Algal extract no.17	0.472	0.573	0.601	0.498	0.495
Algal extract no.18	0.458	0.589	0.633	0.499	0.494
Algal extract no.19	0.457	0.551	0.587	0.523	0.520
Algal extract no.20	0.469	0.573	0.593	0.561	0.559
Algal extract no.21	0.521	0.574	0.584	0.562	0.559
Algal extract no.22	0.453	0.573	0.584	0.562	0.458
Algal extract no.23	0.458	0.578	0.582	0.564	0.455
Algal extract no.24	0.500	0.531	0.593	0.501	0.458
Algal extract no.25	0.501	0.523	0.585	0.463	0.459
Algal extract no.26	0.488	0.511	0.897	0.759	0.622
Algal extract no.27	0.493	0.525	0.588	0.464	0.460
Algal extract no.28	0.452	0.515	0.574	0.461	0.461
Algal extract no.29	0.498	0.593	0.661	0.555	0.543
Algal extract no.30	0.499	0.612	0.752	0.591	0.577
Algal extract no.31	0.452	0.798	0.810	0.600	0.589
Algal extract no.32	0.472	0.59	0.631	0.538	0.581
Algal extract no.33	0.455	0.583	0.611	0.599	0.573

Algal extract no.34	0.480	0.498	0.599	0.450	0.432
Algal extract no.35	0.483	0.523	0.577	0.569	0.555
Algal extract no.36	0.488	0.61	0.619	0.594	0.584
Algal extract no.37	0.479	0.601	0.675	0.597	0.588
Algal extract no.38	0.466	0.593	0.694	0.523	0.493
Algal extract no.39	0.485	0.617	0.703	0.578	0.568
Algal extract no.40	0.461	0.611	0.600	0.547	0.533
Algal extract no.41	0.473	0.598	0.822	0.573	0.567
Algal extract no.42	0.457	0.493	0.593	0.472	0.470
Algal extract no.43	0.456	0.501	0.582	0.463	0.462
Algal extract no.44	0.455	0.522	0.578	0.465	0.463
Algal extract no.45	0.456	0.562	0.577	0.511	0.506
Algal extract no.46	0.459	0.580	0.590	0.500	0.597
Algal extract no.47	0.451	0.593	0.611	0.475	0.470
Algal extract no.48	0.467	0.577	0.621	0.469	0.463
Algal extract no.49	0.478	0.567	0.584	0.467	0.462
Algal extract no.50	0.466	0.589	0.599	0.562	0.572

Table $B_{2:}$ Spectrophotometric measurements of the algal extracts on *B. longum*

Time (Hours)	0	24	48	72	96
Negative control	0.462	0.781	0.874	0.722	0.711
Positive control	0.466	0.982	1.801	1.621	1.301
S. platensis	0.49	1.6	1.811	1.577	1.32
Chlorella	0.495	1.288	1.629	1.581	1.617
D. salina	0.491	1.283	1.367	1.229	1.033
Chlorococcum	0.499	1.271	1.366	1.212	1.038
S. magnus	0.463	0.66	1.728	1.63	1.251
Algal extract no.1	0.463	0.802	0.991	0.831	0.822
Algal extract no.2	0.465	0.955	0.973	0.923	0.899
Algal extract no.3	0.499	0.972	0.989	0.978	0.939
Algal extract no.4	0.467	0.938	0.964	0.921	0.878
Algal extract no.5	0.466	0.953	0.964	0.949	0.922
Algal extract no.6	0.466	0.949	0.953	0.937	0.925
Algal extract no.7	0.469	0.957	0.969	0.942	0.933
Algal extract no.8	0.463	0.969	0.971	0.961	0.753
Algal extract no.9	0.471	0.966	0.971	0.952	0.934
Algal extract no.10	0.461	0.967	0.975	0.952	0.951
Algal extract no.11	0.477	0.988	0.991	0.922	0.977
Algal extract no.12	0.483	0.881	0.891	0.879	0.869
Algal extract no.13	0.491	0.873	0.911	0.87	0.869
Algal extract no.14	0.483	0.79	0.887	0.782	0.78
Algal extract no.15	0.472	0.789	1.22	0.913	0.895
Algal extract no.16	0.477	0.784	0.883	0.737	0.71

Algal extract no.17	0.462	0.79	1.201	0.995	0.879
Algal extract no.18	0.45	0.784	1.221	1.111	0.988
Algal extract no.19	0.55	0.785	0.894	0.794	0.812
Algal extract no.20	0.493	0.788	0.923	0.81	0.815
Algal extract no.21	0.486	0.78	0.982	0.772	0.786
Algal extract no.22	0.481	0.783	0.97	0.753	0.742
Algal extract no.23	0.463	0.787	0.985	0.762	0.753
Algal extract no.24	0.477	0.782	0.899	0.787	0.798
Algal extract no.25	0.455	0.786	0.962	0.821	0.808
Algal extract no.26	0.467	0.784	0.976	0.799	0.789
Algal extract no.27	0.471	0.791	0.953	0.754	0.75
Algal extract no.28	0.481	0.789	1.342	1.031	0.969
Algal extract no.29	0.471	0.455	0.478	0.453	0.451
Algal extract no.30	0.463	0.462	0.522	0.452	0.451
Algal extract no.31	0.465	0.482	0.588	0.473	0.462
Algal extract no.32	0.468	0.494	0.593	0.484	0.463
Algal extract no.33	0.473	0.483	0.49	0.472	0.469
Algal extract no.34	0.471	0.492	0.523	0.478	0.472
Algal extract no.35	0.466	0.458	0.563	0.455	0.45
Algal extract no.36	0.473	0.49	0.497	0.477	0.468
Algal extract no.37	0.469	0.534	0.593	0.493	0.482
Algal extract no.38	0.475	0.478	0.524	0.462	0.459
Algal extract no.39	0.473	0.499	0.573	0.467	0.457
Algal extract no.40	0.476	0.562	0.582	0.459	0.457
Algal extract no.41	0.469	0.551	0.561	0.498	0.485
Algal extract no.42	0.476	0.491	0.564	0.476	0.466
Algal extract no.43	0.465	0.653	0.688	0.591	0.489
Algal extract no.44	0.463	0.663	0.674	0.559	0.482
Algal extract no.45	0.472	0.667	0.675	0.568	0.493
Algal extract no.46	0.481	0.669	0.679	0.599	0.477
Algal extract no.47	0.45	0.665	0.678	0.572	0.469
Algal extract no.48	0.495	1.629	1.783	1.61	1.511
Algal extract no.49	0.490	0.660	0.678	0.577	0.491
Algal extract no.50	0.495	0.671	0.674	0.565	0.499

Table B_3: Spectrophotometric measurements of algal extracts on *L. bulgaricus*

Time (Hours)	0	24	48	72	96
Negative control	0.498	0.58	0.59	0.498	0.478
Positive control	0.5	0.723	0.933	0.877	0.752
S. platensis	0.463	1.284	1.599	1.192	1.163
Chlorella	0.468	0.865	0.911	0.896	0.856

D.salina	0.461	1.12	1.266	1.249	1.222
Chlorococcum	0.465	1.28	1.279	1.268	1.214
S.magnus	0.466	1.421	1.652	1.332	1.111
Algal extract no.1	0.452	0.481	0.491	0.452	0.417
Algal extract no.2	0.477	0.489	0.599	0.466	0.452
Algal extract no.3	0.451	0.533	0.671	0.482	0.433
Algal extract no.4	0.493	0.549	0.554	0.501	0.482
Algal extract no.5	0.485	0.492	0.539	0.481	0.473
Algal extract no.6	0.452	0.478	0.525	0.485	0.472
Algal extract no.7	0.499	0.511	0.573	0.492	0.461
Algal extract no.8	0.488	0.602	0.616	0.529	0.457
Algal extract no.9	0.478	0.493	0.534	0.444	0.453
Algal extract no.10	0.457	0.489	0.604	0.455	0.463
Algal extract no.11	0.467	0.471	0.576	0.478	0.459
Algal extract no.12	0.452	0.457	0.557	0.462	0.451
Algal extract no.13	0.452	0.455	0.492	0.423	0.403
Algal extract no.14	0.491	0.593	0.599	0.511	0.401
Algal extract no.15	0.465	0.582	0.583	0.534	0.452
Algal extract no.16	0.481	0.671	0.674	0.572	0.423
Algal extract no.17	0.477	0.694	0.699	0.555	0.482
Algal extract no.18	0.494	0.622	0.634	0.6	0.481
Algal extract no.19	0.454	0.611	0.677	0.67	0.485
Algal extract no.20	0.452	0.623	0.623	0.592	0.461
Algal extract no.21	0.467	0.666	0.642	0.632	0.477
Algal extract no.22	0.49	0.678	0.673	0.566	0.454
Algal extract no.23	0.478	0.691	0.689	0.625	0.596
Algal extract no.24	0.483	0.684	0.687	0.651	0.612
Algal extract no.25	0.4732	0.682	0.687	0.677	0.668
Algal extract no.26	0.431	0.591	0.674	0.668	0.644
Algal extract no.27	0.46	0.577	0.582	0.511	0.463
Algal extract no.28	0.438	0.577	0.58	0.573	0.473
Algal extract no.29	0.498	0.584	0.589	0.555	0.552
Algal extract no.30	0.479	0.573	0.592	0.56	0.564
Algal extract no.31	0.469	0.584	0.595	0.57	0.573
Algal extract no.32	0.477	0.592	0.596	0.581	0.584
Algal extract no.33	0.466	0.579	0.589	0.564	0.566
Algal extract no.34	0.468	0.589	0.591	0.575	0.578
Algal extract no.35	0.489	0.594	0.601	0.584	0.586
Algal extract no.36	0.47	0.583	0.59	0.572	0.57
Algal extract no.37	0.475	0.594	0.597	0.58	0.578
Algal extract no.38	0.467	0.595	0.599	0.585	0.582
Algal extract no.39	0.481	0.58	0.598	0.579	0.577
Algal extract no.40	0.466	0.586	0.594	0.578	0.573
Algal extract no.41	0.471	0.587	0.592	0.569	0.562
Algal extract no.42	0.475	0.588	0.59	0.579	0.575

Algal extract no.43	0.465	0.681	0.673	0.681	0.679
Algal extract no.44	0.469	0.594	0.589	0.499	0.482
Algal extract no.45	0.493	0.582	0.59	0.501	0.491
Algal extract no.46	0.473	0.573	0.594	0.511	0.497
Algal extract no.47	0.484	0.578	0.601	0.504	0.484
Algal extract no.48	0.462	0.581	1.201	1.003	0.949
Algal extract no.49	0.493	0.585	0.599	0.498	0.488
Algal extract no.50	0.488	0.593	0.595	0.505	0.481

Table $B_{4:}$ The effect of algal extracts on the growth of *B. longum* at 48 hour period

	Log CFU/ml					
Algal extracts	Plate 1	Plate 2	Plate 3	Average	STDev	P values
S. platensis	8.92	8.93	8.91	8.92	0.007939	2.90E-05
S. magnus	8.74	8.76	8.77	8.76	0.015245	5.70E-05
Algal extract no. 48	8.76	8.75	8.76	8.76	0.00762	1.82E-04
Chlorella	8.68	8.72	8.72	8.70	0.02007	4.80E-04
Chlorococcum	8.67	8.69	8.71	8.69	0.017737	3.79E-04
D. salina	8.66	8.66	8.69	8.67	0.015841	2.91E-04
Algal extract no. 28	8.64	8.66	8.65	8.65	0.009653	7.08E-04
Algal extract no. 18	8.64	8.63	8.63	8.64	0.005764	2.96E-03
Algal extract no. 17	8.60	8.64	8.62	8.62	0.020698	5.47E-03
Algal extract no. 15	8.61	8.63	8.59	8.61	0.021204	9.10E-03
Algal extract no. 2	8.59	8.58	8.57	8.58	0.011432	1.68E-02
Algal extract no. 1	8.56	8.58	8.59	8.58	0.017734	4.10E-02
Algal extract no. 21	8.56	8.58	8.54	8.56	0.018151	1.47E-01
Algal extract no. 26	8.52	8.56	8.54	8.54	0.019282	7.86E-01
Positive control (Inulin)	8.89	8.89	8.90	8.89	0.008569	2.70E-05
Negative control (Bacteria only)	8.54	8.52	8.52	8.54	0.014754	-

Values are given as mean ± S.D for triplicates at 48 hours after treatment. A t-test comparing the growth following treatment compared with negative control was performed; p-values each treatment are shown accordingly (p<5E-02 - significant effect of algal extract against the bacterium). NA denotes where the isolate was not tested against the bacterium.

Table $B_{5:}$ The effect of algal extracts on the growth of *L. lactis* at 48 hour period

	Log CFU/ml					
Algal extracts	Plate 1	Plate 2	Plate 3	Average	STDev	P values
S. platensis	9.00	8.99	8.99	8.99	0.004432	8.98E-04
Chlorococcum	8.91	8.92	8.90	8.91	0.010726	5.22E-04
S. magnus	8.79	8.81	8.80	8.80	0.013792	6.31E-04
D. salina	8.76	8.74	8.75	8.75	0.01173	1.37E-03
Chlorella	8.68	8.68	8.69	8.68	0.005170	5.47E-03
Algal extract no. 48	8.64	8.65	8.67	8.66	0.014563	2.82E-03
Algal extract no. 28	8.59	8.58	8.61	8.59	0.016773	7.46E-03
Algal extract no. 18	8.51	8.52	8.46	8.50	0.029312	3.82E-01
Algal extract no. 17	8.49	8.48	8.46	8.48	0.014483	7.98E-01
Algal extract no. 15	8.56	8.52	8.54	8.54	0.019282	3.79E-02
Algal extract no. 25	8.57	8.56	8.53	8.55	0.018737	2.39E-02
Algal extract no. 24	8.49	8.51	8.52	8.51	0.013577	1.84E-01
Algal extract no. 19	8.46	8.45	8.48	8.46	0.014982	6.64E-01
Algal extract no. 26	8.45	8.45	8.46	8.45	0.008799	3.02E-01
Positive control (Inulin)	8.66	8.65	8.66	8.66	0.005511	6.92E-03
Negative control (Bacteria only)	8.51	8.46	8.45	8.47	0.030064	-

Values are given as mean ± S.D for triplicates at 48 hours after treatment. A t-test comparing the growth following treatment compared with negative control was performed; p-values each treatment are shown accordingly (p<5E-02 - significant effect of algal extract against the bacterium). NA denotes where the isolate was not tested against the bacterium

Table $B_{6:}$ The effect of algal extracts on the growth of *L. bulgaricus* at 48 hour period

	Log CFU/ml					
Algal extracts	Plate 1	Plate 2	Plate 3	Average	STDev	P value
S. platensis	9.00	8.99	8.99	8.99	0.006682	5.63E-04
S. magnus	8.91	8.92	8.90	8.91	0.006387	4.87E-04
Chlorococcum	8.79	8.81	8.80	8.80	0.013792	1.31E-04
D.salina	8.76	8.74	8.75	8.75	0.011554	3.76E-04
Algal extract no. 48	8.68	8.68	8.69	8.68	0.011759	4.45E-04
Chlorella	8.64	8.65	8.67	8.66	0.020216	3.19E-01
Algal extract no. 17	8.59	8.58	8.61	8.59	0.015232	1.82E-01
Algal extract no. 24	8.51	8.52	8.46	8.50	0.015965	1.00E+00
Algal extract no. 25	8.49	8.48	8.46	8.48	0.011432	3.74E-02
Algal extract no. 23	8.56	8.52	8.54	8.54	0.018661	9.11E-03
Algal extract no. 26	8.57	8.56	8.53	8.55	0.019859	4.78E-03
Algal extract no. 43	8.49	8.51	8.52	8.51	0.007716	2.53E-03
Positive control (Inulin)	8.46	8.45	8.48	8.46	0.046549	1.75E-02
Negative control (Bacteria only)	8.45	8.45	8.46	8.45	0.015965	-

Values are given as mean ± S.D for triplicates at 48 hours after treatment. A t-test comparing the growth following treatment compared with negative control was performed; p-values each treatment are shown accordingly (p<5E-02 - significant effect of algal extract against the bacterium). NA denotes where the isolate was not tested against the bacterium.

Figure B_1: HPLC chromatogram of galactose standard eluted at 3.27 min

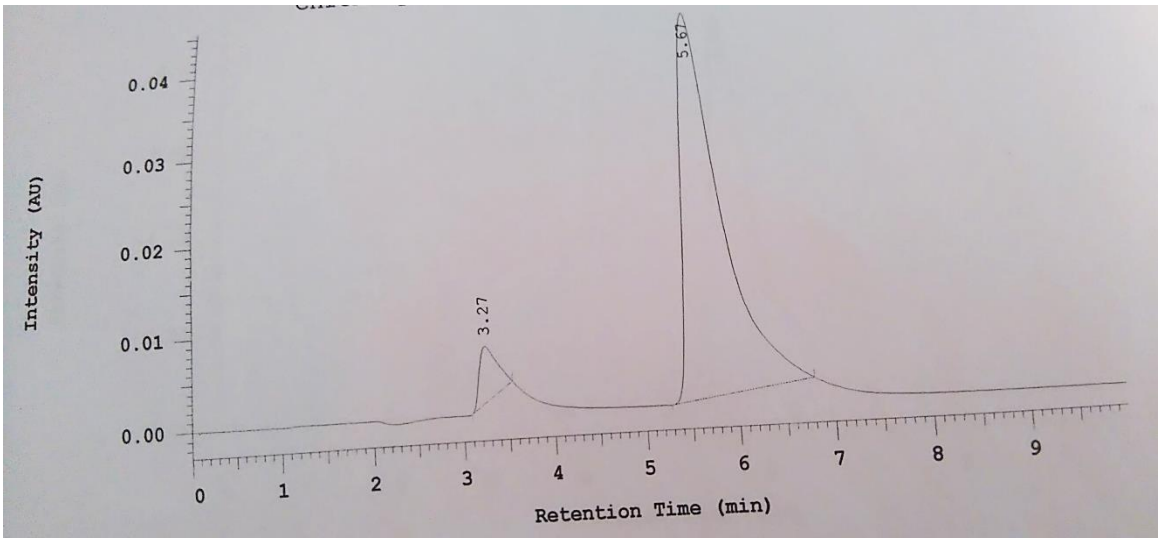

Figure B_2: HPLC chromatogram of xylose standard eluted at 2.56 min

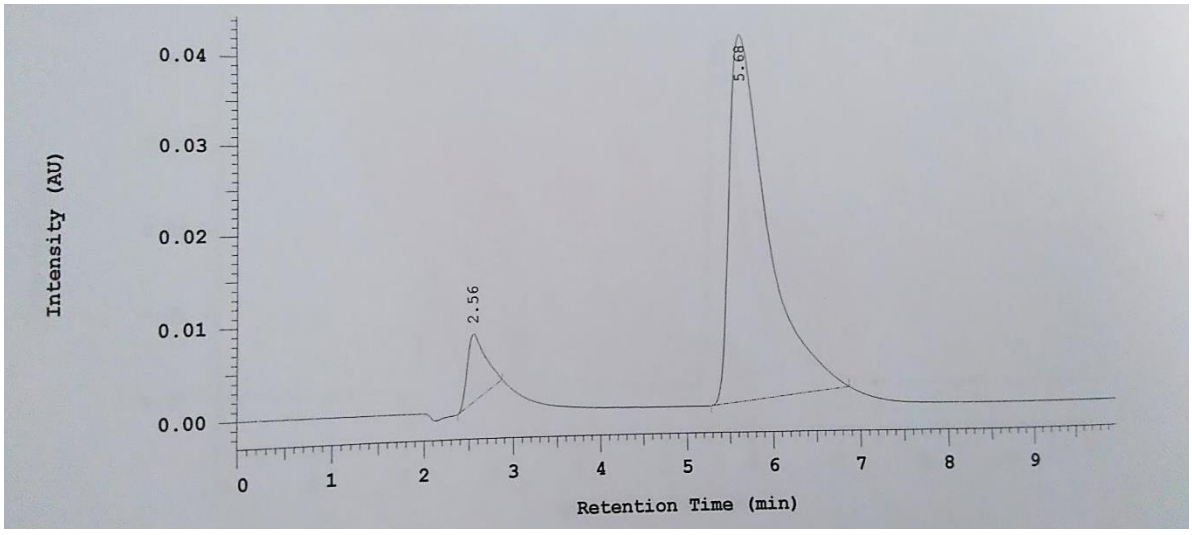

Figure B_3: Galactose standard curve

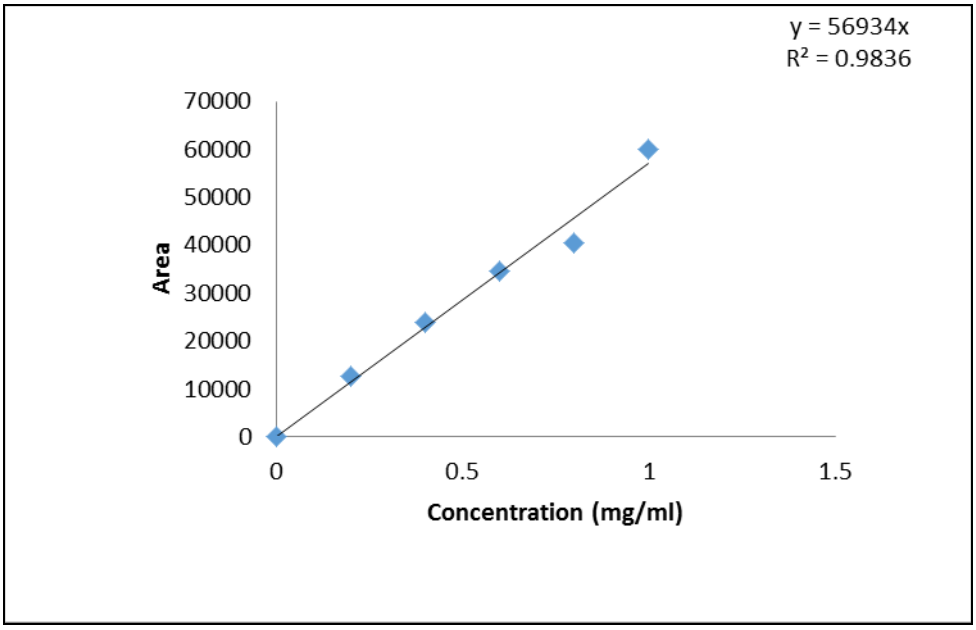

Figure B_4: Xylose standard curve