THE STRUCTURE-FUNCTION RELATIONSHIP BETWEEN *ASCOPHYLLUM NODOSUM* POLYSACCHARIDES AND *IN VITRO* PREBIOTIC ACTIVITY: AN ASSESSMENT OF THE IMPACT OF EXTRACTION TECHNOLOGIES

by

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This thesis is dedicated to God Almighty and all in pursuit of academic excellence.

TABLE OF CONTENT

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

APPENDIX A

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

APPENDIX B

APPENDIX C

ABSTRACT

The impact of four extraction processes (conventional chemical (CCE), microwave-assisted (MAE), ultrasound-assisted (UAE), and enzyme-assisted extraction (EAE)) on the structure-function relationship between polysaccharides from *Ascophyllum nodosum* and prebiotic functioning was investigated. Fucoidan extracts from the MAE method had significantly ($P < 0.05$) higher fucose content and sulphate levels, higher uronic acid content, dispersity index, and lower molecular weight, as compared to other extracts. For sodium alginate, all four extracts had similar dispersity index and M/G ratio, and molecular weight range of 65 – 182 kDa; uronic acid content was highest in the MAE extract. Fucoidan from all four methods significantly improved the growth rate of *Lactobacillus delbruecki* at 0.1% and 0.5% inclusion concentrations, whereas no significant difference was observed for alginate extracts, relative to the un-supplemented strain. Also, fucoidan and alginate supplemented media had no significant impact on the growth rate of *Lactobacillus casei* as compared to the un-supplemented media.

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CHAPTER 1

INTRODUCTION

1.1. THESIS OVERVIEW

The sustainability of our planet is a major topic of interest. This has led to the exploration of alternative sources of basic amenities such as food, energy, and health. This quest for sustainable alternatives is fuelled by reports suggesting a projected spike in global population beyond the carrying capacity of our current resources or production rate. The estimated current global human population, as of October 2017, is 7.6 billion (World population clock (2017)) and is expected to increase to an estimated 9.1 billion people by the year 2050 (FAO, 2009), and 11.2 billion people by the year 2100. An estimated 70% increase in food production will be required to meet the demand of the approximately 2.3 billion additional people by 2050 (Bleakley & Hayes, 2017).

The ocean is the most significant reservoir of both living and non-living marine organisms and makes up about 70% of the earth's surface. Hence, a major source of explorable raw materials. Macroalgae are key members of the coastal ecosystem, with approximately 25,000 – 30,000 species of diverse forms and sizes (Charoensiddhi et al., 2017). They are relatively abundant in coastal regions of the world with fast growth rates and do not require arable land, fresh water, or fertilizers to grow (Charoensiddhi et al., 2017). Some seaweeds are traditionally consumed as food (sea vegetables) and have been used as herbal remedies in the treatment of stomach ailments, eczema, cancer, psoriasis, lung disease, gall stones, asthma and heart disease (Peng et al., 2015). The cultivation of seaweeds, currently practised in about 50 countries, has increased tremendously in recent times as a result of their wide range of commercial applications. In 2003, about 8 million tons of seaweed

were harvested worldwide; in 2006, 15.1 million harvested tons were recorded, and in 2014, 28.5 million tons of seaweed were harvested, to be used for dietary purposes, or as starting materials for food production, fertilizers, animal nutrition, cosmetics, hydrocolloids, pharmaceutical ingredients, and other purposes (FAO, 2016; Peng et al., 2015). Recently, the annual global production of value-laden seaweed hydrocolloids, such as agar, alginate, and carrageenan reached about 100,000 tonnes and a gross market value of about USD 1.1 billion (Charoensiddhi et al., 2017). These hydrocolloids have a wide range of applications in the pharmaceuticals, food, and biotechnological industries. Thus, seaweeds are an exploitable, sustainable and alternative source of starting materials for a wide range of commercial and industrial applications.

Seaweeds are grouped into three classes based on their pigmentation: the Chlorophyta or green seaweeds with chlorophyll a and b as accessory pigments; the Phaeophyta or brown seaweeds with fucoxanthin as the major pigments; and the Rhodophyta or red seaweeds, with phycocyanin and phycoerythrin as key pigments (O'Sullivan et al., 2010). Seaweeds contain nutrients such as proteins, minerals, vitamins, dietary fibres and lipids, as well as secondary metabolites such as terpenes, phloro-tannins, steroids, pigments and mycosporine-like amino acids (MAA) (Peng et al., 2015). These bioactive ingredients provide both nutritional and beneficial properties for humans including: anti-microbial, anti-diabetic, anti-coagulant, anti-cancer, anti-HIV, anti-viral, immunomodulatory, cholesterol-lowering effect and prebiotic activities (Charoensiddhi et al., 2017). The chemical composition of these nutrients in seaweeds varies across groups and species. For example, species of the genus *Ulva* (Chlorophyta) contain about 15 – 65 % polysaccharides, 4 - 44% total proteins, 0.3 – 1.6 % lipids, and 11 – 26 % ash or minerals; *Ascophyllum*, a phaeophyte, contains approximately 42 – 70 % polysaccharides, 1.2 – 12%

protein, 1.2 – 4.8 % lipids and 18 – 27% minerals; Members of the Rhodophyta e.g. *Porphyria* species contain about 40 – 76 % polysaccharides, 7 – 50 % proteins, 0.1 – 2.8 % lipids and 7 – 21 % minerals (Rioux & Turgeon, 2015). From the aforementioned data, it is evident that certain seaweeds may be rich in polysaccharides, particularly in the brown and red seaweeds. Of interest to this study, are the polysaccharides from brown seaweeds, and in particular *Ascophyllum nodosum*. This seaweed is one of the most well used macroalgae belonging to the family, fucales which are a rich source of fucoidan and alginates. It is readily available, commercially cultivated, and has a wide range of applications (Brebion, 2013; Charoensiddhi et al., 2016; Okolie et al., 2017).

Some polysaccharides extracted from phaeophytes have promising prospects for humans and animals as both health promoting functional ingredients and starting materials for industrial applications. The three major polysaccharides found in brown seaweeds include laminarin or laminaran, fucoidan, and alginate. Laminarins are water-soluble storage polysaccharides made up of about 20 – 25 glucose moieties with β-(1,3) and β-(1,6) linkages and have been reported to possess anti-bacterial, anti-oxidative, anti-coagulant and immunomodulating potentials (Kadam et al., 2015; Walsh et al., 2013; Zhang & Row, 2015). Fucoidans are a group of heterogenous fucose-rich sulphated polysaccharides located in cell walls with varying amounts of monosaccharides such as glucose, xylose, galactose, and mannose (Zhao et al., 2016). Fucoidans have been utilized in various industrial sectors such as cosmetics, animal feed supplements, and functional foods. In addition to these industrial benefits, fucoidans also possess health-promoting potentials including anti-thrombotic, anti-coagulant, anti-viral, anti-tumour, anti-cancer, immunomodulating and prebiotic activities (Ale et al.,, 2011; Fitton et al., 2015).

Alginates are the most abundant polysaccharides extracts from brown seaweeds. They exist in the cell wall of brown seaweeds as alginic acids and are extracted as alginates, in most cases, complexed with a multivalent ion (Na^{+}, Ca^{2+}, and Mg^{2+}). They are linear copolymers of β - (1,4) -D-mannuronic acids and α-L- guluronic acids. This hydrocolloid, with well-defined gelling properties in the presence of multivalent ions (Na^{+}, Ca^{2+}, and Mg^{2+}) or at pH less than 3.5, is an attractive commercial product with global value of about US$ 300 million. Alginates are of significant value to both food and pharmaceutical industries and have been used as gelling agents, thickeners, and stabilizers (Rhein-Knudsen et al., 2017). In the medical field, they have been used as wound dressing, appetite modulators, cell immobilizers, and as drug delivery agents (Dettmar et al., 2011).

The commercial applications of brown seaweed polysaccharides (BSP) have attracted millions of dollars from industries and as such maximization and large-scale production with advanced technological inputs appears to be the next step, to further exploit the many benefits of seaweed polysaccharides. The conventional method is to extract polysaccharides from brown seaweed with a heat source (usually at 70 ºC), such as hot plate. Technological applications such as microwave, ultrasound, and enzyme technologies are processes that can be employed in aiding the conventional extraction of BSP. The expectation is that these methods would help to improve yield, maintain the structural integrity/properties and extract these polysaccharides in a shorter time period and with greater efficiency as compared to the conventional methods. This may not always be the case, as high energy inputs in the form of microwave heating and sound energy may negatively influence the structural properties and by extension, the functional properties of these polysaccharides, hence the need to comparatively evaluate the influence of extraction processes and conditions on the structural and functional properties of BSP.

Of the many health prospects of BSP, its prebiotic potential correlates with brown seaweed being a reservoir of long chain molecules . Prebiotics are dietary fibres or non-digestible food components with the capacity to escape hydrolytic activity of digestive enzymes as a result of non-hydrolysable linkages between monosaccharides, while selectively stimulating the activities of beneficial gut microbes (e.g. *Lactobacilli* and *Bifidobacteria*) by serving as a carbon source. Multiple health benefits arise from the stimulation of beneficial gut microbiota including immunomodulation, anti-microbial activities, short chain fatty acid (SCFA) production, decrease in cell permeability, and the improvement of the overall health of the intestinal epithelial cells (Rajendran et al., 2017). BSP is an emerging prebiotic due to its *in vitro* capacity to meet the requirements for its regulatory classification as a prebiotic at least as demonstrated by *in vitro* studies (Deville et al., 2007; Lynch et al., 2010; Charoensiddhi et al., 2016). These requirements include non-digestibility, fermentation by intestinal microbes and selective stimulation of beneficial microbial populations (Gibson & Roberfroid, 1995). The high content of prospective prebiotic poly- and oligo-saccharides in seaweeds make them an attractive and exploitable resource for prebiotic applications as functional food ingredients in the future.

This project therefore investigated the influence of conventional and more recent extraction technologies e.g MAE, UAE, and EAE processes on the structural properties and *in vitro* prebiotic potential of polysaccharides extracted from the brown seaweed, *Ascophyllum nodosum*.

1.2. THESIS OBJECTIVES AND ORGANIZATION

This study focused on three main objectives. The first objective was the sequential extraction of fucoidan and alginate fractions from brown seaweed polysaccharides using four extraction processes (CCE, MAE, UAE, and EAE). The second objective was to comparatively evaluate the structural properties of both fucoidan and alginate fraction obtained from the four extraction processes. The third and final objective was to investigate the strain-specific prebiotic activity of extracts obtained from these extraction processes by testing their capacity to improve the growth of *Lactobacillus casei* and *Lactobacillus delbruecki* subsp: *bulgaricus.*

This thesis is organized in manuscript format with five chapters, including the present chapter. Chapter 2, the literature review, is a published paper on the prospects of brown seaweed polysaccharides as prebiotics and potential immunomodulators. Chapters 3 and 4 are original manuscripts to be submitted for publication which address the structure-function relationship of fucoidan- and alginate-prebiotic activities respectively. The thesis concludes with Chapter 5 which summarizes the research, and is followed by an appendix.

CHAPTER 2

LITERATURE REVIEW

PROSPECTS FOR BROWN SEAWEED POLYSACCHARIDES (BSP) AS PREBIOTICS AND POTENTIAL IMMUNOMODULATORS

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2.1. ABSTRACT

Prebiotics enhance immune responses through the modulation of intestinal microbial activities, production of short chain fatty acids (SCFA), direct interaction with toll-like receptors and mucin production. These non-digestible food components are known to be resistant to enzymatic hydrolysis by digestive enzymes and are utilized as a carbon source for the growth of beneficial bacterial population through the process of fermentation. Brown seaweed polysaccharides (BSP) have been described as emerging prebiotics due to their potential to stimulate gut microbiota activities at *in vitro* and *in vivo* stages. This

review examines evidence of the relationship between the prebiotic capacity of BSP, their structure, extraction, and possible mechanisms of immunomodulation.

2.2. INTRODUCTION

Our world is currently experiencing a plethora of degenerative and terminal diseases, which have led to an increase in morbidity and mortality. The latter was estimated by the World Health Organization (WHO) at 57 million in 2015 with an expected increase to about 70 million by 2030 (WHO, 2013). The immune system is a major target for a wide range of diseases. The pathogenesis of HIV/AIDS, cancer and diabetes mellitus (Type 1), currently ranked as sixth, seventh, and eighth leading causes of death respectively worldwide, has been closely associated with the immune system (Shurin, 2012; WHO, 2013; Zhernakova et al., 2009). Hence, the role of the defensive machinery of the immune system in disease prevention and health promotion is of great significance. Other immune-related diseases include rheumatological disease, allergy, psoriasis, asthma, ulcerative colitis and inflammatory bowel disease (Zhernakova et al., 2009). Conventional treatments for immune-related diseases are faced with the challenges of impaired immune responsiveness, hypersensitivity, immunogenicity, and drug resistance (Aher & Wahi, 2012; Hansel et al., 2010). In this regard, attention is shifting to functional foods (with little or no side effects) such as prebiotics, as alternatives, due to their potential to strengthen the immune system and prevent degenerative diseases (Choque Delgado et al., 2011; Peshev & Van den Ende, 2014).

Prebiotics, as defined by the Food and Agricultural Organization (FAO), are “non-viable food components that confer health benefits on the host, resulting from its association with the modulation of intestinal microbiota” (Pineiro et al., 2008). They may effect immune

functions through stimulation of the activities and growth of beneficial bacteria such as: *Bifidobacteria* and *Lactobacilli* populations in the gut microbiota, production of short-chain fatty acids (SCFA) and mucin. Additionally, direct interaction with pattern recognition receptors (PRR) such as β-glucan receptors and dectin receptors can also stimulate immune-protective activities including macrophage activation and cytokine production (Laparra & Sanz, 2010; Schley & Field, 2002; Song et al., 2014). BSP (laminarin, fucoidan, and alginate) have received a lot of attention in recent years as emerging prebiotics due to their gut microbiota stimulatory activities and reported multiple bioactivities including anti-viral, anti-inflammatory, anti-coagulant, anti-oxidant, anti-obesity, and immunomodulatory properties.

This review, therefore, contributes to the literature by providing extensive discussion on the prospects of BSP as potential prebiotics and their role in immunomodulation.

2.3. SEAWEEDS

Seaweeds are marine macroalgae. Certain seaweeds contain a large proportion of polysaccharides, low lipid content, high amounts of minerals, and are considered a major source of food and shelter for marine life (Brebion, 2013; Gupta & Abu-Ghannam, 2011a). Seaweeds also contain several bioactive compounds: secondary metabolites - phlorotannin, diterpenes, and bromophenol; polysaccharides (ulvan, agars, carrageenan, laminarin, fucoidan, and alginates) (Gupta & Abu-Ghannam, 2011a; O'Sullivan et al., 2010). The medicinal properties exhibited by these bioactive compounds include anti-inflammatory, anti-oxidant, anti-coagulant, anti-viral, anti-microbial, and immunomodulatory activities (Fitton et al., 2015; Gupta & Abu-Ghannam, 2011a; O'Sullivan et al., 2010).

Seaweeds are subdivided into three phyla based on their pigmentation; Chlorophyta or green seaweeds (chlorophyll a and b pigments), Phaeophyta or brown seaweeds (brown nature attributed to the presence of fucoxanthin, a carotenoid), and Rhodophyta or red seaweed (with major accessory pigments as phycoerythrin and phycocyanin) (O'Sullivan et al., 2010). These classes also possess different polysaccharides. For instance, ulvans (composed of sulphated rhamnose, xylose, glucuronic acid, and iduronic acid) are the major polysaccharides in the class Chlorophyta; agars (agaropectin and agarose) and carrageenans can be extracted from red seaweeds (Rhodophyta); laminarin, fucoidan, and alginate in brown seaweeds. Of major interest to this review are the polysaccharides of brown seaweeds, since they possess structural features with health prospects such as immunomodulatory, anti-inflammatory, anti-cancer, and prebiotic activities.

The brown seaweed family is further divided into 13 orders (Gupta & Abu-Ghannam, 2011a), about 300 genera (Silberfeld et al., 2014), and consists of an estimated 1836 known species (Wehr, 2015). However, only three Orders, Laminarales, Fucales, and Dictyotales, have been widely explored in terms of their phytochemical content (Gupta & Abu-Ghannam, 2011a). Prominent members of the Laminarales and Fucales, which have been explored for their chemical composition and/or bioactivity, include: Laminarales (*Laminaria digitata*, *L. saccharina, L. japonica, L. religosa, L. angustata, Ecklonia kurome, E. radiata*, and *Macrocystis pyrifera)* (Ale et al., 2011; Charoensiddhi et al., 2016; Fitton et al., 2015; Li et al., 2008); Fucales (*Ascophyllum nodosum, Fucus vesiculosus, F. evanescens, F. serratus, F. spiralis, F. distichus, Sargassum fusiforme, and S. kjellmanianum*) (Ale et al., 2011; Fitton et al., 2015; Li et al., 2008).

Among the brown seaweed species, *L. digitata* and *A. nodosum* are the most common because they are readily available and harvested from wild populations on a commercial

scale, rich in polysaccharides, and possess multiple bioactivities (Brebion, 2013; Charoensiddhi et al., 2016).

2.4. BROWN SEAWEED POLYSACCHARIDES

As mentioned earlier, laminarin, fucoidan, and alginates are the major polysaccharides of brown seaweed (Zvyagintseva et al., 1999). Laminarin (also called laminaran) is a group of low molecular weight (about 5 kDa) water-soluble polysaccharides composed of β-(1,3)-linked glucans with β-(1,6)-linked side chains of varying distributions and length (about 20–25 glucose moieties) (Walsh et al., 2013). These polysaccharides are located in cell vacuoles, constitute about 35% dry weight of certain brown seaweeds, and also serve as storage polysaccharides (Kadam et al., 2015; O'Sullivan et al., 2010). Studies on laminarin have shown promising results with respect to antibacterial, immunomodulating, antioxidative, and anticoagulant properties (Kadam et al., 2015; Zhang & Row, 2015). Interestingly, gamma-radiated degradation of laminarin showed increased antioxidant activity and inhibition of melanin synthesis when compared to non-irradiated laminarin (Choi et al., 2012). Figure 2.1 presents the structures of the two types of laminarin; chains ending with mannitol residue and chains terminated by glucose residue.

Fucoidans are a class of fucose-rich sulphated homo- or hetero-polysaccharides composed mainly of α - (1,2) and/or (1,3)-linked fucose (Zvyagintseva et al., 1999). They are found in the fibrillar cell walls and intercellular spaces of brown seaweeds. Sulphates may be substituted at the C2 and C4 positions of L-fucopyranosyl residues but rarely on C3 depending on the source (Ale et al., 2011). In addition to fucose, other monomers found in fucoidan extracts include galactose, mannose, xylose, glucose, and glucuronic acid (Ale et al., 2011). Fucoidans are soluble in water and dilute acids and have a molecular weight

of between 100 and 1,600 kDa (Zhang & Row, 2015). Fucoidans have several industrial applications including cosmetics, functional foods, dietary supplements, livestock and aquaculture feed supplements. They have also been reported to possess multiple bioactivities such as: anti-viral, anti-inflammatory, immunomodulatory, anti-thrombotic, anti-coagulant, anti-oxidative, anti-tumour, anti-bacterial, and anti-cancer (Ale et al., 2011; Gupta & Abu-Ghannam, 2011b; Fitton et al., 2015; Zhang & Row, 2015). In addition, they are known to specifically contribute resistance toward diseases associated with the kidney, liver, cardiovascular system, cerebral ischemia, urinary system, and also Alzheimer's disease (Ale et al., 2011; Gupta & Abu-Ghannam, 2011b; Fitton et al., 2015; Zhang & Row, 2015). Although there has been remarkable progress in identifying the *in vitro* biological activities of fucoidan, there is a dearth of information on their approved use in biomedical applications either within biomaterials or via direct intravenous, intraperitoneal, intramuscular, or subcutaneous administration (Fitton et al., 2015). Furthermore, research on its prospects as a biomaterial in drug delivery, topical, and orally delivered agent for a variety of pathologies appears to be emerging (Fitton et al., 2015). Molecular weight, sulphate levels, and monosaccharide composition are the major structural characteristics of fucoidans associated with reported bioactivities (Ale et al., 2011; Fitton et al., 2015; Vo & Kim, 2013). Figure 2.2 presents fucoidan structures obtained from different sources.

Alginates occur as alginic acids in the cell wall of brown seaweeds. They are also found in some bacteria as capsular polysaccharides (Draget & Taylor, 2011). They are copolymers of (1,4)-linked β-D-mannuronic acid and a-L-guluronic acid joined in a linear form, and the distribution of these uronic acids (mannuronate and guluronate) in alginate chains give rise to three different block types: poly-M, poly-G and alternating MG blocks

(Draget & Taylor, 2011). The chemical composition and monomeric sequence of extracted alginate vary with algal species, part of the algae used, seasonal variations, and environmental conditions in the ocean (Dettmar et al., 2011; Draget & Taylor, 2011; Rioux, Turgeon et al., 2007). Alginates are in abundance in the cell wall of brown seaweeds (O'Sullivan et al., 2010). They have a well-established gelling property in the presence of multivalent cations (Na^{+}, Ca^{2+}, Mg^{2+}) or at a pH less than 3.5 (Draget & Taylor, 2011; O'Sullivan et al., 2010). The gel-forming potential is associated with the high content of guluronic acids (G). Alginates are widely used in the food and textile industries as thickeners, stabilizers, gel formers, and film formers. In the health sector, they are applied in wound dressing to absorb exudates and in drug delivery for controlled and sustained release of drugs 1(Draget & Taylor, 2011). Additionally, their biomedical applications include immobilization of cells, modulation of appetite and energy intake. Bioactivities reported for alginates are largely dependent on their monomeric structural arrangement. The mechanical and swelling property of the gel depends primarily on the monomeric composition, block structure, and molecular weight of the alginate. Alginates require a monomeric composition above 70% guluronic acid to exert their desired mechanical stability (Draget & Taylor, 2011). Conversely, mannuronate-rich alginate induces immunogenic response through its interaction with PRR especially toll-like receptor (TLR) 4 or TLR 2 together with CD 14 (Espevik et al., 2009). The structure of mannuronate and guluronate are presented in Figure 2.3.

2.4.1. EXTRACTION METHODOLOGIES OF BSP

Extraction methodology has been closely linked with the biological activity of polysaccharides of brown seaweed (Ale et al., 2011; Fitton et al., 2015). Water or diluted

acid extraction has been the conventional method for extracting seaweed polysaccharides (Ale et al., 2011; Rioux et al., 2007). As a result of the biodiversity, bioactive, and commercial relevance of BSP, technology (microwave and ultrasonication)-aided and enzymatic methods have been applied in the extraction of polysaccharides from brown seaweed. These techniques also aim at maintaining the structural integrity and properties of BSP. Thus far, methods employed in the extraction of BSP include conventional chemical extraction (CCE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and enzyme-assisted extraction (EAE).

2.4.1.1. CONVENTIONAL CHEMICAL EXTRACTION

Chemical extraction is one of the earliest and widely used methods for the extraction of BSP. It dates back to 1913 when Kylin, in his first seminal report, observed that extract obtained with dilute acetic acid contained mainly fucose with mannitol, alginic acid, and laminarin (Kylin, 1913). In 1915, a more appropriate step for alginate isolation was proposed when Hoagland and Lieb used a sodium carbonate soaking step and addition of HCl for the extraction of BSP (Hoagland & Lieb, 1915). In recent years, the step-wise methodology outlined by Rioux et al., (2007) has been cited a number of times for the extraction of BSP (Rioux et al., 2007). This method involves a pre-extraction step with ethanol, chloroform, or formaldehyde to remove proteins, pigments, terpenes and polyphenols, and prevent contamination of target compounds (Hahn et al., 2012). The pre-extracted seaweed is then treated with mild acid or water in the presence of heat to hydrolyze the cell walls and extract the polysaccharides. Calcium chloride may be used in this step to precipitate alginic acids extracted along with other polysaccharides. Sodium carbonate is also commonly used to extract alginate from seaweeds. Since BSP are

insoluble in polar solvents, ethanol and acetone are commonly used in the precipitation step (Rioux et al., 2007; Yuan & Macquarrie, 2015).

2.4.1.2. MICROWAVE-ASSISTED EXTRACTION

Microwave heating has been described as a faster and more efficient method for extraction of BSP as compared to the conventional extraction methods (Yuan & Macquarrie, 2015). It has a good performance for biomass extraction, hydrolysis, and pyrolysis. MAE involves the vibration of water molecules induced by microwave heating. Molecular vibrations result in an increase in the temperature of intracellular material and evaporation of water, which in turn exerts pressure on the cell wall leading to its lysis and release (Hahn et al., 2012). Extraction of fucoidan from *F. vesiculosis* with microwave heating produced yields (18.2%) comparable to those of multiple extraction steps and in shorter time (pressure of 120 psi for 1 min) (Rodriguez-Jasso et al., 2011). Fucoidan extracted from *A. nodosum* using microwave technology exhibited strong antioxidative potential (Yuan & Macquarrie, 2015). Although the MAE method is a faster and more effective method of extraction, it offers mainly laboratory scale extraction and has not been used in large-scale commercial extraction (Fitton et al., 2015).

2.4.1.3. ULTRASOUND-ASSISTED EXTRACTION

The UAE method uses sound waves migrating through a medium, inducing pressure variation and creating small vacuum bubbles or voids which collapse violently (cavitation), resulting in localized pressure and heat which helps to solubilize polysaccharides (Kadam et al., 2015). Comparing the efficiency of UAE with MAE and CCE, Kadam et al. (2015) discovered that extraction with ultrasound-assisted technology

(10.79%) gave a better yield when compared to MAE (9.56%) and conventional solvent extraction (4.67%) in shorter time (Kadam et al., 2015; Ying et al., 2011). However, the high energy input and pressure required for UAE and MAE could be detrimental to the integrity of the extracts, as these technologies have been associated with cleavage of sulphate esters. Hence, moderation of extraction conditions is advised to avoid structural alteration of sulfated polysaccharides and other unintended damages (Hahn et al., 2012; Kadam et al., 2015).

2.4.1.4. ENZYME-ASSISTED EXTRACTION

The complex nature of the seaweed cell wall and its rigid nature present a major obstacle to efficient extraction of polysaccharides and other bioactive compounds. This has led to the use of enzymes in the extraction of potent BSPs (Charoensiddhi et al., 2016). Application of cell wall-degrading carbohydrases led to the breakdown of cell walls to release polysaccharides of desirable bioactivities at optimal temperature and pH. These carbohydrases include Viscozyme, pH 4.5, 50 ºC (Sigma Aldrich, St. Louis, MO), Celluclast, pH 4.5, 50 ºC (Sigma Aldrich), and Ultraflo, pH 7.0, 60 ºC (Sigma Aldrich) (Rodrigues et al., 2015). The EAE method has been described as one of the best, as it generates high yield and has favourable biological properties such as antioxidant, antidiabetic and prebiotic properties. A recent study reported that a high amount of sulfated polysaccharides was obtained from *O. pinnatifida* using EAE with Viscozyme (Rodrigues et al., 2015). Notable prebiotic, antioxidant, and antidiabetic properties of EAE from *Sargassum muticum* and *Osmundia pinnatifida* have also been reported (Kadam et al., 2013; Rodrigues et al., 2015).

2.5. PREBIOTIC ACTIVITY OF BSP

Various definitions have been proposed for prebiotics since 1995. Gibson and Roberfroid (1995) defined prebiotics as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or more limited number of *Bifidobacteria* in the colon and thus improving host health". Although this definition provided a breakthrough in the knowledge and understanding of prebiotics, it lacked certain aspects, such as the fact food components are fermented by the intestinal microflora and selectively stimulate bacterial growth and not just *Bifidobacteria* sp. (Brebion, 2013). Valcheva and Dieleman (2016) also argued that this definition restricted the prebiotic class to only a few carbohydrate compounds, notably the short and long chain fructans, galactoligosacharides (GOS) and lactulose (Valcheva & Dieleman, 2016). The definition by Gibson and Roberfroid was however updated nine years later to address the aforementioned limitations. A prebiotic was then defined as a: "selectively fermented ingredient that allows specific changes both in the composition and/or activity of the gastrointestinal microflora that confers benefits on the host well-being and health" (Gibson et al., 2004). Figure 2.4 presents an illustration of the interaction between prebiotics and beneficial bacteria strains. In 2007, the FAO of the United Nations defined a prebiotic as: "a non-viable food component that confers a health benefit on the host associated with the modulation of microbiota." This definition, though silent on some specifics outlined in the 2004 updated definition, summarizes the concept of prebiotics and gives room for the classification of more compounds as prebiotics other than the conventional inulin, fructo-oligosaccharides (FOS), GOS, and lactulose. The same FAO technical report which defined prebiotics also presented three criteria for the classification of prebiotics, which included: (a) non-digestibility—resistance to gastric acidity, hydrolysis by mammalian

enzymes and gastrointestinal absorption (Gibson et al., 2004); (b) fermentation by intestinal microflora (Gibson et al., 2004); and (c) selective stimulation of growth and/or activity of beneficial intestinal bacteria (Gibson et al., 2004). An illustration of the interaction between fucoidan and TLR's of macrophages is presented in Figure 2.5.

In a good number of reviews on prebiotics (Akhter et al., 2015; Al-Sheraji et al., 2013; Choque Delgado et al., 2011; Song et al., 2014), there has been little or no mention of BSP as a prebiotic. There is a perceived delay in the acceptance of BSP as prebiotics despite existing evidence of *in vitro* prebiotic activity of BSP. This may be attributed to limited *in vivo* studies and human clinical trials on the prebiotic potential of BSP, as well as the lack of commercialization of bioactive polysaccharides of brown seaweed. Part of the focus of this review is to extensively evaluate the progress made in the prebiotic classification of BSP by documenting evidence of their prebiotic activities *in vitro*, *in vivo*, and in clinical trials. The source, stage of trial, and evidence of prebiotic activity of BSP are presented in Table 2.1. Although available evidence provides basic information and background knowledge for the classification of BSPs as prebiotics, more *in vivo* and clinical trials need to be conducted to solidify the health claim.

2.5.1. METHODS FOR EVALUATING PREBIOTIC ACTIVITY

The different methods for determining prebiotic activity are best described and understood under the three major requirements for the classification of a compound or substance as a prebiotic; non-digestibility, fermentation by gut bacteria, and stimulation of activity/growth of intestinal bacterial populations.

2.5.1.1. NON-DIGESTIBILITY

The non-digestibility criterion requires that a proposed prebiotic is resistant to gastric acidity, hydrolysis by digestive enzymes, and gastrointestinal absorption (Roberfroid, 2007). *In vitro* methods used for evaluating non-digestibility include resistance of the candidate prebiotic to both acid hydrolysis and enzymatic degradation by salivary, pancreatic, and intestinal enzymes. Laminarin, obtained from *Laminaria saccharina* was subjected to acid hydrolysis by incubating the laminarin solution (1 mg/mL) with a HCl (150 mM)/KCl (17 mM) solution at physiological condition (37 °C) (Deville et al., 2004). In the same study, fresh homogenates of human organ (stomach, small intestine, colon, and pancreas) and saliva were also incubated with laminarin, allowing enzymes from these organs and saliva to hydrolyze laminarin. Results obtained from the study indicated that laminarin was resistant to both acidic and enzymatic degradation. In another study, Wang et al. (2006) reported that alginate oligosaccharides suspended in phosphate buffer and incubated with amylases and proteases at 37 ºC, resisted hydrolysis (Wang et al., 2006). In animal models, a proposed prebiotic can be recovered from faecal samples, after oral administration of a diet containing the prebiotic. This can be used to evaluate non-digestibility. Experimental animals must be germ-free or pre-treated with antibiotics to suppress the intestinal microbiota (Roberfroid, 2008). Also, incubation within the gastrointestinal system of living anaesthetized rats has been used to evaluate non-digestibility (Nilsson et al., 1988). The stability of BSP has not been assessed using this approach. At the clinical trial stage, recovery of undigested molecules from the faeces and distal ileum of human volunteers has been used as a direct approach to studying non-digestibility. It can also be assessed indirectly by measuring blood glucose and insulin levels in the serum of human subjects (Roberfroid, 2007).

2.5.1.2. FERMENTATION BY INTESTINAL BACTERIA

The fermentation process is one of the most commonly used methods for investigating prebiotic activity. Prebiotics undergo microbial fermentation in the distal end of the intestinal tract, stimulating the activity and growth of intestinal bacteria, especially beneficial bacteria, as well as the production of SCFA. *In vitro* methods of fermentation use batch and continuous fermentation systems to investigate anaerobic fermentation. Faecal samples are a major source of mixed bacterial populations, although pure culture of selected bacteria could also be used. Fermentation is measured *in vitro* through pH determination, enumeration of bacterial populations (turbidometry or colony count), SCFA quantification and utilization of candidate prebiotics in the fermented solution. All of these indices have been widely used in studying the prebiotic activity of BSP with the exception of the utilization of the candidate prebiotic in fermented sample (Charoensiddhi et al., 2016; Deville et al., 2007; Kong et al., 2016; Zhao & Cheung, 2011). *In vivo* methods use faecal samples and the content of gastrointestinal tracts collected from anaesthetized experimental animals after oral administration of diets containing a proposed prebiotic. Heteroxenic rats, which harbour human faecal flora, are good experimental models for evaluating prebiotic activity *in vivo* (Roberfroid, 2008). Faecal samples obtained are screened for fermentation products such as gases and SCFA. Gastro-intestinal tract (GIT) and digesta samples from the caecum and proximal colon of pigs were aseptically removed and screened for colonic pH, bacterial population, and SCFA. Prior to slaughtering, the pig diet was supplemented with laminarin and fucoidan and fed to pigs for a period of 14 days (Lynch et al., 2010). In humans, fermentation by intestinal bacteria is investigated indirectly at time intervals through the amount of gases, especially hydrogen, present in

breath air. A more direct method involves collection of faecal samples from volunteers and measuring the recovery of candidate prebiotics (Roberfroid, 2007).

2.5.1.3. STIMULATION OF THE ACTIVITY/GROWTH OF INTESTINAL BACTERIA

A strong indicator of a prebiotic's activity is its potential to stimulate the activity or increase the population of beneficial bacterial. Faeces is used for gut microbiota assessment due to its representation of a wide range of bacteria species in the distal colon of experimental models (Roberfroid, 2007). The various methods for studying microbial population can be classified as either culture-dependent or culture-independent (Gong & Yang, 2012).

Culture-dependent techniques are based on the principle of culturing selected bacteria species on agar media, which support their growth, followed by morphological, biochemical, and physiological assays (Gong & Yang, 2012; Roberfroid, 2008). This method is able to examine the physiological function of living strains of bacteria, detect specific intestinal pathogens, and determine genotypes of cultured isolates (Gong & Yang, 2012). The use of culture-mediated techniques dates back to 1901 and has thus contributed immensely to the understanding of the gut microbiota. However, there are certain drawbacks. The culture-dependent method is quite selective to culturable bacteria and cannot account for the 40–90% of intestinal bacteria that are not culturable under laboratory conditions (Gong & Yang, 2012). Also, this approach does not simulate the interplay between beneficial bacteria, non-beneficial bacteria and host cells in the gut. Additionally, it is time consuming and associated with errors especially in the counting of bacteria (Gong & Yang, 2012). This method has been widely used for studying the

prebiotic activity of BSP since beneficial bacterial populations, *Bifidobacteria* and *Lactobacilli*, are culturable (Kong et al., 2016; Lynch et al., 2010; Wang et al., 2006; Zhao & Cheung 2011).

Culture-independent techniques emerged as a result of the increased interest in prebiotic research and the need for more efficient and reliable methods for quantifying microbial populations (Roberfroid, 2008). These methods operate on the principle of quantifying genetic elements or gene expression and do not require the culturing of bacteria; they qualitatively and quantitatively study a wide range of bacterial species.

Culture-independent techniques include fluorescent *in situ* hybridization (FISH), quantitative polymerase chain reaction (Q-PCR), DNA profiling, flow cytometry, DNA microarray and DNA sequencing. Culture-independent methods target the 16S ribosomal RNA, a gene found in all Eubacteria, with relatively small size, and enough variations to distinguish different bacterial species. Alternatively, cpn60 can serve as the target gene since it distinguishes closely related species. FISH (Ramnani et al., 2012) and Q-PCR (Charoensiddhi et al., 2016; Deville et al., 2007) are common culture-independent methods for evaluating prebiotic activity of BSP. FISH uses specific oligonucleotide probes that target the highly conserved region of rRNA (16S RNA or cpn60) to identify and distinguish bacterial species (Roberfroid, 2008). Conversely, fluorescence-labelled groups, strain-specific probes, or a non-sequence specific DNA binding dye can be used to target 16S rRNA or cpn60 genes during amplification process (Gong & Yang, 2012). A detailed review on these methodologies for intestinal bacterial population determination is available (Gong & Yang, 2012).

2.6. PREBIOTICS, BSP AND IMMUNOMODULATION: EVIDENCE AND MECHANISM

Early studies on the immunomodulatory potential of prebiotics recorded improved immune health through its interaction with gut microbiota (Schley & Field, 2002). Direct contact of lactic acid bacteria or bacterial metabolic products with intestinal immune cells, production of SCFA from fermentation of prebiotics, and modulation of mucin production were proposed as possible mechanisms for immunomodulation over a decade ago (Schley & Field, 2002). Table 2.2 presents various evidence of the immunostimulatory potential of BSP. Recent studies have shown that prebiotics do not only interact with cells of the intestinal microflora, but can bind to specific membrane receptors (TLR) to initiate a cellular immune response at a systemic level (Peshev & Van den Ende, 2014).

In the interaction of prebiotics with gut microbiota, the composition of the gut microbiome is known to directly influence immune status and disease susceptibility (Hemarajata & Versalovic, 2013). It has been established that prebiotics can alter the composition and activity of intestinal bacteria *in vitro* (Charoensiddhi et al., 2016). Prebiotic formulations can be selectively utilized (as a carbon sources) by beneficial microbes as a result of the differences in their microbial machinery (Barrangou et al., 2003). Recent studies on the interaction between prebiotics and gut microbes have shown that they stimulate the growth and activities of intestinal bacteria, especially beneficial bacterial strains, *Bifidobacteria* and *Lactobacilli*, which are known to modulate immune response (Charoensiddhi et al., 2016; Delgado et al., 2010; Kong et al., 2016; Lynch et al., 2010). In addition, metabolic products from prebiotics have suppressed the growth of certain bacteria as well (Shibata et al., 2003). Thus, early understanding of the role of prebiotics in immune response was elucidated from the interaction of probiotics (beneficial bacterial strains introduced into

the body) with intestinal immune cells. The mechanism proposed was that few species of bacteria can cross the intestinal epithelial barrier into the Peyer's patches leading to the activation of other immune cells (Schley & Field, 2002).

2.6.1. SCFA PRODUCTION

Prebiotics are broken down by the activities of the gut microbiota to produce SCFA such as acetate, propionate and butyrate (Gibson & Roberfroid, 1995). These SCFA directly modulate immune response (Correa-Oliveira et al., 2016). SCFA administered orally to rats significantly increased natural killer (NK) cell activity (Pratt et al., 1996). Intravenous administration of acetate to healthy subjects and cancer patients increased production of peripheral blood antibodies, NK cell activity, and allogeneic mixed lymphocyte reaction (Pratt et al., 1996). Also, SCFA were able to enhance the activation of G protein-coupled receptors (GPR 41 and GPR 43) in mice, triggering mitogen activated protein kinase (MAPK) signalling, which in turn modulates the activity of transcription factors and subsequently, the production of chemokines and cytokines (Kim et al., 2013). In an *in vitro* study examining the influence of SCFAs on pro- and anti-inflammatory cytokines, acetate and propionate cultured with rat mesenteric lymph node lymphocytes increased the production of IL-10 and partly prevented the inhibitory activity of butyrate on IL-2 production (Cavaglieri et al., 2003).

2.6.2. MODULATION OF MUCIN PRODUCTION

Mucin is responsible for the maintenance of the intestinal barrier and regulates permeability for microbial translocation. The layer of mucus that mucin constitutes overlays the GIT, preventing microbe adherence and subsequent translocation across the

epithelial cell wall (Schley & Field, 2002). The number of studies directly linking prebiotics with mucin modulation is limited. However, it is well established that SCFA arising from the metabolism of prebiotics stimulates the proliferation of intestinal epithelial cells, which in turn are the primary sources of mucin and antimicrobial peptide secretions (Correa-Oliveira et al., 2016). SCFA, especially butyric acid, are utilized as a source for ATP production in these epithelial cells (Donohoe et al., 2011). The fermentation of pectin, gum arabic, and cellulose stimulated mucin secretion with a concurrent production of acetate and butyrate (Barcelo et al., 2000). Fermentation of dietary fibre in this same study did not stimulate mucin release, suggesting that mucin production may occur in response to the production of SCFA (Barcelo et al., 2000).

2.6.3. INTERACTION WITH TLR OF IMMUNE CELLS

In recent times, emerging areas in prebiotics research have expanded to include studies on the immunomodulatory potential of prebiotics and understanding of the mechanism of action. Prebiotics interact directly with TLRs of immune cells to activate immune response (Ale et al., 2011). TLRs are pattern recognition receptors (PRR) designed to recognize pathogen-associated microbial patterns that signal the activation of nuclear factor kappa-light-enhancer of activated B cells (NF-κB) and other transcription factors, thus increasing the secretion of cytokines (de Kivit et al., 2014). Fructans primarily activate TLR-2, and to a lesser degree, TLR-5, 7, 8, and also the nucleotide-binding oligomerization domain containing proteins 2 (NOD 2). This led to the stimulation of NF-κB/Activator Protein 1 (AP-1) in human peripheral blood mononuclear cells (Vogt et al., 2013). The immune-enhancing potential of prebiotics (GOS, FOS, mannanoligosaccharides, inulin, and

arabinoxylan) in fish models have been extensively reviewed by Akhter et al., (2015) and Song et al., (2014).

Preliminary evidence supporting the immune enhancing activities of BSP have been well documented by Ale et al., (2011) and Fitton et al., (2015), with focus on fucoidan activity. It has been reported that fucoidans induce immune response via macrophage activation mediated by membrane receptors (Toll-Like Receptor 4 (TLR 4), cluster of differentiation 14 (CD 14), competent receptor-3 (CR-3), scavenging receptor (SR)) leading to the signal transduction via mitogen-activated protein kinase (MAPK) and the activation of transcription factors that induce the production of cytokines, which control and regulate other activities such as activation of natural killer (NK) cells and T lymphocytes (Ale et al., 2011). Jin and colleagues reported that a *Fucus vesiculosus* fucoidan extract treatment up-regulated pro-inflammatory cytokines (IL-6, IL-12, and tumour necrosis factor (TNF)-α) in serum and spleenocytes of C57BL/6 mice after 3 hr of administration (Jin et al., 2014). Another study found that *Laminaria japonica, L. cichorioides,* and *F. evanescens* polysaccharides served as TLR ligands that interacted with TLR-2 and TLR-4 *in vitro* to activate NF-κB. This, in turn, led to the expression of the defence effector mechanism of innate immunity including secretion of cytokines, chemokines, and expression of MHC class I and II molecules, which are necessary for defence against foreign invaders and activation of adaptive immune response (Makarenkova et al., 2012). Negishi et al., (2013) conducted a clinical trial on adult male and female volunteers supplementing their diet with 1 g/day of fucoidan from *Undaria pinnatifida* for 24 weeks (Negishi et al., 2013). This study reported enhanced immune response to seasonal influenza vaccine through antibody production. Guluronate oligosaccharides obtained from the enzymatic degradation of alginate induced TNF-α, nitric oxide (NO), and reactive oxygen species

(ROS) production, whereas mannuronate oligosaccharides from enzymatic degradation showed no significant effect on ROS and NO production, but significantly increased the production of TNF- α in RAW 264.7 cells (Xu et al., 2014). Iwamoto et al., (2005) also reported that unsaturated alginate oligomers from enzymatic depolymerization stimulated the secretion of TNF- α in RAW 264.7 cells, while saturated alginate polymers from acid hydrolysis induced secretion of trace levels (Iwamoto et al., 2005). Based on these studies, there seems to be evidence of various mechanisms by which prebiotics modulate immune activities. This is achieved primarily through: (a) interaction with intestinal cells of the gut microbiota, and (b) direct stimulation of immune cells through the TLRs.

2.7. CONCLUSION

This review highlighted the structural and chemical properties of BSP (laminarin, fucoidan, and alginate), their extraction, mechanism and evidence of prebiotic and immunomodulatory activities. Prebiotics exert immunostimulatory effects through the stimulation of beneficial gut bacterial populations/activity and direct interaction with pattern recognition receptors of immune cells. From the *in vitro, in vivo,* and limited clinical evidence on both prebiotic and immunomodulatory potential, BSP has strong prospects for applications in prebiotic formulations as well as immune boosting supplements. However, more clinical trials are encouraged to consolidate these claims for the respective health claim. Future studies should also focus on the influence of extraction methods on the structural properties and prebiotic potential of BSP.

Delivery mechanisms of BSP and possible incorporation into functional foods should also be considered. Active research of the future perspectives will enhance the commercialization prospects of BSP.

2.8. ACKNOWLEDGMENTS

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TABLE 2.1. EVIDENCE OF PREBIOTIC ACTIVITY OF BROWN SEAWEED POLYSACCHARIDES

BSP	Source	Stage of trial	Evidence of prebiotic activity	References
β glucans	*Laminaria digitata*	*in vitro*	Supported the growth of *Bifidobacteria* species, increased SCFA (propionate and butyrate) levels, and decreased pH.	Zhao & Cheung, 2011
Laminarin	*Laminaria digitata*	*in vivo*	Increased acidic mucin production indicative of potential modulation of mucus production in Wistar rats	Deville et al., 2007
Laminarin and fucoidan	*Laminaria hyperborea*	*in vivo*	Stimulated the growth of *Lactobacilli* while reducing the enterobacteria population in the gut of pigs.	Lynch et al., 2010
Fucoidan	*Laminaria japonica*	*in vitro*	Reduced pH level, increased population of beneficial bacteria, and production of SCFA.	Kong et al., 2016
LMW polysaccharides from alginate bearing seaweed	Commercial source alginate powder (FMC Biopolymer, UK) & *Ascophyllum nodosum*	*in vitro*	Enhanced the growth of *Bifidobacteria* and production of SCFA	Ramnani et al., 2012
Brown seaweed polysaccharides	*Ecklonia radiata*	*in vitro*	Promoted the growth of beneficial bacterial populations (*Bifidobacteria* and *Lactobacilli*) as well as SCFA production	Charoensiddhi et al., 2016
Alginate oligosaccharide derived from enzymatic hydrolysis	Purchased from Qingdao Yijia Huayi Import and Export Co Ltd, China	*in vitro* *in vivo*	Resistant to digestive enzymes (amylases and proteases) in the upper gastrointestinal tract; and stimulated the growth of *Bifidobacteria* and *Lactobacilli* Selectively increased the number of *Bifidobacteria* and *Lactobacilli* in rats	Wang et al., 2006

TABLE 2.2. EVIDENCE OF IMMUNE STIMULATORY ACTIVITIES OF BROWN SEAWEED POLYSACCHARIDES

Evidence of immune response	BSP/fraction/extracts	Source	Stage of trial	References
Stimulated the production of TNF-α in human monocytes	β-1,3 glucan oligomer (from Laminarin)	*Laminaria digitata*	*in vitro*	Miyanishi, Iwamoto, Watanabe, & Oda, 2003
Decreased TNF-α and nitrite levels in *E. coli* lipopolysaccharide challenged male Wistar rats	Laminarin	Supplied by Goemar (St. Malo, France)	*in vivo*	Neyrinck, Mouson, & Delzenne, 2007
Increased the expression of IL-1β, IL-8, and TLR-2 in serum of *Epinephelus coioides* fish model	Laminarin	Commercially produced by Addison Biological Technology (Beijing, China)	*in vivo*	Yin et al., 2014
Extracts from both sources supplemented (individually or in combination) had no significant influence on TNF α, IL- 1α, IFN γ, IL 4, IL 6 of pigs. However, supplementation with both *L. hyperborea* and *L. digitata* extracts increased IL-8 expression in pigs	Seaweed extract (laminarin + fucoidan)	*L. hyperborea* *L. digitata*	*in vivo*	Reilly et al., 2008
Interaction with TLR 2 and TLR 4 to activate NF-κB in HEK293 eukaryotic cells	Sulphated polysaccharides	*Laminaria japonica, Laminaria cichorioides, Fucus evanescens*	*in vitro*	Makarenkova et al., 2012
Enhanced dendritic cells (DC) maturation in human monocytes	Fucoidan	*F. vesiculosus*	*in vitro*	M. Yang et al., 2008
Up regulation of TNF-α induced secretion of matrix metalloproteinase – 9 (MMP-9) (an enzyme necessary for migration of immune cells) in monocytic cell line U937	Fucoidan	*F. vesiculosus*	*in vitro*	Jintang et al., 2010
Increased phagocytosis, lysozyme activity and production of nitric oxide (NO), hydrogen peroxide, TNF-α, and IL-6 in splenic lymphocytes of BALB/c mice	Fucoidan	*F. vesiculosus*	*in vitro*	E.-M. Choi, Kim, Kim, & Hwang, 2005
Suppression of anti-inflammatory cytokines – IL-4, IL-5, IL-13 in male BALB/c mice	Fucoidan	*Undaria pinnatifida*	*in vivo*	Maruyama, Tamauchi, Hashimoto, & Nakano, 2005

TABLE 2.2. EVIDENCE OF IMMUNE STIMULATORY ACTIVITIES OF BROWN SEAWEED POLYSACCHARIDES

Increased production of TNF-α, IL-12, and maturation of dendritic cells via NF-κB signalling pathway in C57BL/6 mice	Fucoidan	*F. vesiculosus*	*in vivo*	Mi Hyoung Kim & Joo, 2008
Upregulation of pro-inflammatory cytokines (IL-6, IL-12, TNF-α) in serum and spleenocytes of C57BL/6 mice; maturation of dendritic cells and antibody production in C57BL/6 mice.	Fucoidan	*F. vesiculosus*	*in vivo*	Jin et al., 2014
Increased antibody production in serum of elderly Japanese men and women after they were subjected to influenza vaccination.	Fucoidan	*U. pinnatifida*	Clinical trial	Negishi et al., 2013
Stimulated the production of NO, reactive oxygen species (ROS), TNF-α, and the expression of inducible nitric oxide synthase (iNOS) in RAW264.7 murine macrophages cell line.	Alginate (Unsaturated guluronate oligosaccharides from enzymatic degradation)	Purchased from Nuotai, (Shangai, China)	*in vitro*	Xu et al., 2014
Increased TNF-α production, with no significant effect on the NO and reactive oxygen species production.	Alginate (Unsaturated mannuronate oligosaccharide from enzymatic degradation)	Purchased from Nuotai, (Shangai, China)	*in vitro*	Xu et al., 2014
Enhanced the production of TNF-α, IL-1α, IL-1β, and IL-6 in RAW264.7 cell line	Unsaturated alginate oligomers (Guluronate and mannuronate oligomers)	Purchased from Nacalai Tes-que Incorporation. (Kyoto, Japan)	*in vitro*	Iwamoto et al., 2005
Produced pro-inflammatory cytokines (IL-1β, IL-6, IL-12 and TNF-α) in RAW 264.7 cell line	Alginate (M/G ratio = 1.96 MW = 9500 kDa)	Purchased from Sigma (Ontario, Canada)	*in vitro*	D. Yang & Jones, 2009
Stimulated immunological activity of intestinal cells through the Peyer's patch cells of C3H/HeJ mice	β – D – Mannuronate residue of Alginate	*L. japonica*	*in vivo*	Suzuki, Christensen, & Kitamura, 2011

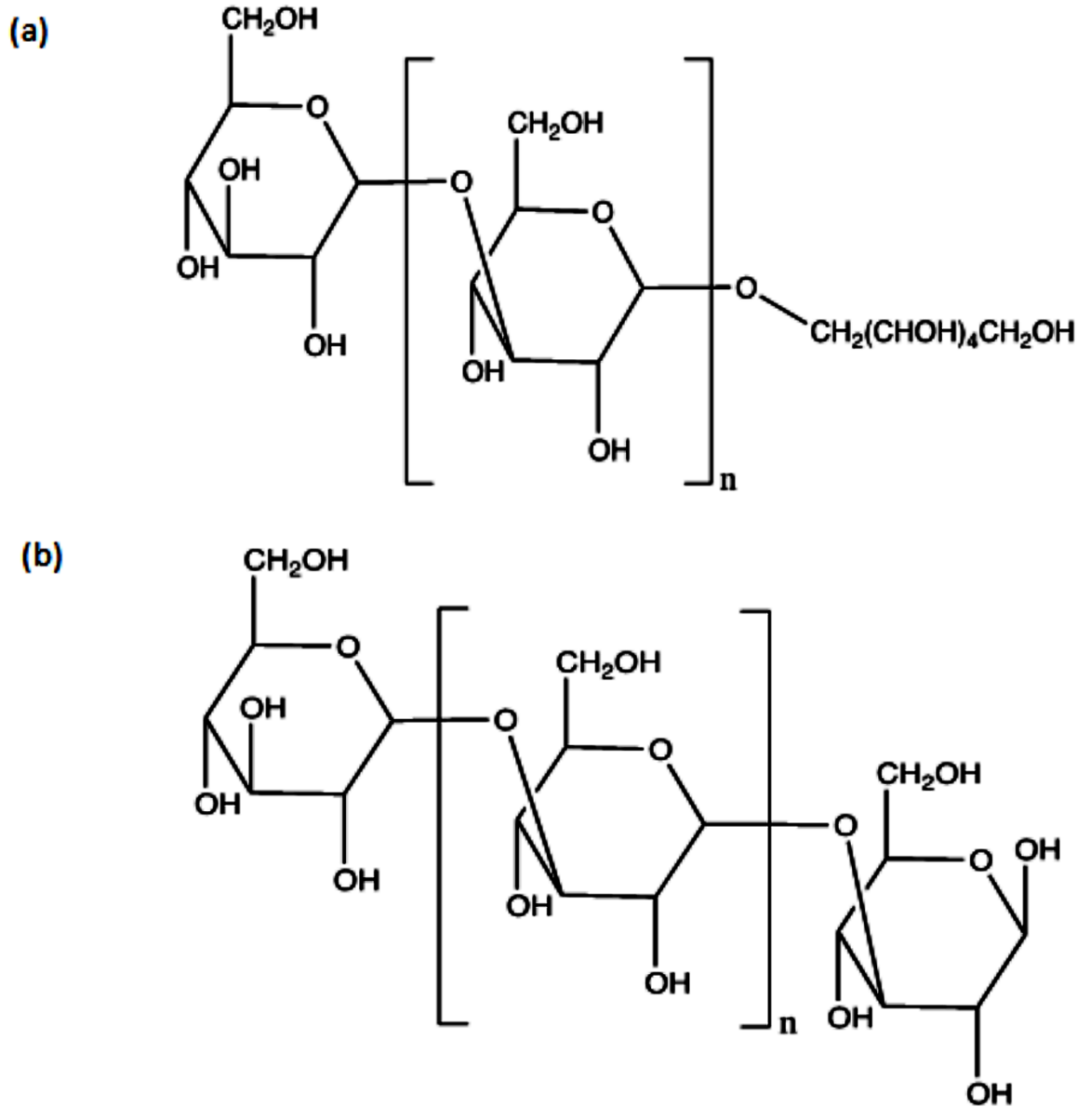


Fig. 2.1. (a) Mannitol chain structure of Laminarin. (b) Glucose chain structure of Laminarin (from Kadam et al., 2015)

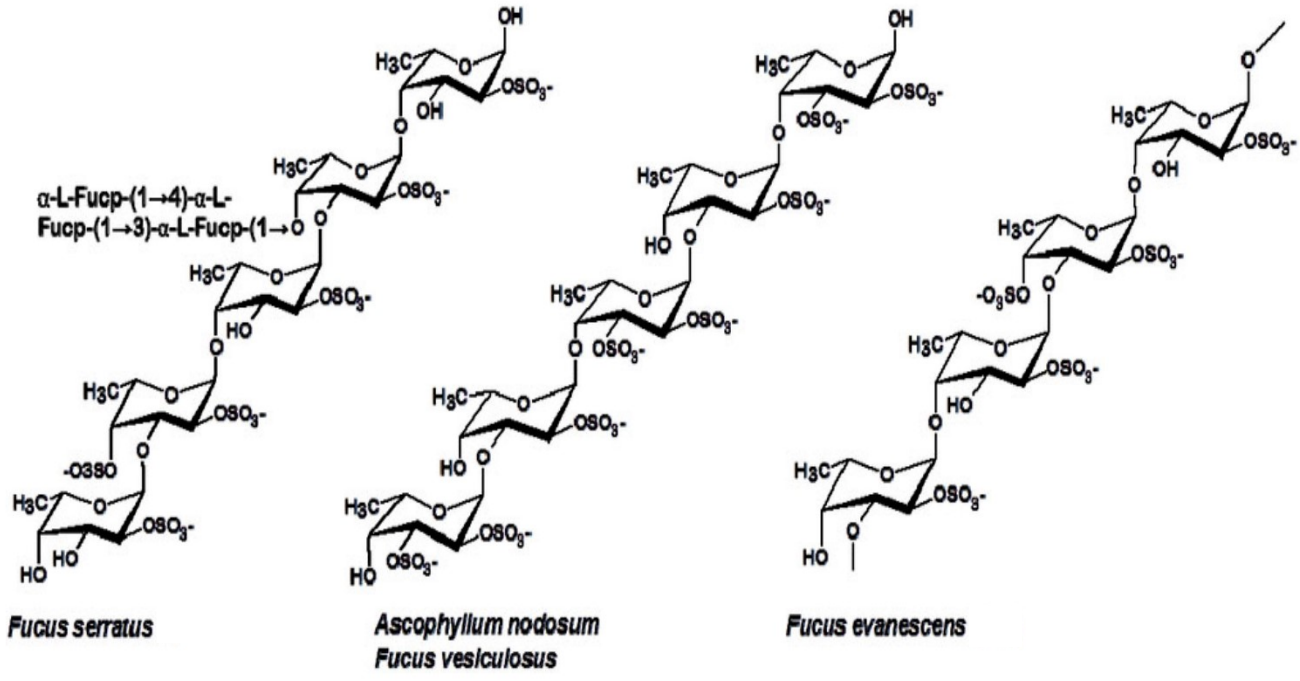


Fig 2.2. Fucoidan structure from various sources showing the changes in position of sulphate group (from Ale et al., 2011)

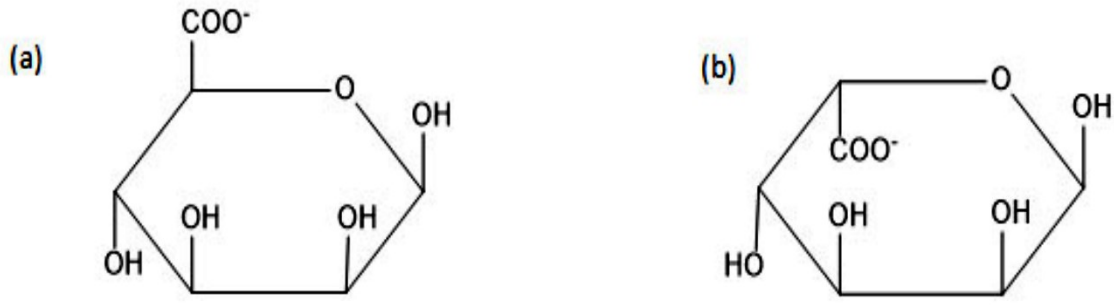


Fig 2.3. (a) Mannuronate structure of alginate. (b) Guluronate structure of alginate (from Draget & Taylor, 2011)

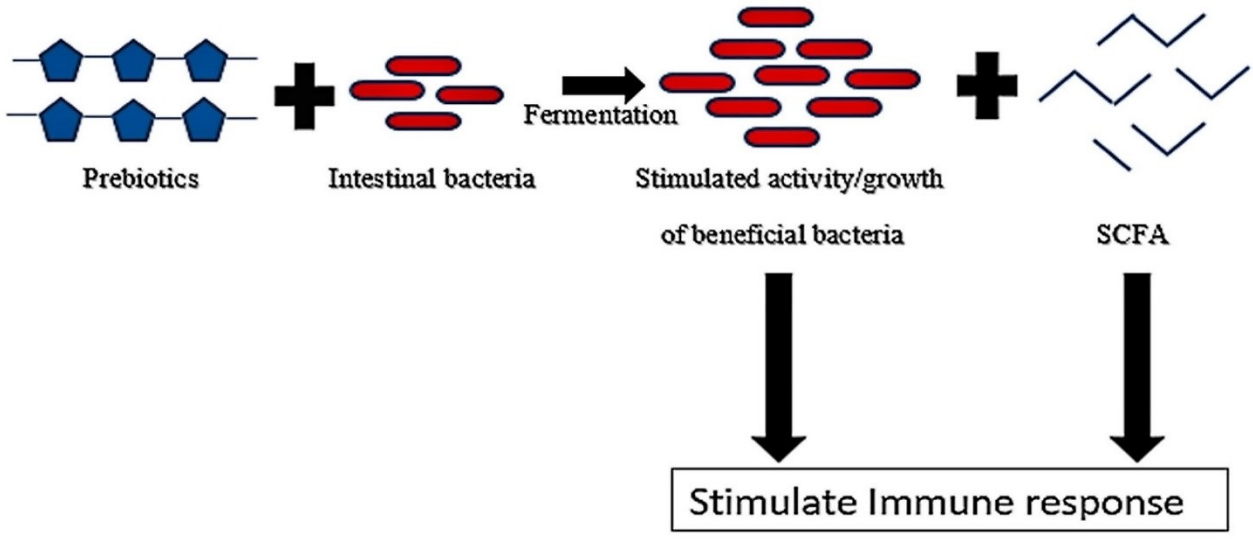


Fig 2.4. Prebiotics enhance immune response through its association with intestinal bacteria

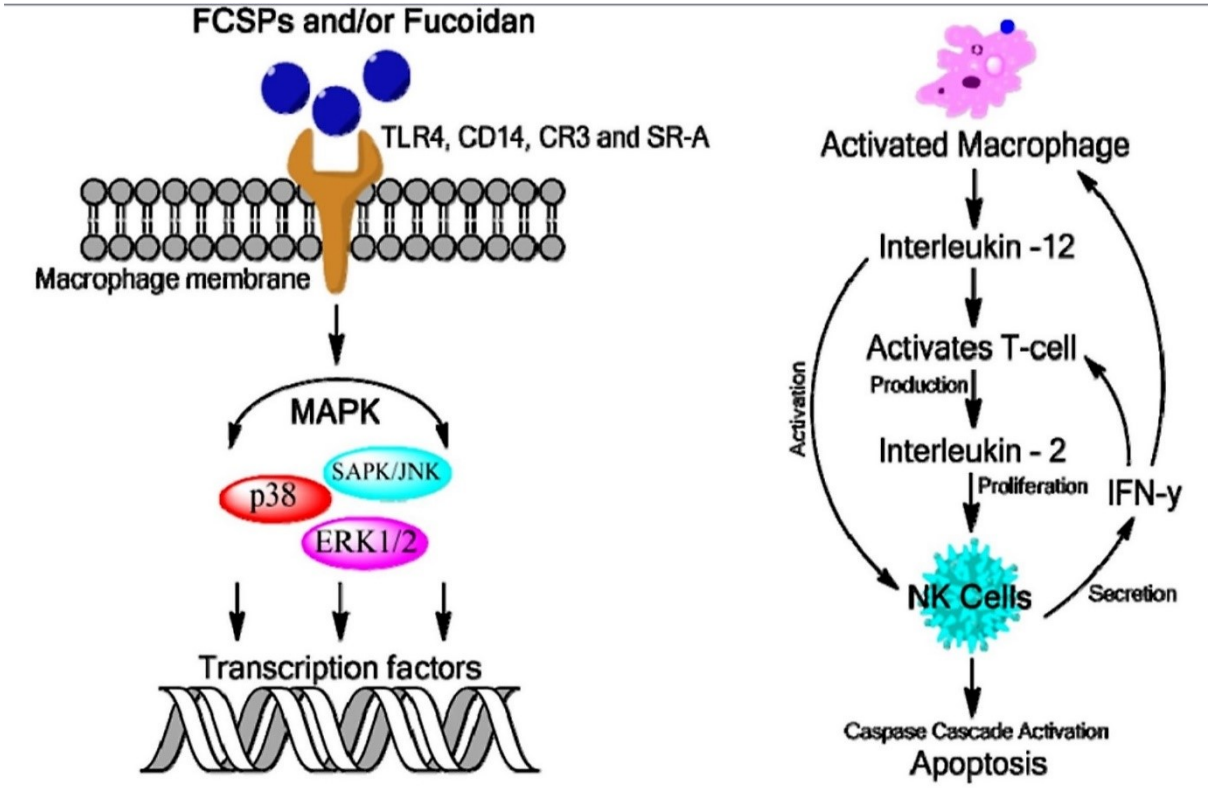


Fig 2.5. Fucoidan interaction with macrophages (from Ale et al., 2011)

CHAPTER 3

THE STRUCTURE-FUNCTION RELATIONSHIP BETWEEN FUCOIDAN EXTRACTS FROM *ASCOPHYLLUM NODOSUM* AND *IN VITRO* PREBIOTIC ACTIVITY: AN ASSESSMENT OF THE IMPACT OF EXTRACTION TECHNOLOGIES

3.1. ABSTRACT

The influence of conventional and novel extraction technologies on the structure-prebiotic activity relationship of fucoidan was investigated. Fucoidan extracted using conventional chemical (CCE), microwave-assisted (MAE), ultrasound-assisted (UAE) and enzyme-assisted (EAE) extraction were characterized and their prebiotic activity was determined. Single (1×) and three times (3×) extractions were performed using CCE, MAE, and UAE, but only EAE (1×) was performed. The CCE (3×) had significantly higher extract yield as compared to MAE (3×), UAE (3×), and EAE (1×). UAE (3×) fucoidan extract had significantly ($p < 0.05$) lower fucose content and no significant difference was observed in the galactose content among the extracts. The sulphate levels in MAE (3×) and CCE (3×) were significantly higher than UAE (3×) and EAE (1×). Average molecular weight range for CCE (3×), MAE (3×), UAE (3×) and EAE (1×) were 97.5, 81.2, 136.3 and 115.2 kDa, respectively. Although fucoidan supplemented MRS from all methods had significantly ($p < 0.05$) higher growth rates of *Lactobacillus delbruecki*, there was no significant difference in prebiotic activity amongst the extracts. The prebiotic activity of fucoidan extracts was comparable to the commercial prebiotic, inulin, thus confirming its capacity to improve growth rate of the probiotic strains. The results indicated that the MAE extracts had better physicochemical characteristics (fucose & galactose, sulphate,

molecular weight and dispersity index) than the other extracts, but had no significant advantage in prebiotic activity.

3.2. INTRODUCTION

Seaweeds are a rich source of nutrients, bioactive molecules, and industrial starting materials, with a vast array of applications in the food, cosmetics, pharmaceutical, and textile industries (Ale et al., 2011; Bleakley & Hayes, 2017; Charoensiddhi et al., 2017; Peng et al., 2015; Rioux & Turgeon, 2015). Among the nutrients and bioactives present in seaweeds (proteins, polysaccharides, vitamins, minerals, polyphenols, peptides, amino acids, pigments, secondary metabolites) polysaccharides are the most prominent and widely explored for commercial relevance (Marudhupandi et al., 2015; Rioux & Turgeon, 2015; Youssouf et al., 2017). The economic value of seaweeds is estimated at about $US 7.4 billion (FAO, 2014; Youssouf et al., 2017), with annual global production of seaweed hydrocolloids alone valued at approximately $US 1.1 billion (Charoensiddhi et al., 2017). Brown seaweeds, especially, *Ascophyllum nodosum,* are abundant and a readily available source of polysaccharides (Rioux & Turgeon, 2015).

Fucoidan is a term used to describe a group of sulphated polysaccharides with high fucose content and lower amounts of other monosaccharides such as galactose, mannose, glucose and uronic acids (Lim et al., 2016; Zhao et al., 2016). These biomolecules are embedded in the fibrillar cell walls and intercellular spaces of brown seaweed. Fucoidans consist primarily of linear or alternating α-(1,3) and α-(1,4) linked L-fucopyranose residues that may be sulphated at C-2 or C-4 but rarely on C-3 (Ale et al., 2011). Fucoidans have been investigated for a number of pharmacological potentials and have shown promising

prospects as functional ingredients. Among the many health ameliorating prospects are anti-cancer (Marudhupandi et al., 2015) anti-inflammatory (Sanjeewa et al., 2017), anti-thrombotic (Zhao et al., 2016), anti-viral (Thuy et al., 2015), anti-oxidant (Wang et al., 2010), anti-diabetic (Shan et al., 2016), neuro-protective (Meenakshi et al., 2016), and prebiotic (Charoensiddhi et al., 2017) potentials. The physicochemical properties, such as monosaccharide composition, sulphate levels, and molecular weight are important determinants of the biological function of fucoidan, hence the need to maintain its structural integrity during extraction and purification (Ale et al., 2011; Fitton et al., 2015).

The industrial and fast-growing economic relevance of various seaweeds has attracted the application of technological methods, such as MAE, UAE, and EAE, in enhancing existing methods. These technologies, with different *'modus operandi'* target degrading the cell walls of brown seaweeds where most of the bioactive molecules reside, including fucoidan. These technological applications, although still primarily applied at the laboratory-scale, are expected to improve extraction efficiencies & yield in a shorter time, and maintain the structural integrity fucoidan extracts. However, this may not always be the case, as high energy inputs in the form of microwave heating and sound energy, may have adverse effects on the structural stability of heat-labile components. Hence, investigating the impact of extraction technologies on key structural components will help industries to better understand the pros and cons of these methods and a possible upscale of either of these extraction processes.

Of the various health-promoting characteristics, prebiotic activity seems to be promising, since fucoidan could be utilized as carbon source for the growth of beneficial gut microbial populations. Prebiotics are non-digestible food components fermented preferentially by

intestinal microbes in the gut and selectively stimulate the growth of beneficial bacterial populations, e.g *Lactobacilli* and *Bifidobacteria* (Gibson et al., 2004). Fucoidans are prospective prebiotics as they meet the requirements for the classification of prebiotics which are: non-digestible, fermented by gut microbiota, and selectively stimulate beneficial bacteria strains (Gibson & Roberfroid, 1995) There appears to be limited evidence supporting the prebiotic activity of fucoidan *in vitro* and *in vivo*, and sparingly at human trials (Kong et al., 2016; Lynch et al., 2010; Okolie et al., 2017). Hence, more validatory tests are required before the possible commercialization of fucoidan products as prebiotics. *Lactobacillus casei* and *L. delbruecki* are two probiogenic facultative anaerobes that can be found in the human hind gut. They have been reported to improve gut health when consumed in dairy products (Cats et al., 2003).

This study therefore investigated the influence of four extraction processes, CCE, MAE, UAE, and EAE, on the structure and prebiotic activity of fucoidan extracts using *L. casei* and *L. delbruecki* ss. *bulgaricus* as probiotic strains.

3.3. MATERIALS AND METHODS

3.3.1. MATERIALS

Air-dried whole *Ascophyllum nodosum* was provided by Acadian Seaplants Limited, Dartmouth, NS, Canada. The whole harvested seaweed was chopped and pulverized into fine powder prior to pre-extraction, with a food processor (Black and Decker, China FP2500C) using the stainless-steel chopping blade (5½ inches). Monosaccharide standards (glucose, fucose, fructose, mannose, mannitol, galactose), short-chain fatty acid standards

(propionic, butyric, iso-butyric, valeric and iso-valeric acids), sodium carbonate, Celluclast, sodium phosphate, and de Man, Rogosa, and Sharpe (MRS) broth were all purchased from Sigma Aldrich (St. Louis, USA). Hydrochloric acid, sulphuric acid, and ethanol were purchased from Fisher Chemicals (Belgium). *Lactobacillus delbruecki* ss *bulgaricus* and *L. casei* were both obtained from Ward Science (USA). Sodium acetate was purchased from OmniPur® (Germany), acetic acid from BDH VWR Analytical (USA) and a prebiotic standard, inulin, from Beneo GmbH (Germany).

3.3.2. EXTRACTION OF FUCOIDAN FROM BROWN SEAWEED

3.3.2.1. PRE-EXTRACTION

Milled *Ascophyllum nodosum* was pre-extracted with ethanol to remove interfering compounds such as pigments and proteins, following the method reported by Rioux et al., (2007) with some modifications. The milled seaweed (100g) was hydrated in 1 L of 80% (v/v) ethanol and kept under constant mechanical stirring for 20 hours at room temperature (23 °C). Afterwards, the temperature of the mixture was increased to 70 °C and stirred for another 5 hours. This step was done twice, and residual seaweed was separated from the mixture via vacuum filtration using Whatman® grade 1 qualitative filtration paper.

3.3.2.2. CONVENTIONAL CHEMICAL EXTRACTION (CCE)

Extraction of fucoidan from *A. nodosum* using the conventional chemical method was followed as outlined by Rioux et al., (2007) and Yuan & Macquarrie, (2015) with some

modifications. Pre-extracted seaweed (10g) was added to 100 mL of 0.01M HCl and heated at 70 °C for 3 hours. Another 10g of pre-extracted seaweed was also added to a separate 100 mL of 0.01M HCl, heated at 70 °C for 3 hours, and the chemical treatment applied for three separate extractions on the same residue. Afterwards, filtrates from the three separate extractions were then combined in a separate beaker. Equal volumes (1:1) of 2% (w/v) calcium chloride ($CaCl_2$) were added to the filtrates of both single and triple extraction for alginate removal. The mixtures were kept overnight at 4 °C after which they were centrifuged (3000 ×g, 20 min) and the liquid fractions, precipitated with 95% ethanol (4:1). The precipitated fucoidan samples from both single and triple extraction were freeze dried, ground with a ball mill (for 20 min with medium-size spherical balls), and stored in Falcom sterile air-tight containers at room temperature, for further analysis..

3.3.2.3. MICROWAVE-ASSISTED EXTRACTION (MAE)

The microwave-assisted extraction of *A. nodosum* polysaccharides was performed as described by Yuan and Macquarrie, (2015). Pre-extracted seaweed (1g each) was added to three 35 mL reactor tubes, each containing 10 mL of 0.01M HCl. Microwave irradiation of the mixtures was done at 90 °C for 15 min for fucoidan extraction, using CEM Discover and Explorer microwave (MARS 6 230/60 910900, USA). Following irradiation, the mixtures were centrifuged (3000 ×g; 20 min), and the supernatants precipitated with 2% (w/v) $CaCl_2$ (1:1) and kept over-night at 4 °C for alginate removal. The colloidal mixtures were then centrifuged, and the filtrates precipitated with 95% ethanol (4:1). Another 1g of pre-extracted seaweed underwent all the above-mentioned steps, this time, for three separate extractions consecutively, on the same residue, after which, the filtrates were

combined prior to precipitation with $CaCl_2$ (1:1). Fucoidan precipitates (with 95% ethanol) from both single and triple extraction were freeze dried, ground using a ball mill (for 20 min with medium-size spherical balls), and stored at room temperature in air-tight containers at room temperature.

3.3.2.4. ULTRASOUND-ASSISTED EXTRACTION (UAE)

The ultrasound extraction of *A. nodosum* polysaccharides was performed as outlined by Kadam et al., (2015) with some modifications. Pre-extracted (10g) seaweed was suspended in 100 mL of 0.01 M HCl and sonicated (20 kHz; 35 min) continuously with a half inch (13 mm in diameter) probe with removable tip, at an amplitude of 40%, using Sonics and Materials Inc. Ultrasonicator (USA VCX750). The sonicated mixture was then centrifuged (3000 ×g; 20 min) and 2% (w/v) $CaCl_2$ (1:1) was added to the filtrate and stored overnight at 4 °C for alginate precipitation and removal. The colloidal mixture was then centrifuged, and the filtrate precipitated with 4 volumes of 95% ethanol. A separate 10g of pre-extracted seaweed was used for a triple extraction process, with three separate extractions on the same residue, using the same method and conditions outlined above. Filtrates from the three separate extractions were combined prior to precipitation with $CaCl_2$. Fucoidan precipitates (with 95% ethanol (4:1)) from both single and triple extractions were freeze dried, ground with a ball mill (for 20 min with medium-size spherical balls) and stored in Falcom sterile air-tight containers for further analysis at room temperature.

3.3.2.5. ENZYME-ASSISTED EXTRACTION (EAE)

A. nodosum polysaccharides were extracted using cellulase enzyme according to the method described by Charoensiddhi et al., (2016). Pre-extracted seaweed (10g) was suspended in 100 mL of sodium acetate buffer (pH 4.5) and heated to optimum temperature (50 °C) in a water bath with an orbital shaker. Celluclast enzyme (1 mL) was added to the mixture and enzymatic hydrolysis went on for 24 hours at optimum enzyme conditions (pH 4.5, 50 °C). At the end of the hydrolysis, the temperature was increased to 100 °C for 10 min to inactivate the enzyme and cooled immediately in an ice bath. The mixture was then centrifuged (3000 ×g, 20 min), and the supernatant precipitated with 2 % (w/v) $CaCl_2$ and kept overnight at 4 °C for alginate removal. The supernatant after alginate removal was precipitated with ethanol (4:1), after which the filtrate was freeze-dried, ground (for 20 min with medium-size spherical balls) and stored in Falcom sterile air-tight containers at room temperature.

3.3.3. MONOSACCHARIDE PROFILING OF FUCOIDAN EXTRACTS

3.3.3.1. HYDROLYSIS OF FUCOIDAN EXTRACTS

Fucoidan extracts from all four extraction methods were hydrolysed as prescribed by Chen et al., (2016) and Templeton et al., (2012)**.** In brief, 5 mg of fucoidan extracts were dissolved in 2 mL of 4 % sulphuric acid and hydrolysed in a sealed glass ampoule at 121 °C for 1 hour. At the end of the hydrolysis, samples were centrifuged at 3000 ×g for 1 min, after which 1 mL of the hydrolysed fucoidan extracts were added to HPLC vials for monosaccharide quantification.

3.3.3.2. HPLC ANALYSIS OF FUCOIDAN MONOSACCHARIDE CONTENT

The monosaccharide content of the fucoidan extracts were determined using a Perkin Elmer HPLC system using a refractive index (RI) detector. The data were obtained using Chromera software manager provided by Perkin Elmer Company (USA). Monosaccharide standards (glucose, fucose, xylose, galactose, mannose, mannitol, fructose) and fucoidan extracts were analysed using a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) with 0.01 M sulphuric acid as mobile phase, at a flow rate of 0.6 mL/min, column temperature of 60 °C, and an injection volume of 50 µL (Templeton et al., 2012). Monosaccharide standards with concentrations of 0.5, 1.0, 1.5, 2, and 3 mg/mL were analysed and plotted against their respective peak areas to obtain a standard curve and equation used to quantify the detected monosaccharides in fucoidan extracts.

3.3.4. SULPHATE CONTENT ANALYSIS OF FUCOIDAN EXTRACTS

The sulphate content of fucoidan extracts was determined using the Perkin Elmer series II CHNS/O Analyser 2400 (USA) which gives the percentage carbon, hydrogen, nitrogen and sulphur content of a given sample. Briefly, fucoidan extracts were weighed using the Perkin Elmer Autobalance AD6000 (USA), with sample weights maintained between 1.5 and 2.5 mg. The weighed samples were inserted into the CHNS/O analyser for sample combustion (at 975 °C with adequate oxygen supply and 500 ºC for reduction) and detection via a thermal conductivity detector. The data were collected using Perkin Elmer 2400 Data Manager. The percentage sulphate content of fucoidan extracts was calculated

from the percentage sulphur content of the extracts using a conversion factor of: percentage sulphate = percentage sulphur × 3.22 (Roger et al., 2004).

3.3.5. DETERMINATION OF URONIC ACID CONTENT OF FUCOIDAN EXTRACTS

The uronic acid content of fucoidan extracts was determined as described by Cesaretti et al., 2003. Different concentrations (250, 125, 62.5, 31.25, 15.625, 7.813 and 0 µg/L) of standards (guluronic acid, mannuronic acid, and glucuronic acid) and fucoidan extracts (1 mg/mL) were prepared prior to the analysis. Standard solutions and samples (50 µL) were added to a 96 well plate. Subsequently, 25 mM of sodium tetraborate in concentrated sulphuric acid (200 µL) was added. The plate was then heated in an oven (100 ºC, 15 min), after which it was left to cool for 15 min. After cooling, 0.125 % of carbazole in absolute ethanol was added and heated in an oven at 100 ºC for another 15 min. The microplate was then left to cool for 15 min and the absorbance readings taken at 550 nm. The glucuronic acid equivalent of the samples was obtained from the respective standard curves.

3.3.6. MOLECULAR WEIGHT DETERMINATION OF FUCOIDAN EXTRACTS

The molecular weight distribution of fucoidan extracts was analysed using the Agilent Technologies 1260 Infinity II Gel Permeation Chromatography (GPC) System according to steps outlined by Lim et al., (2016). The GPC system consist of an Agilent pump, an Agilent autoinjector and a refractometer. The GPC system was connected to a multi-angle laser light scattering detector (MALLS) and an RI detector. The PL – aquagel-OH

MIXED-M 8 µm 300 x 7.5 mm (PL1149-6801) column was used for the molecular weight analysis. Sodium acetate (0.1 M) prepared with ultrapure water and filtered through a 0.45 µm filter was used as mobile phase. The GPC/SEC system calibration and performance were verified using Agilent GPC/SEC Calibration kits containing pullulan polysaccharides with molecular weights ranging from 0.18 kDa – 642 kDa. A differential refractive index increment (dn/dc) value of 0.129 for galacto-fucans was used and the data obtained were analysed using the Agilent GPC/SEC software manager. The average molecular weight, number average molecular weight, polydispersity index and peak area data were obtained from the analysis.

3.3.7. DETERMINATION OF SODIUM, POTASSIUM AND CALCIUM CONTENTS OF FUCOIDAN EXTRACTS

The sodium, potassium and calcium content of fucoidan extracts were quantified using the Thermo-Fischer M-Series Flame Atomic Absorption Spectrometer (AAS). The AAS was equipped with a deuterium lamp for background correction and air-acetylene flame for the quantification of the minerals. The wavelength and bandwidth used for the analysis were 589 nm and 0.2 nm for sodium; 766.5 nm and 0.5 nm for potassium; 422.7 and 0.5 nm for calcium. Sodium, potassium and calcium equivalents of sodium chloride, potassium chloride and calcium chloride were prepared at different concentrations (0, 1, 3, 5, 7, and 10 ppm) and measured. The absorbance of fucoidan samples (10 and 100 ppm) were measured and the concentrations of each of the trace elements were determined from their respective standard curves.

3.3.8. PREBIOTIC ACTIVITY OF FUCOIDAN EXTRACTS

3.3.8.1. GROWTH RATE AND DOUBLING TIME

The effect of fucoidan extracts on the growth rate of two strains of *Lactobacilli*, *Lactobacillus casei* and *Lactobacillus delbruecki* ss *bulgaricus,* was investigated in accordance with steps described by Chen et al., (2016) with some modifications. Briefly, both *L. casei* and *L. delbruecki* strains were cultured on MRS agar for about 48 – 96 hours. The MRS agar was prepared by adding 1.2% agar to a MRS broth (5 % w/v). The mixture was subjected to mechanical stirring until the agar was completely dissolved in the solution, after which it was sterilized at 121 °C for 25 min. After sterilization, the MRS was cooled, poured into petri dishes and left over-night in the fume hood. Isolated strains from both bacterial cultures were inoculated in freshly prepared and sterilized MRS broth and incubated until the turbidity (optical density at 600 nm) of the culture was 1.0.

Inulin (positive control), glucose (negative control) and fucoidan extracts were added to MRS broths separately, at concentrations of 0.1, 0.3 and 0.5 % (w/v). Supplemented and un-supplemented MRS broths (200 µL) were incubated with *L. casei* and *L. delbruecki* cultures (7.5 µL) in a sealed 96-well plate for 24h. The optical density (OD at 600 nm) measurement was recorded every 0.5 hour and the temperature maintained at 37 °C throughout the experiment. MRS broth and supplemented samples without inoculation were also added as blank control. At the end of the incubation period, the optical densities of the blank samples were subtracted from those of the inoculated samples, and the resulting data were plotted against time to obtain a growth curve. Growth rates and doubling times were calculated from the exponential phase of the growth curve using the formula below:

$$\text{Growth rate} = \frac{\log Nt - \log No}{0.301 \times t}$$

Where, Nt = cell number or optical density at time, t

No = cell number or optical density at initial time

t = difference between final time and initial time

Doubling time = 1/growth rate (Neidhardt et al., 1990)

3.3.8.2. SHORT CHAIN FATTY ACIDS QUANTIFICATION

The short chain fatty acids (SCFA), often referred to as volatile fatty acids (VFA), were quantified in cultured samples after 24-hours of incubation period as described by Chen et al., (2016). Cultured samples were centrifuged (3000 ×g; 5 min) to decant bacterial debris. The top layers were collected and analysed for SCFA. SCFA standards (acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, and iso-valeric acid) and samples were analysed using the Perkin Elmer HPLC system and Bio-Rad Aminex HPX-87H column, with 0.005 M sulphuric acid as mobile phase at a flow rate of 0.6 mL/min and column temperature of 50 °C. The data were collected and analysed using Chromera software manager.

3.3.8.3. pH CHANGES DURING MRS INCUBATION

The pH of the MRS broth and all cultured samples was measured prior to a 24-hour incubation period and after the incubation period using a pH meter (Thermo Fischer Scientific, USA). The pH meter was calibrated with standard solutions of pH 4, 7, and 10, prior to sample pH measurement.

3.3.9. STATISTICAL ANALYSIS

All experiments, apart from the pH measurements, were performed in triplicate. The results were expressed as mean ± standard deviation and analysed for statistical significance at $p < 0.05$, using the statistical package for social sciences (SPSS) version 20 (IBM SPSS statistics for windows, Armonk, New York, USA). The Levene's and Welch's tests for homogeneity of sample distribution were performed, followed by a one-way analysis of variance (ANOVA) using the Duncan's multiple comparison test. A linear regression analysis (Table 3.7) was performed to show the relationship between structural properties and prebiotic activity. A factorial analysis to study the impact of extraction methods and number of extractions on structural properties was also performed.

3.4. RESULTS AND DISCUSSION

3.4.1. STRUCTURAL ANALYSIS OF FUCOIDAN EXTRACTS AND THE IMPACT OF EXTRACTION PROCESSES

3.4.1.1. EXTRACT YIELD

The yield of fucoidan from four extraction processes, CCE (Fuc-CCE), MAE (Fuc-MAE), UAE (Fuc-UAE), and EAE (Fuc-EAE), was compared in the present study. Three times extraction (of all methods except EAE) was performed for exhaustive recovery of fucoidan extracts. Although some studies have reported 3× extraction for the conventional method, MAE, UAE, and EAE protocols have mostly reported 1× extraction of polysaccharides from brown seaweeds (Chandía & Matsuhiro, 2008; Lim et al., 2016; Rioux et al., 2007). For effective comparison, 1× and 3× extraction processes were done for CCE, MAE and UAE. Enzymes are specific in action, and as such, the recommended 24-hour extraction period can be considered as an exhaustive time frame, given the catalytic action of enzymes. Therefore, repeating the EAE three times may be redundant.

The results (Table 3.1) indicated that there was significant ($p < 0.05$) improvement in the yield of Fuc-CCE (3×) and Fuc-UAE (3×) when compared to Fuc-CE (1×) and Fuc-UAE (1×). Although not statistically significant ($p < 0.05$), a similar trend was observed for Fuc-MAE, i.e. 3× tended to have higher fucoidan yield than 1×. Also, the yield of Fuc-CE (3×), was significantly ($p < 0.05$) higher than Fuc-MAE (3×), Fuc-UAE (3×), and Fuc-EAE (1×). Extraction processes with high extract yield are among the major targets for industries aiming to maximize production. While extract yield may, in most cases, correlate with increased amounts of active ingredients, this may not always be true, as extracts may also contain high amount of impurities and low amounts of the compound of

interest. Pre-extraction of seaweeds was conducted in this study for the removal of excipients such as pigments, proteins, and lipids (Fletcher et al., 2017), however this does not account for minerals, which has been reported to make up approximately 18-27% of *Ascophyllum nodosum* seaweed (Rioux & Turgeon, 2015). The impact of both extraction methods and number of extractions (independent factors) on extract yield (fixed factor) was also investigated. The results (Appendix A, Table A1) indicated that the choice of extraction method and number of extractions, independently had a significant ($p < 0.05$) impact on the yield. The extract yield increased significantly ($p < 0.05$) from single to triple extraction, and thus confirmed that 3× extraction produced more yield. The factorial analysis also revealed that there were significant differences between the yields obtained from the different methods. However, there was no significant ($p < 0.05$) impact of the interaction between extraction method and number of extractions on the yield of the extracts.

Similar yield for CCE extracts (Table 3.6) in this study have been reported for *A. nodosum* (20.5% w/w) (Yuan & Macquarrie, 2015a) in the literature, however some other studies recorded significantly low yields 1.75% w/w for *A. nodosum* (Yuan & Macquarrie, 2015b) and 2.75% (w/w) for *S. polycistum* (Thuy et al., 2015). This may be attributed to differences in extraction conditions, source, and species. With MAE, 14.9% (w/w) (Yuan & Macquarrie, 2015b) and 14.9% (w/w) (Yuan & Macquarrie, 2015a) were reported for *A. nodosum* species. There are limited comprehensive studies on the characterization of fucoidan from brown seaweeds using UAE and EAE within the literature.

$$\text{Extract yield (\% w/w)} = \frac{\text{Weight of extract}}{\text{Weight of pre-extracted seaweed}} \times 100$$

3.4.1.2. MONOSACCHARIDE COMPOSITION

The monosaccharide content of fucoidan is considered to be one of the most important structural properties since most of the bioactivities of fucoidan from some seaweed species have been attributed to the presence of fucose-rich sulphated polysaccharides. Thus, the impact of extraction technologies on the recovery of fucoidan monosaccharides was investigated in the present study. The results (Table 3.1) from this study showed that the extracts were made up of fucose and galactose. As expected, fucose had the highest percentage composition of monosaccharides. Amongst the extraction processes, more fucose was extracted in 3× than 1×, with a significant ($p < 0.05$) increase in Fuc-UAE (3×) as compared to Fuc-UAE (1×). Similarly, the galactose content for Fuc-UAE increased significantly in 3×, rather than the 1×. However, there were no significant increases in the galactose content of 3× and 1× extractions for the other extraction methods applied in this study. Of all the extracts from the different extraction processes, the Fuc-MAE (3×) had the highest fucose and galactose content. There were no significant differences between the yield (% w/w of fucoidan extracts), fucose (% w/w of fucoidan extracts) and galactose contents (% w/w of fucoidan extracts) of MAE (3×) and MAE (1×) extracts. Thus, suggesting that triple extraction had no significant impact on quantitative output of the aforementioned properties. The fucose content of Fuc-MAE (3×) was higher than Fuc-CCE (3×), Fuc-UAE (3×), and Fuc-EAE (1×) by 35.04%, 36.53%, and 27.15% respectively. Comparing the impact of extraction method and number of extractions (as independent factors) on fucose and galactose contents (Appendix A, Table 1), it was observed that there was no significant ($p < 0.05$) impact of the number of extractions on fucose and galactose contents. However, the choice of extraction method significantly ($p < 0.05$) influenced the fucose and galactose contents. The factorial analysis data suggested

that a 3× extraction may not have improved the fucose and galactose contents, however, the extraction methods differentially influenced the content of both monosaccharides.

Various fucoidan molecules are localized in the cell walls of different brown seaweeds and as such the degradation of their thick cell walls is required for the release of these biomolecules into extraction media. The MAE, UAE, and EAE are recent techniques with different mechanisms of action, aimed at improving the conventional chemical extraction method, which uses heat, under aqueous/mild acidic conditions, at about 70 – 80 ºC to extract fucoidan (Rioux et al., 2007; Zhang & Row, 2015).

The microwave technique uses microwave energy as a volumetrically distributed heat source generated by ionic conduction of dissolved ions and dipole rotation of polar solvents (Rodriguez-Jasso et al., 2011; Yuan & Macquarrie, 2015a). This rapid internal heating process results in the lysis of the cell wall, releasing its content into the extraction medium (Yuan & Macquarrie, 2015a).

The ultrasonication technique applied physical forces, generated by acoustic cavitation, such as shear, shockwaves, microjets and acoustic streaming in the extraction of molecules (Feng et al., 2017). Acoustic cavitation results in the rapid formation and collapse of cavitation bubbles within irradiated liquid medium, leading to intense stress and irreversible chain splitting (Yan et al., 2016).

The enzymatic method uses the cell-wall degrading enzyme, such as cellulase, to rupture seaweed cell walls and release its content into liquid solutions (Charoensiddhi et al., 2017). The capacity to efficiently hydrolyse cell walls is attributed to the fact that enzymes are specific in their actions. Cellulase for instance, will rupture the cell walls by hydrolysing cellulose, a major component of the algal cell wall structure.

The monosaccharide composition of the extracts from this study, suggested that the rapid heating technique offered by microwave was the most effective at extracting fucoidan. Also, Fuc-UAE had a significantly ($p < 0.05$) lower fucose content as compared to the other extraction processes. This suggest that the extraction conditions applied in this study may have resulted in the degradation of some of the monosaccharides. The extraction conditions (amplitude, probe size, duration, sample volume, concentration of solution) for ultrasonication in this study has previously been applied with success in the extraction of polysaccharides from *A. nodosum* as reported by Kadam et al., (2015). However, these conditions may not have been the optimal for our seaweed of interest, since seaweed composition, variability in *Ascophyllum* species from different geographies, and structural properties varies depending on factors such as seasonal change, location, source, and species. Hence, optimization of the various extraction parameters is recommended to obtain the best fit for the seaweed of interest. It is necessary for future studies to carry-out an optimization study on the seaweed species, prior to apply the same conditions from other studies. The results in the present study show that, although the conditions applied was effective for the extraction of polysaccharides from Irish *A. nodosum*, it was not as effective on the *A. nodosum* used in this study. Extraction at a lower intensity and longer duration may have improved the extraction yield and monosaccharide content, considering that high intensity may damage the structural integrity of fucoidan. Similar concerns have been expressed by Yip et al., (2016) who reported that UAE may not be the best method for the extraction of medicinal polysaccharides from herbal sources and recommended an optimization study before extraction to find a favourable range of extraction condition for the molecule of interest in addition to quantitative and qualitative analysis of extracts from

UAE. Table 3.6 compares the structural properties of fucoidan extracts in the present study, to reported literature values.

It is noteworthy to mention that the significantly ($p < 0.05$) higher extract yield of Fuc-CCE 3× rather than the 1× extraction did not translate to a significantly ($p < 0.05$) higher content of fucose and galactose. Furthermore, Fuc-CCE and Fuc-UAE, both had low galactose content, thus suggesting that more excipients (on a w/w basis), particularly minerals, were extracted in the Fuc-CCE and Fuc-UAE samples.

3.4.1.3. SULPHATE LEVELS

The sulphate content of fucoidans has been identified as an important structural determinant of its biological activity (Ale et al., 2011), and since most studies have correlated increased in sulphate content with increased biological function (Rodriguez-Jasso et al., 2011), then the influence of various extraction methods on sulphate content was examined. The sulphate content (Table 3.1) in all the triple extraction processes was higher than in single extraction. Fuc-CCE and Fuc-MAE sulphate contents were both significantly ($p < 0.05$) higher than Fuc-EAE. Comparing the impact of extraction method and number of extractions on sulphate levels (Appendix A, Table A1), both extraction method and the number of extractions independently influenced sulphate levels significantly ($p < 0.05$). The factorial analysis revealed that 3× extraction contributed significantly to increasing the sulphate content and the extraction methods differentially influenced sulphate levels. The sulphate content observed in this study were indicative of the presence of sulphated fucose in all of the extracts.

3.4.1.4. TOTAL URONIC ACID CONTENT

Fucoidan extracts contain uronic acids such as glucuronic and galacto-uronic acids, which may serve as an additional carbon source for bacterial growth. Hence, the impact of extraction processes on total uronic acid content was investigated using glucuronic acid as the standard. The results (Table 3.1) showed that higher uronic acids were obtained at 1× rather than 3× extraction for CCE, MAE and UAE, with a significant ($p < 0.05$) increase in Fuc-CCE and Fuc-UAE samples. Triple extraction may have resulted in the extraction of other components, which may have reduced the % w/w value of the uronic acids. Also, the uronic acid content was highest in Fuc-MAE for both 1× and 3× extractions and lowest in the Fuc-EAE, thus suggested that the Fuc-MAE technique was the most effective of all four methods. Also, the factorial analysis study showed that there was a significant of both extraction method and number of extractions (Appendix A, Table A1), as independent factors, on the total uronic acid content. The implication is that the uronic acid content decreased significantly ($p < 0.05$) in 3× extracts than 1× extract, and thus confirmed that 3× extraction may not best for the recovery of uronic acids from *A. nodosum*. However, the choice of extraction method influenced the uronic acid content significantly.

3.4.1.5. MOLECULAR WEIGHT

The molecular weight range for fucoidan extracts (Table 3.2) was analysed using GPC which provided information on the weight average molecular weight (Mw), the number average molecular weight (Mn) in the region of 1-500 kDa, and the dispersity index (Đ) (Mn provides information on the statistical average of all polymer chains within a sample, whereas Mw represents the molecular size of the sample). The Mw would be more

influenced by high molecular weights, while the Mn would be influenced by the lower molecular weight. The Đ measures the broadness or heterogeneity of molecular weight distributions within a polymer sample. It is represented as Mw/Mn, thus a higher difference between Mw and Mn indicates a more heterogenous or wider molecular weight distribution. Fucoidans are polydisperse and contain individual components with different molecular weights. The results in the present study had a minimum of two peaks within the molecular weight range of 0.6 kDa to 642 kDa (system calibration range). The percentage peak area, of peaks above 0.2 kDa (lowest possible disaccharide molecular weight) within the extract, was calculated to estimate the percentage composition of the different molecular weight distributions within the various extracts. Peak 1, with average molecular weight distribution between 80 and 131 kDa, all had a higher percentage than peak 2, with Mw between 2 and 8 kDa, in the data presented in this study. A comparison of the distribution of molecular weights across the various extraction methods, revealed a more heterogenous distribution in MAE (3×) and CCE (3×) relative to all other extracts, as depicted by their Đ values. This suggests that both techniques may have resulted in the splitting of the cell wall polysaccharides into short-chain oligo/monosaccharide short chains. Also observed, the weight average molecular weight increased significantly from Fuc-MAE (3×) to Fuc-CCE (3×) to Fuc-EAE (1×) to Fuc-UAE (3×). This indicated that of the four methods, the Fuc-UAE (3×) and Fuc-EAE (3×) extracts had the higher average molecular weight, as compared to the Fuc-MAE and Fuc-CCE samples. Also, a higher peak area and peak percentage was observed in the MAE (3×) and CCE (3×) compared to UAE (3×) and EAE (1×). The Mw for peak 1 reported in this study fell within the range of reported fucoidan molecular weight values, 54 kDa to 1600 kDa (Fitton et al., 2015; Zhang & Row, 2015). A factorial analysis of the impact of extraction method and number

of extractions (Appendix A, Table A1), revealed that molecular weight was significantly ($p < 0.05$) influenced by extraction methods, but not number of extractions. This data suggested that 3× extraction had no major impact on molecular weight, however, the extraction methods may have differentially influenced molecular weight. It also indicated that there was a significant impact of the interaction between both independent variables on the average molecular weight. For dispersity index, both extraction methods and number of extractions significantly ($p < 0.05$) influenced the dispersity index values, as well as the interaction between both extraction methods and number of extractions.

The differences in molecular weights when compared to literature values, may be attributed to seasonal variation, source, location and extraction methods/conditions (Ale et al., 2011; Fitton et al., 2015). A broader molecular weight distribution in both Fuc-MAE and Fuc-CCE appeared to be in agreement with the quantitative advantage of these methods in other structural properties reported in this study. A possible mechanism for the more heterogenous distribution observed in Fuc-MAE may be that, in addition to degrading the cell wall, microwave heating and dipole rotation of polar solvents may have contributed significantly to splitting poly/oligo-saccharides within the extraction medium. Oligosaccharides with short chain length and lower molecular weight may be more desirable for prebiotic activity as these may be more accessible to beneficial bacteria populations for stimulated growth and activities.

3.4.1.6. SODIUM, POTASSIUM AND CALCIUM CONTENTS

The sodium, potassium, and calcium contents were investigated to ascertain the quantity of these minerals in the extracts any possible interference with the active ingredient of

interest. From the results presented in Table 3.3, both sodium and potassium content were very low (< 1%) compared to the calcium content which was within the range of 2 - 5%. Since 2% calcium chloride was used, as a purification step, to remove alginate molecules that may have been extracted together with the fucoidan fraction, thus, may be responsible for the calcium content observed in this study as unbound calcium may have been retained in the extract. Also, calcium content was significantly higher in 1× than 3× for Fuc-CCE and Fuc-UAE. For sodium content, Fuc-EAE (1×) was highest compared to all other extracts. The EAE method used sodium phosphate buffer for fucoidan extraction, this may be responsible for the high sodium content relative to other extracts. The potassium content, on the other hand, was significantly higher in 1× than 3×, except for UAE. Potassium content was significantly highest in EAE (1×) compared to other extracts. Potassium plays a crucial role in muscle contraction, nerve conduction, and acid-alkaline balance, and is one of the major minerals found in seaweeds. Reported levels of some brown seaweed species for sodium, potassium, and calcium content rages from 0.54 - 0.66% (w/w), 0.81 - 0.89% (w/w), and 0.26 - 0.29% (w/w) respectively. The potassium content of fucoidan extracts from 1× extraction in this study were within the range of reported literature, and the sodium content below the reported range. The calcium content was well above the reported range. This may be attributed to a probable calcium retention in the extract from purification step with 2 % calcium chloride. The results (Appendix A, Table A2) in this study also revealed that both extraction methods and number of extractions significantly ($p < 0.05$) influenced the sodium, potassium and calcium contents. This significant impact revealed that their contents decreased in 3× extraction. The factorial analysis also showed that there was a significant impact of the interaction

between extraction methods and number of extractions on the sodium, potassium and calcium contents.

3.4.2. PREBIOTIC ACTIVITY OF FUCOIDAN EXTRACTS FROM VARIOUS EXTRACTION METHODS

Fucose-rich sulphated polysaccharides are potential prebiotics with proven *in vitro* and *in vivo* prospects (Zhao & Cheung, 2011; Deville et al., 2007; Lynch et al., 2010). This is attributed to its ability to escape the activities of digestive activities (hydrolytic and enzymatic) and undergo fermentation by gut bacteria while stimulating the growth and activity of beneficial bacterial populations. Fucose and other monosaccharides present in the fucoidan matrix are utilized by these bacteria for growth and stimulated activities such as microbial balance, nutrient absorption, immune functions, and improved digestion (Linares et al., 2016). Hence, the effect of extraction procedures and fucoidan extracts on prebiotic activity was investigated. For fucoidan from the 3× extraction of CCE, MAE, and UAE were used for the prebiotic assay as they had better structural properties (such as fucose, galactose, and molecular weight contents) than the 1× extraction products. The results show that supplementing MRS with fucoidan extracts significantly ($p < 0.05$) improved the growth of the *L. delbruecki* (Fig 3.1) strain at 0.1 and 0.5% inclusion rates, relative to the un-supplemented medium. There was no dose- or treatment-dependent response observed for *L. casei* (Fig 3.2), as extracts at all concentrations maintained similar growth rates, except for Fuc-CCE. Although there were no significant differences between the growth rates of *L. casei* supplemented fucoidan extracts, a 24.5 % increase in growth rate was observed in Fuc-MAE at the 0.5 % inclusion rate.

The growth rates of fucoidan extracts was comparable to that of inulin, a standard prebiotic with no significant difference in activity, thus indicating that fucoidan extracts are as effective as inulin in improving growth rates *in vitro* for these specific bacterial strains. The negative control (glucose), although not a prebiotic, had similar growth rates as inulin. This is because, glucose serve as a carbon source, like inulin and other extracts *in vitro*, hence provides nutrient for bacterial growth and stimulation. At *in vivo* and human trials, it is expected that glucose would be absorbed into the blood stream and will not be accessible for bacterial growth stimulation in the large intestine. Hence, its role as a negative control. Both *L. casei* and *L. delbruecki* strains are commensal, beneficial gut bacteria and have been applied as probiotics in many dairy products.

In addition to the prebiotic potential demonstrated in this study, the results also indicated that the fucoidan extracts could be used in synbiotic relationship with *L. delbruecki* and *L. casei* strains to maintain the viability of these bacteria in dairy products such as yoghurts. The same trend is observed with the doubling time data, although the Fuc-MAE, glucose, and inulin supplemented media had lower doubling time compared to other media. This data supported the claim of higher growth rate activity for Fuc-MAE media compared to the other extracts supplemented media. Overall, the results from this study suggested some prebiotic potential, although more *in vitro, in vivo* and clinical testing would be required for further confirmation. This is required, as the efficacy of prebiotics *in vivo* may be influenced by challenges of a much more complex microbiota comprising both beneficial and non-beneficial bacteria and enzymatic degradation of fucoidan extracts.

The promotion of the growth of specific *Lactobacillus* strains has been associated with increased SCFA production. The metabolic activities of gut microbiota result in the

breakdown of poly/oligosaccharides to SCFA. The production of SCFA has singularly been associated with health promoting effects such as antimicrobial activity, modulation of bowel inflammation, reduction of carcinogenesis and improvement in gut health through intestinal epithelial cells via increase in mucin production leading to decrease in cell permeability. Of the three most prominent SCFA (acetate, propionate, and butyrate) two were detected in this study, acetate, and propionate. Higher concentrations of acetate (Fig 3.3 and 3.5) than propionate (3.4 and 3.6) was detected in the samples of both *L. delbruecki* and *L. casei* thus suggesting acetic acid as the major SCFA produced under these substrate/microbe condition. This finding agree with reports from Charoensiddhi et al., (2016), Chen et al., (2016) and Li et al., (2015). However, results from this study suggest that there was no significant impact of fucoidan, inulin, and glucose supplementation on acetic and propionic acid concentrations. An increase in SCFA production will be accompanied by a decrease in pH which is an indirect indicator of a balance between beneficial and harmful gut bacteria. Table 3.5 contains the pH of fucoidan and inulin supplemented growth media showing a higher reduction in pH of fucoidan supplemented MRS broths in both *L. casei* and *L. delbruecki* strains than un-supplemented strains. The data suggested an increase in the production of SCFA, although not reflected in the acetic and propionic acid concentration. A decrease in the pH of fucoidan supplemented media as compared to the un-supplemented media may be indicative of substantial utilization of these monosaccharides by intestinal microbes.

3.4.3. STRUCTURE – PREBIOTIC ACTIVITY RELATIONSHIP OF FUCOIDAN EXTRACTS

There is a lack of specific information in the literature on the relationship between the structural properties of fucoidans and potential prebiotic function. Part of the focus in this study was to understand the structure-function relationship between fucoidan from different extraction processes and their relationships to prebiotic activity. The structure function relationship was explored using regression analysis (Table 3.7). The coefficient of determination value (R^2) was used to test the fit to linear causative relationship (obtained when *in* vitro growth rate data were plotted against structural properties). This is measure of the strength of the relationship between prebiotic activity and a structural property. The closer the R^2 value to 1, the more the approximate linear correlation.

For fucose content, an R^2 value of 0.9169, at 0.5% (w/v) concentration, indicated a strong linear relationship with growth rate of *L. casei* amongst the different extraction groups. The relationship between fucose content and growth rate of *L. delbruecki* at 0.5% (w/v) inclusion concentration was non-linear, with a high R^2 value (0.8372). Similarly, the relationship between galactose content and growth rate of *L. casei* at 0.5% and *L. delbruecki* at 0.5 % (w/v) (0.6808), were linear (0.7124) and non-linear (0.7124) respectively. The same relationship was applicable to the uronic acid content versus growth rate of 0.5% (w/v) *L. casei* and 0.5% (w/v) *L. delbruecki*.

There was a linear relationship between sulphate content and growth rates at 0.3 % *L. casei* (0.8119), 0.1% (w/v) *L. delbruecki* (0.8964), and 0.3% (w/v) *L. delbruecki* (0.7615). This may be indicative of a possible negative influence of the sulphate content on prebiotic activity. However, this is not a definite/conclusive assumption, since the data simply

suggest relationships and not a significant impact. The molecular weight – growth rates relationship had a linear correlation at 0.3% (w/v) *L. casei* (0.7006), 0.3% (w/v) *L. delbruecki* (0.6156), and 0.5% (w/v) *L. delbruecki* (0.6979), however, the Đ relationship with prebiotic activity showed a linear relationship in 0.3% (w/v) *L. casei* (0.9577) and 0.3% (w/v) *L. delbruecki* (0.7818). These trends suggested increased prebiotic activity with increased molecular weight, and decreased activity with increase in Đ . There is a dearth of information on the structure function relationship between structural properties and prebiotic activity for literature comparison.

Comparing the impact of extraction processes on prebiotic activity, extracts from the different processes all significantly ($p < 0.05$) improved *L. delbruecki* growth and maintained a similar growth rate as that of *L. casei*. The Fuc-MAE which had the highest fucoidan content improved the growth rate of *L. casei* by 24.5%. For the *L. delbruecki* strain, extract from all extraction methods significantly ($p < 0.05$) stimulated growth, as compared to the un-supplemented broth. However, there were no significant differences between extracts and the prebiotic activity of the extracts from various extraction processes. These results suggested that the fucoidan monosaccharide composition is of major significance to the growth of beneficial bacteria. The results also provided evidence that fucoidan monosaccharides were able to be utilized as carbon sources for the growth of these specific beneficial bacteria. Interestingly, the Fuc-UAE, which had significantly ($p < 0.05$) low concentration of fucose compared to the rest of the extracts, improved the growth of the *L. delbruecki* strain significantly, as compared to the un-supplemented *L. delbruecki* strain. These data suggested that the monosaccharide contents of the extracts in this study may have been sufficient for bacterial growth. The trend observed for the molecular weight revealed that the reported average molecular weight ranges in this study

were suitable for prebiotic functioning. Fuc-MAE with the lowest molecular weight and highest Đ value produced a 24.5% increase in the *L. casei* strain and significantly ($p < 0.05$) improved the growth of *L. delbruecki*. Most of the studies on the bioactive properties of fucoidans have associated lower average molecular weight with increased fucoidan activity (Ale et al., 2011; Fitton et al., 2015). The role of the level of sulphation in prebiotic activity is yet to be fully understood, however the sulphate content has proven to be an important structural parameter in anti-cancer and immunostimulatory activities with well-defined mechanisms, which include interaction with toll-like receptors and interleukin-mediated apoptosis (Ale et al., 2011; Fitton et al., 2015).

3.5. CONCLUSION

The impact of four fucoidan extraction processes on the structural properties and *in vitro* prebiotic activity indicators were investigated in the present study. The results indicated that Fuc-MAE had significantly ($p < 0.05$) higher fucose content and sulphate levels, higher uronic acid content, broader molecular weight distribution, and lower average molecular weight. The factorial analysis of the impact of extraction methods and number of extractions, as independent variables, was also investigated. The results revealed that the number of extractions significantly influenced extract yield, uronic acid content, sulphate levels and dispersity index, whereas the choice of extraction method significantly influenced the extract yield and the other structural properties. The sodium, potassium, and calcium contents were significantly influenced by both extraction methods, number of extractions and the interaction between both independent variables. The factorial analysis data suggested that both choice of extraction method and number of extractions play major

roles in determining the properties of these extracts. All four extraction methods significantly improved the growth rate of *L. delbruecki* at 0.1% and 0.5% (w/v) inclusion concentrations, while maintaining similar growth rates of *L. casei* strains, relative to the un-supplemented *L. casei* strain. The Fuc-MAE extract however had a 24.5% increase at the 0.5% (w/v) rate of inclusion concentration. The fucoidan extracts from this study had comparable efficiency with the standard prebiotic, inulin, in stimulating the growth of the beneficial bacterial strains and as such may support the prebiotic potential of fucoidan extracts. Advanced testing, such as an *in vivo* digestibility studies of bacterial populations in faecal samples/intestinal content are needed to consolidate these claims. Also, the use of technological extraction methods, MAE and UAE, had similar efficiency as the conventional technique in both the characterization and prebiotic phase as seen from the results. However, application of these technologies (MAE and UAE) have the advantage of lesser extraction time and are likely more eco-friendly. From an economic perspective, although MAE performed best in the characterization section of this study, the cost of setting up and maintaining a microwave unit may be expensive. However, this may be worth the risk, since the *in vitro* prebiotic study suggested that Fuc-MAE extracts were very effective in. Hence, if its prebiotic efficacy is confirmed *in vivo* and in humans, MAE may be recommended for large scale extraction, since it has the advantage of efficiency, and saves time. The may translate into increased revenue for industries. Whilst extraction conditions influenced the outcome of extraction products qualitatively and quantitatively, the raw material, seasonal variation, location and species are also factors which influence the final extraction products. Optimization of extraction conditions may help to determine the best fit for efficient extraction of bioactive molecules, such as polysaccharides. Although some purification measures were employed in this study to obtain the bioactive

compound of interest, further purification measures such as ultrafiltration for molecular weight cut off, and anion exchange chromatography could be employed to improve the purity of the extracts. Findings from this study suggested that MAE was the most effective of all the extraction methods considering its effect on structural properties and prebiotic function.

TABLE 3.1. THE EXTRACT YIELD AND STRUCTURAL PROPERTIES OF *ASCOPHYLLUM NODOSUM* FUCOIDAN EXTRACTS FROM VARIOUS EXTRACTION PROCESSES

	Extract yield (%w/w of pre-extracted *A. nodosum*)		Monosaccharide composition (% w/w of fucoidan extract)				Uronic acid (% w/w of fucoidan extract)		Sulphate levels (% w/w of fucoidan extract)	
			Fucose (% w/w)		Galactose (% w/w)					
	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)
Fuc-CCE	5.58 ± 0.10 [c]	11.9 ± 2.93 [a, b]	26.1 ± 6.83 [e]	27.4 ± 3.27	13.6 ± 5.13	6.56 ± 0.92	3.35 ± 0.13 [g]	0.59 ± 0.13	15.3 ± 1.25	21.7 ± 1.71 [i, j]
Fuc-MAE	3.76 ± 2.65	5.71 ± 1.01	34.6 ± 6.72 [e]	37.0 ± 6.82	11.0 ± 3.46	13.0 ± 4.08	4.14 ± 2.17	3.59 ± 0.76	11.9 ± 4.39	18.8 ± 0.39 [i]
Fuc-UAE	0.88 ± 0.20	4.56 ± 0.63 [d]	14.9 ± 6.92	27.1 ± 2.04 [f]	1.84 ± 0.59	8.53 ± 0.85	1.55 ± 0.23 [h]	0.49 ± 0.09	12.3 ± 1.89	17.3 ± 2.18 [k]
Fuc-EAE	3.89 ± 0.55	-	29.1 ± 1.42 [e]	-	10.6 ± 1.06	-	0.39 ± 0.19		15.4 ± 1.49	-

[a] Fuc-CCE (3×) was significantly higher than Fuc-MAE (3×), Fuc-UAE (3×), and Fuc-EAE (1×); [b] Fuc-CCE (3×) was significantly higher than Fuc-CCE (1×); [c] Fuc-CCE (1×) and Fuc-MAE (1×) were significantly higher than Fuc-UAE; [d] Fuc-UAE (3×) was significantly higher than Fuc-UAE (1×); [e] Fuc-CCE (1×), Fuc-MAE (1×), and Fuc-EAE (1×) were significantly higher than Fuc-UAE (1×); [f] Fuc-UAE (3×) was significantly higher than Fuc-UAE (1×); [g] Fuc-CCE (1×) was significantly higher than Fuc-CCE (3×); [h] Fuc-UAE (1×) was significantly higher than Fuc-UAE (3×); [i] Fuc-CCE (3×) and Fuc-MAE (3×) were significantly higher than Fuc-EAE (1×); [j] Fuc-CCE (3×) was significantly higher than Fuc-CCE (1×); [k] Fuc-UAE (3×) was significantly higher than Fuc-UAE (1×) (Results are presented as mean ± standard deviation; at $p < 0.05$)

TABLE 3.2.THE MOLECULAR WEIGHT DISTRIBUTION OF *ASCOPHYLLUM NODOSUM* FUCOIDAN EXTRACTS FROM VARIOUS EXTRACTION PROCESSES

Extraction method	Component	Number average molecular weight, Mn (kDa)	Weight average molecular weight, Mw (kDa)	Polydispersity index (Đ)	Peak area	% Peak area
Fuc-CCE (1×)	Peak 1	63.2 ± 9.28	90.1 ± 13.9	1.42 ± 0.01	184.5 ± 34.2	65.0 ± 14.8
	Peak 2	8.05 ± 0.41	8.46 ± 0.51	1.05 ± 0.01	15.61 ± 2.92	5.44 ± 0.74
	Peak 3	2.51 ± 0.03	2.69 ± 0.59	1.08 ± 0.01	71.77 ± 21.9	24.9 ± 6.07
Fuc-CCE (3×)	Peak 1	40.2 ± 3.57	97.5 ± 7.80 [c]	2.47 ± 0.29	318.3 ± 144.6	95.4 ± 0.21
	Peak 2	2.62 ± 0.12	2.79 ± 0.16	1.07 ± 0.01	12.05 ± 6.72	3.52 ± 0.61
Fuc-MAE (1×)	Peak 1	97.3 ± 27.7	116 ± 13.7	1.22 ± 0.20	336.9 ± 254.8	90.2 ± 0.02
	Peak 2	11.0 ± 14.5	6.40 ± 6.36	0.77 ± 0.48	32.76 ± 24.59	8.79 ± 0.07
Fuc-MAE (3×)	Peak 1	30.8 ± 1.99	81.2 ± 8.07	2.64 ± 0.19	335.3 ± 116.2	86.5 ± 1.70
	Peak 2	2.55 ± 0.08	2.86 ± 0.10	1.12 ± 0.007	40.31 ± 13.6	10.4 ± 0.18
Fuc-UAE (1×)	Peak 1	97.04 ± 0.03	105.2 ± 0.02	1.084 ± 0.01	90.3 ± 8.86	35.6 ± 0.89
	Peak 2	7.75 ± 0.42	8.00 ± 0.37	1.03 ± 0.008	13.1 ± 1.98	5.23 ± 1.42
Fuc-UAE (3×)	Peak 1	121.1 ± 3.94	136.3 ± 4.39[a]	1.13 ± 0.07	239.6 ± 65.6	79.8 ± 0.56
	Peak 2	2.58 ± 0.07	2.70 ± 0.05	1.05 ± 0.008	57.5 ± 17.9	19.0 ± 1.21
Fuc-EAE (1×)	Peak 1	100.1 ± 15.3	115.2 ± 8.87[b]	1.16 ± 0.09	227.4 ± 108.7	59.7 ± 4.00
	Peak 2	2.57 ± 0.02	2.73 ± 0.02	1.07 ± 0.002	27.62 ± 21.6	4.11 ± 0.02

[a] Significantly higher than Fuc-EAE (1×), Fuc-CCE (3×), and Fuc-MAE (3×); [b] Significantly higher than Fuc-MAE (3×) and Fuc-CCE (3×); [c] Significantly higher than Fuc-MAE (3×) (Results are presented as mean ± standard deviation; at $p < 0.05$)

TABLE 3.3. THE SODIUM, POTASSIUM, AND CALCIUM CONTENTS OF *ASCOPHYLLUM NODOSUM* FUCOIDAN EXTRACTS

Fucoidan extracts	Sodium (% w/w of fucoidan extract)		Potassium (% w/w of fucoidan extract)		Calcium (% w/w of fucoidan extract)	
	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)
Fuc-CCE	$0.151 \pm 0.003^{c,e}$	0.069 ± 0.007	$0.783 \pm 0.00^{k,l}$	0.476 ± 0.02	4.919 ± 0.027^{o}	2.399 ± 0.027
Fuc-MAE	0.059 ± 0.005	0.079 ± 0.003^{f}	$0.816 \pm 0.02^{j,m}$	0.719 ± 0.01^{g}	3.083 ± 0.000	3.177 ± 0.000
Fuc-UAE	0.132 ± 0.005^{d}	0.134 ± 0.008^{a}	0.338 ± 0.01	$0.543 \pm 0.02^{h,n}$	6.693 ± 0.027^{p}	3.737 ± 0.000
Fuc-EAE	0.357 ± 0.003^{b}	-	0.872 ± 0.00^{i}	-	4.577 ± 0.000	

[a] Fuc-UAE (3×) was significantly higher than Fuc-MAE (3×) and Fuc-CCE (3×); [b] Fuc-EAE (1×) was significantly higher than Fuc-MAE (1×) and Fuc-CCE (1×); [c] Fuc-CCE (1×) was significantly higher than Fuc-UAE (1×) and Fuc-MAE (1×); [d] Fuc-UAE (1×) was significantly higher than Fuc-MAE (1×); [e] Fuc-CCE (1×) was significantly higher than Fuc-CCE (3×); [f] Fuc-MAE (3×) was significantly higher than Fuc-MAE (1×); [g] Fuc-MAE (3×) was significantly higher than Fuc-UAE (3×) and Fuc-CCE (3×); [h] Fuc-UAE (3×) was significantly higher than Fuc-CCE (3×); [i] Fuc-EAE (1×) was significantly higher than Fuc-MAE (1×), Fuc-CCE (1×) and Fuc-UAE (1×); [j] Fuc-MAE (1×) was significantly higher than Fuc-CCE (1×) and Fuc-UAE (1×); [k] Fuc-CCE (1×) was significantly higher than Fuc-UAE (1×); [l] Fuc-CE (3×) was significantly higher than Fuc-CCE (1×); [m] Fuc-MAE (1×) was significantly higher than Fuc-MAE (3×); [n] Fuc-UAE (3×) was significantly higher than Fuc-UAE (1×); [o] Fuc-CCE (1×) was significantly higher than Fuc-CCE (3×); [p] Fuc-UAE (3×) was significantly higher than Fuc-UAE (1×) (Results are presented as mean ± standard deviation; at $p < 0.05$)

TABLE 3.4. THE DOUBLING TIME OF *Lactobacillus casei* AND *Lactobacillus delbruecki* MEDIA WITH SUPPLEMENTED *ASCOPHYLLUM NODOSUM* FUCOIDAN EXTRACTS

Extraction method	*L. casei* doubling time (hr)				*L. delbruecki* doubling time (hr)			
	MRS + Lb.cs	0.1 %	0.3 %	0.5 %	MRS + Lb.db	0.1 %	0.3 %	0.5 %
Inulin	2.426 ± 0.239	2.171 ± 0.291	2.051 ± 0.017	2.149 ± 0.157[a]	3.90 ± 2.19	3.031 ± 0.298	2.888 ± 0.558	2.806 ± 0.479
Glucose	2.426 ± 0.239	2.015 ± 0.087	2.005 ± 0.063	2.046 ± 0.140[a]	3.90 ± 2.19	3.057 ± 0.339	2.863 ± 0.267	2.960 ± 0.486
Fuc-CCE (3×)	2.426 ± 0.239	2.393 ± 0.051	2.701 ± 0.075	2.586 ± 0.124	3.90 ± 2.19	1.597 ± 0.082	1.726 ± 0.240	1.843± 0.124
Fuc-MAE (3×)	2.426 ± 0.239	2.723 ± 0.011	2.646 ± 0.031	1.942 ± 0.032[a]	3.90 ± 2.19	1.956 ± 0.249	1.901 ± 0.119	2.008 ± 0.114
Fuc-UAE (3×)	2.426 ± 0.239	2.540 ± 0.115	2.379 ± 0.097	2.410 ± 0.072	3.90 ± 2.19	1.898 ± 0.127	1.704 ± 0.076	1.785 ± 0.167
Fuc-EAE (3×)	2.426 ± 0.239	2.287 ± 0.012	2.369 ± 0.155	2.494 ± 0.062	3.90 ± 2.19	1.668 ± 0.165	1.790 ± 0.263	1.937 ± 0.327

[a] Fuc-MAE (3×), glucose, and inulin are significantly lower than Fuc-CCE, Fuc-UAE, and Fuc-EAE (The results are presented as mean ± standard deviation; all at $p < 0.05$)

TABLE 3.5. THE CHANGES IN pH OF *Lactobacillus delbruecki* AND *Lactobacillus casei* GROWTH MEDIA AFTER 24 HOURS INCUBATION

Extraction Method	Inclusion concentration	Change in pH of *L. delbruecki* media	Change in pH of *L. casei* media
MRS	-	-0.036	-0.036
L. delbruecki/*L. casei*	-	-1.275	-1.839
Inulin	0.1	-2.014	-1.876
	0.3	-1.984	-1.872
	0.5	-2.003	-1.927
Glucose	0.1	-2.010	-1.854
	0.3	-1.990	-1.877
	0.5	-1.996	-1.868
Fuc-CCE (3×)	0.1	-1.110	-2.314
	0.3	-0.987	-2.208
	0.5	-1.080	-2.216
Fuc-MAE (3×)	0.1	-1.419	-2.257
	0.3	-1.015	-2.143
	0.5	-1.150	-2.280
Fuc-UAE (3×)	0.1	-1.455	-2.277
	0.3	-1.103	-2.129
	0.5	-1.204	-2.171
Fuc-EAE (3×)	0.1	-1.261	-2.095
	0.3	-1.247	-2.114
	0.5	-0.997	-2.268

TABLE 3.6. A COMPARISON OF THE STRUCTURAL PROPERTIES OF FUCOIDANS FROM THE PRESENT STUDY WITH LITERATURE VALUES

Extraction method	Seaweed	Extract yield (%w/w of pre-extracted *A. nodosum*)	Fucose (% w/w of fucoidan extract)	Galactose (% w/w of fucoidan extract)	Sulphate (% w/w of fucoidan extract)	Molecular weight (kDa)	References
Conventional chemical extraction	*A. nodosum* (Present study)	11.9	27.4	6.56	21.7	97.5	
	A. nodosum	1.75	52.1	6.10	19.0	420 and 47	Foley et al. 2011
	A. nodosum	20.1	42.5	1.12	29.3	40.2	Yuan & Macquarrie, 2015a
	U. pinnatifida	-	39.2	26.5	15.0	171	Mak et al. 2013
	S. Polycistum	2.75	20.3	13.7	23.4	-	Thuy et al. 2015
	S. binderi	-	34.5	12.1	7.66	47.8	Lim et al. 2016
	Sargassum sp.	-	3.14	1.39	38.4	-	Ale et al. 2011
	F. vesiculosis	-	13.87	2.79	34.2	-	Ale et al. 2011
Microwave-assisted extraction	*A. nodosum* (Present study)	5.71	37.0	13.0	18.8	81.2	
	A. nodosum	14.1	41.3	5.95	27.1	34.4	Yuan & Macquarrie, 2015b
	A. nodosum	11.9	42.3	5.69	27.8	37.5	Yuan & Macquarrie, 2015a
Ultrasound-assisted extraction	*A. nodosum* (Present study)	4.56	27.1	8.53	17.3	136	
Enzyme-assisted extraction	*A. nodosum* (Present study)	3.89	29.1	10.6	15.4	115	

TABLE 3.7. THE LINEAR REGRESSION ANALYSIS COMPARING THE RELATIONSHIP BETWEEN *IN VITRO* GROWTH RATES AND STRUCTURAL PROPERTIES USING THE COEFFICIENT OF DETERMINATION (R^2) AND THE LINEAR EQUATIONS

Structural properties	Growth rates of *L. delbruecki*			Growth rates of *L. casei*		
	0.1% (w/v)	0.3% (w/v)	0.5% (w/v)	0.1% (w/v)	0.3% (w/v)	0.5% (w/v)
Fucose	0.0002 (y = -1.084x + 30.73)	0.0416 (y = -22.10x + 42.16)	0.8372 (y = -154.6x + 112.6)	0.4906 (y = -107.5x + 73.58)	0.1612 (y = -66.90x + 56.77)	0.9169 (y = 76.61x - 2.752)
Galactose	0.1989 (y = 22.94x + 2.506)	0.0541 (y = 14.97x + 1.535)	0.6808 (y = -82.87x + 53.88)	0.2461 (y = -45.27x + 27.96)	0.0057 (y = 7.489x + 6.692)	0.7124 (y = 40.13x + 7.565)
Sulphate	0.8964 (y = 46.84x + 43.17)	0.7615 (y = 54.06x + 47.68)	0.0010 (y = 2.992x + 16.70)	0.0548 (y = 20.55x + 26.60)	0.8119 (y = 85.84x + 52.47)	0.0000 (y = 0.454x + 18.49)
Molecular weight	0.1343 (y = 160.4x + 22.28)	0.6156 (y = 429.9x – 126.3)	0.6979 (y = 713.9x – 273.5)	0.1384 (y = 288.9x – 9.286)	0.7006 (y = 705.3x – 173.3)	0.3568 (y = 241.7x + 211.2)
Dispersity index	0.5087 (y = -10.84x + 7.607)	0.7818 (y = 16.83x +10.99)	0.3208 (y = -16.82x + 10.82)	0.2365 (y = -13.12x + 7.149)	0.9577 (y = 28.64x + 13.25)	0.2543 (y = 7.032x + 1.170)

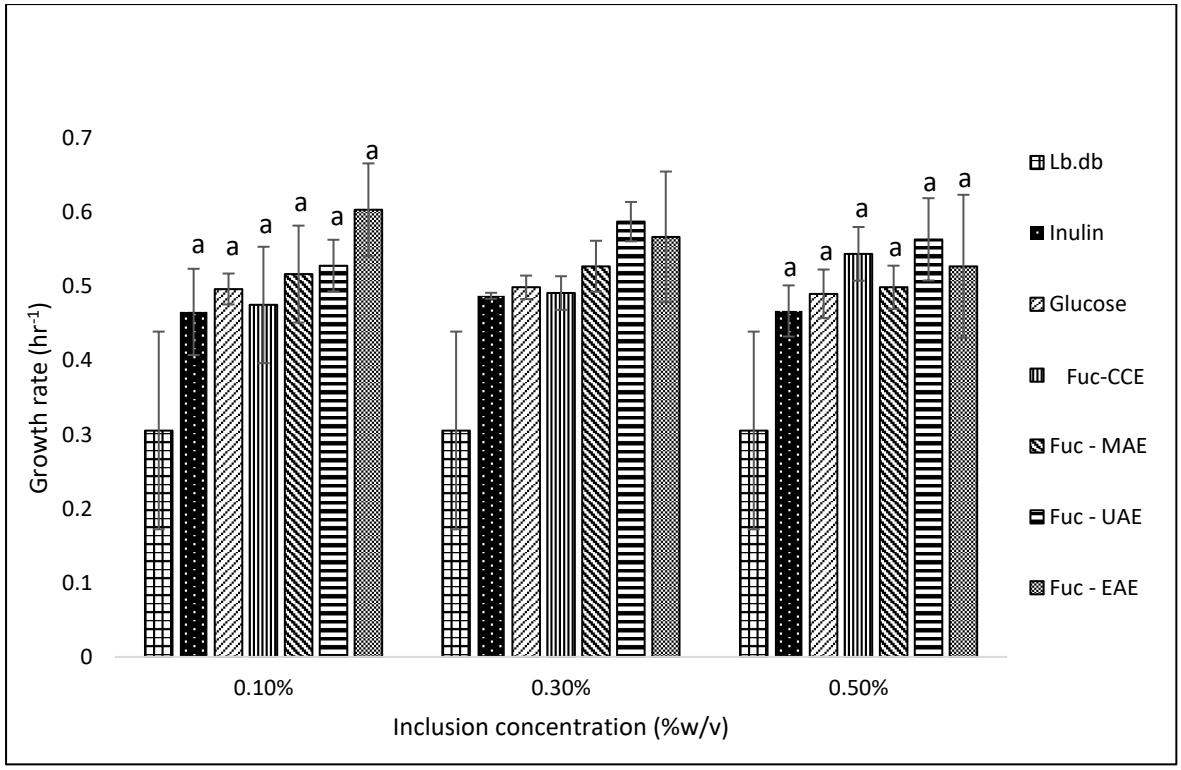


FIG. 3.1. THE *Lactobacillus delbruecki* GROWTH RATE

[a] Significantly different from Lb.db at $p < 0.05$
Lb.db – *L. delbruecki*
Fuc-CCE – Fucoidan from conventional chemical extraction
Fuc-MAE – Fucoidan from microwave-assisted extraction
Fuc-UAE – Fucoidan from ultrasound-assisted extraction
Fuc-EAE – Fucoidan from enzyme-assisted extraction
(The results are presented as mean ± standard deviation (error bars))

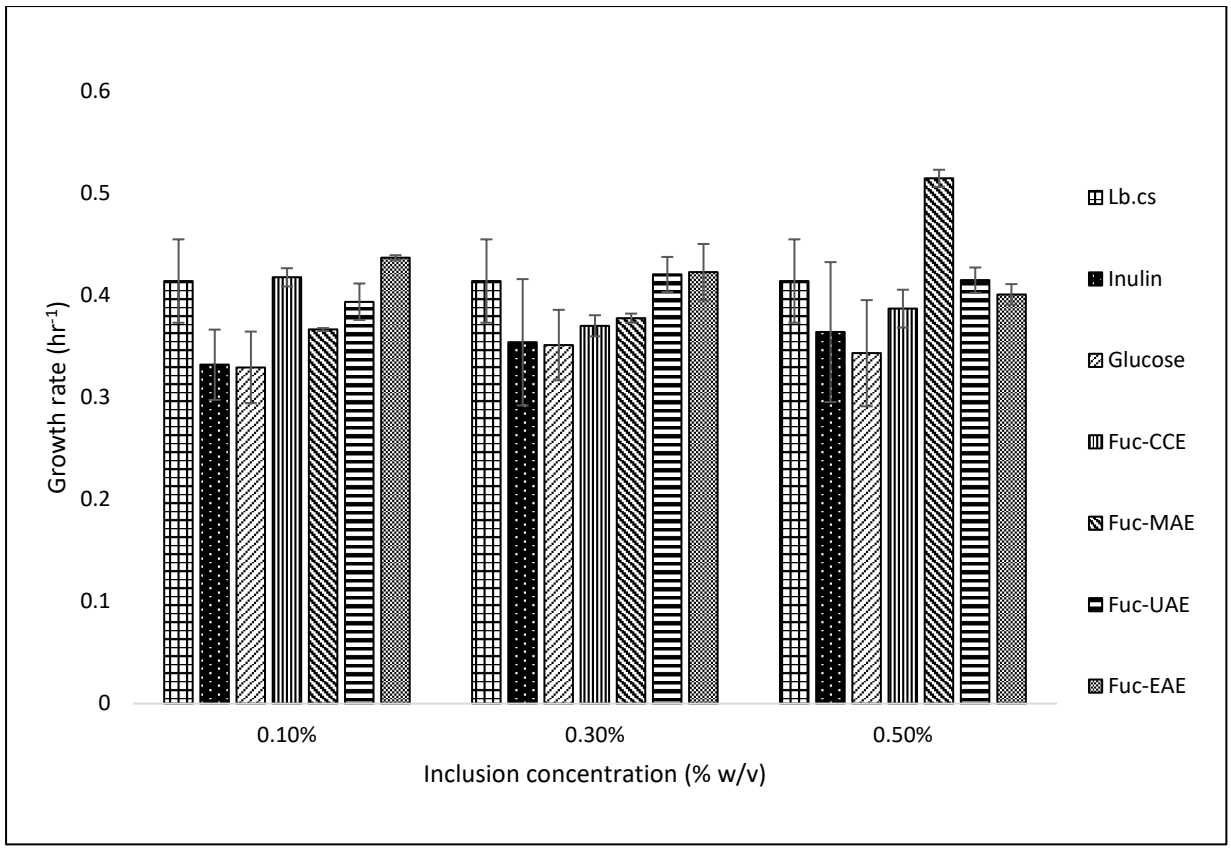


FIG. 3.2. THE *Lactobacillus casei* GROWTH RATE

[a] Significantly different from Lb.db at $p < 0.05$
Lb.cs – *L. casei*
Fuc-CCE – Fucoidan from conventional chemical extraction
Fuc-MAE – Fucoidan from microwave-assisted extraction
Fuc-UAE – Fucoidan from ultrasound-assisted extraction
Fuc-EAE – Fucoidan from enzyme-assisted extraction
(The results are presented as mean ± standard deviation (error bars))

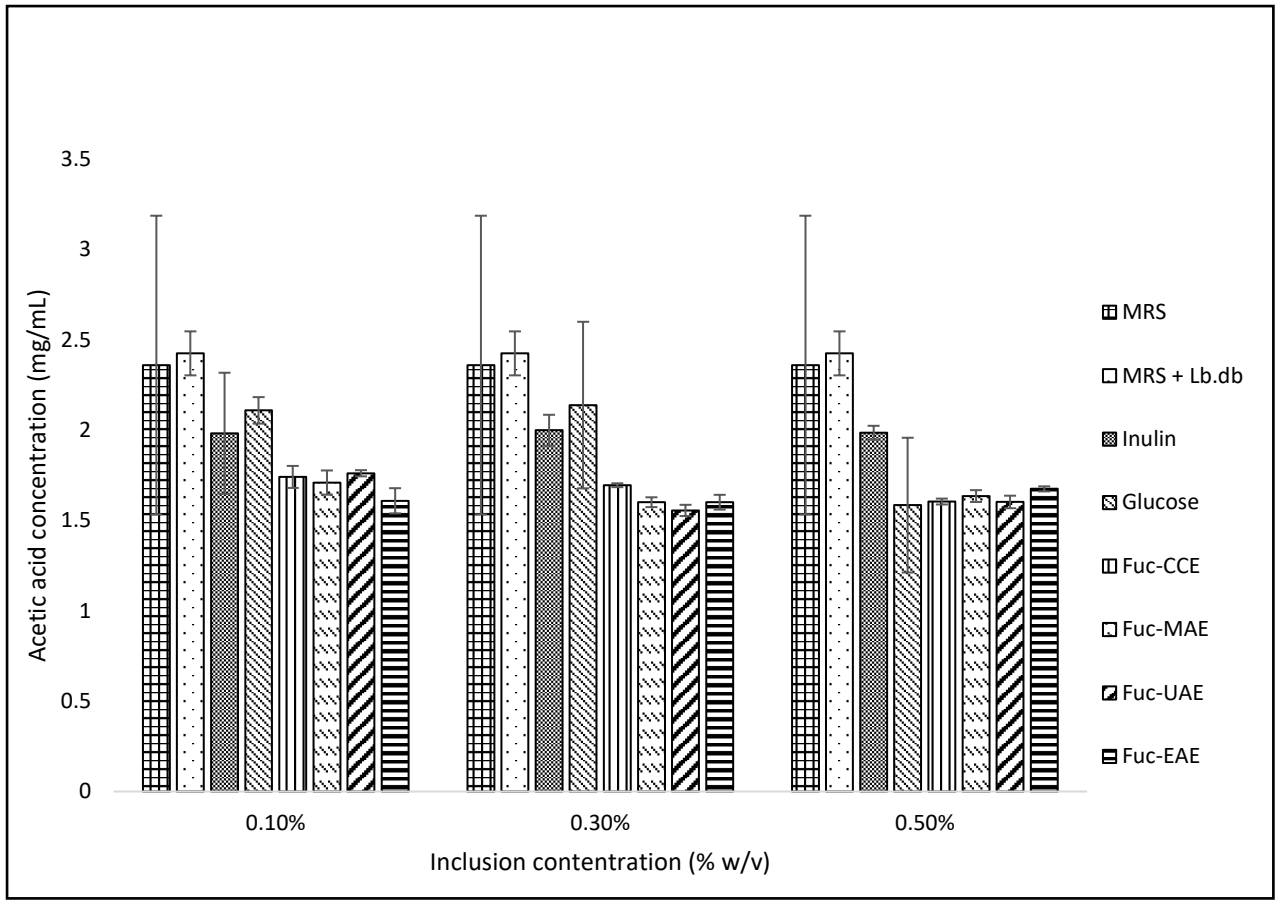


FIG. 3.3. THE ACETIC ACID CONCENTRATION IN *Lactobacillus delbruecki* GROWTH MEDIA

Lb.db – *L. delbruecki*
MRS + Lb.db – *L. delbruecki* in MRS broth
Fuc-CCE – Fucoidan from conventional chemical extraction
Fuc-MAE – Fucoidan from microwave-assisted extraction
Fuc-UAE – Fucoidan from ultrasound-assisted extraction
Fuc-EAE – Fucoidan from enzyme-assisted extraction
(The results are presented as mean ± standard deviation (error bars))

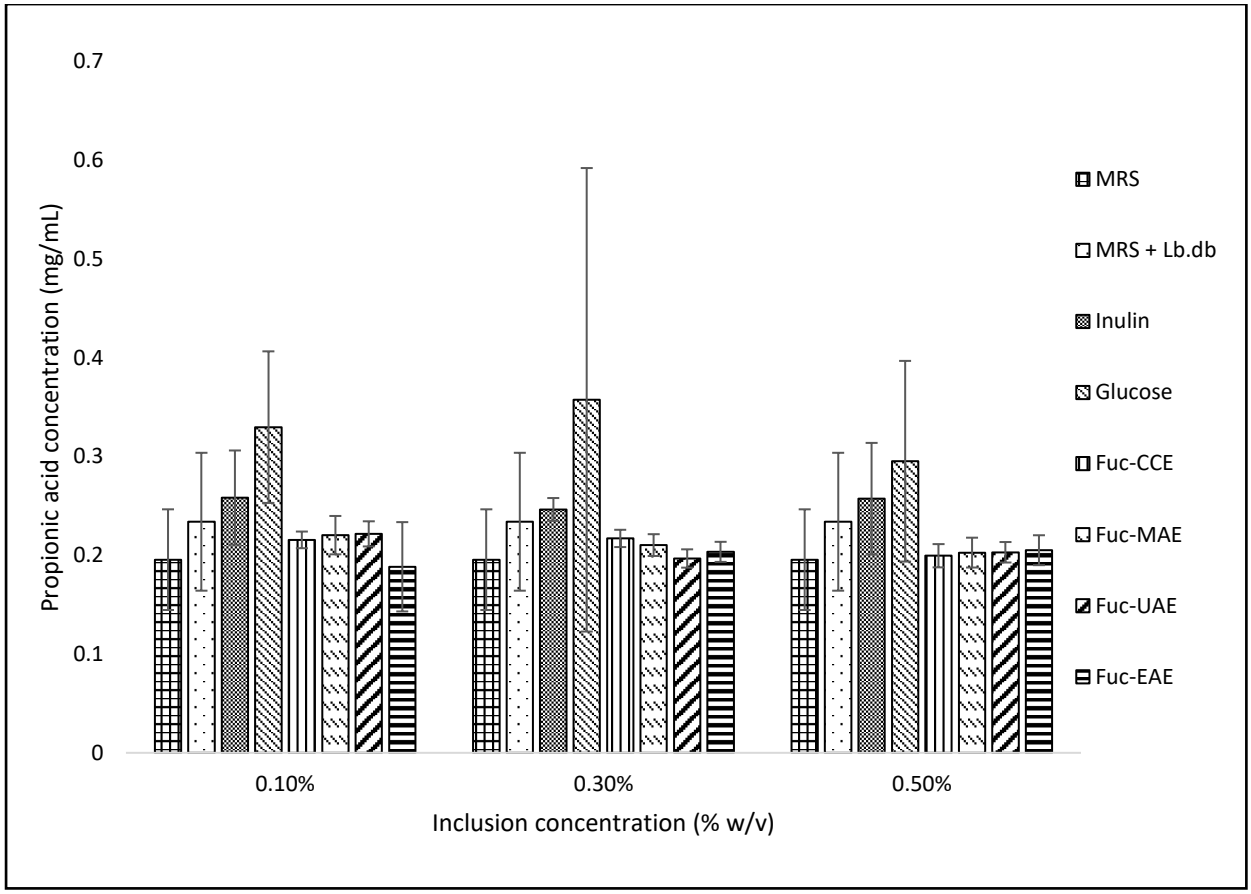


FIG. 3.4. THE PROPIONIC ACID CONCENTRATION IN *Lactobacillus delbruecki* GROWTH MEDIA

Lb.db – *L. delbruecki*
MRS + Lb.db – *L. delbruecki* in MRS broth
Fuc-CCE – Fucoidan from conventional chemical extraction
Fuc-MAE – Fucoidan from microwave-assisted extraction
Fuc-UAE – Fucoidan from ultrasound-assisted extraction
Fuc-EAE – Fucoidan from enzyme-assisted extraction
(The results are presented as mean ± standard deviation (error bars))

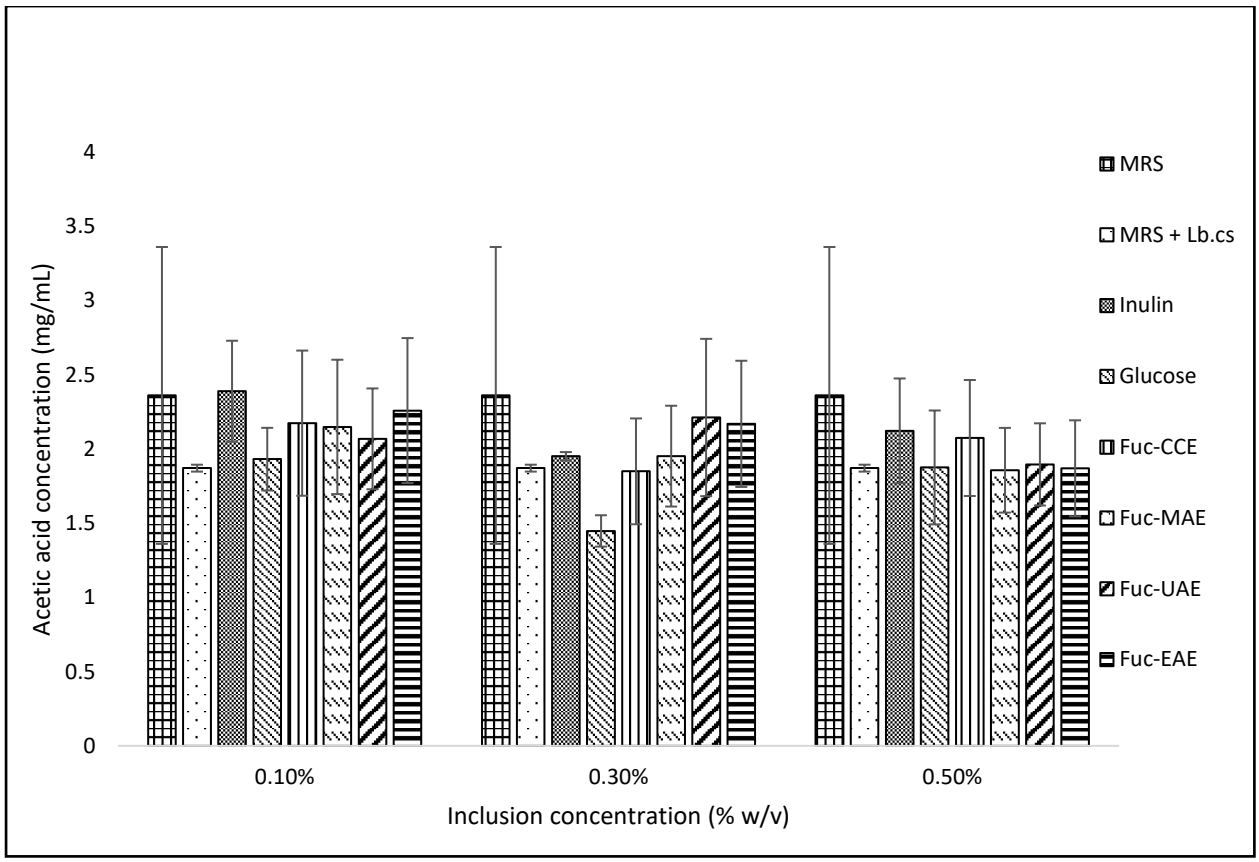


FIG. 3.5. THE ACETIC ACID CONCENTRATION IN *Lactobacillus casei* GROWTH MEDIA

Lb.cs – *L. casei*
MRS + Lb.cs – *L. casei* in MRS broth
Fuc-CCE – Fucoidan from conventional chemical extraction
Fuc-MAE – Fucoidan from microwave-assisted extraction
Fuc-UAE – Fucoidan from ultrasound-assisted extraction
Fuc-EAE – Fucoidan from enzyme-assisted extraction
(The results are presented as mean ± standard deviation (error bars))

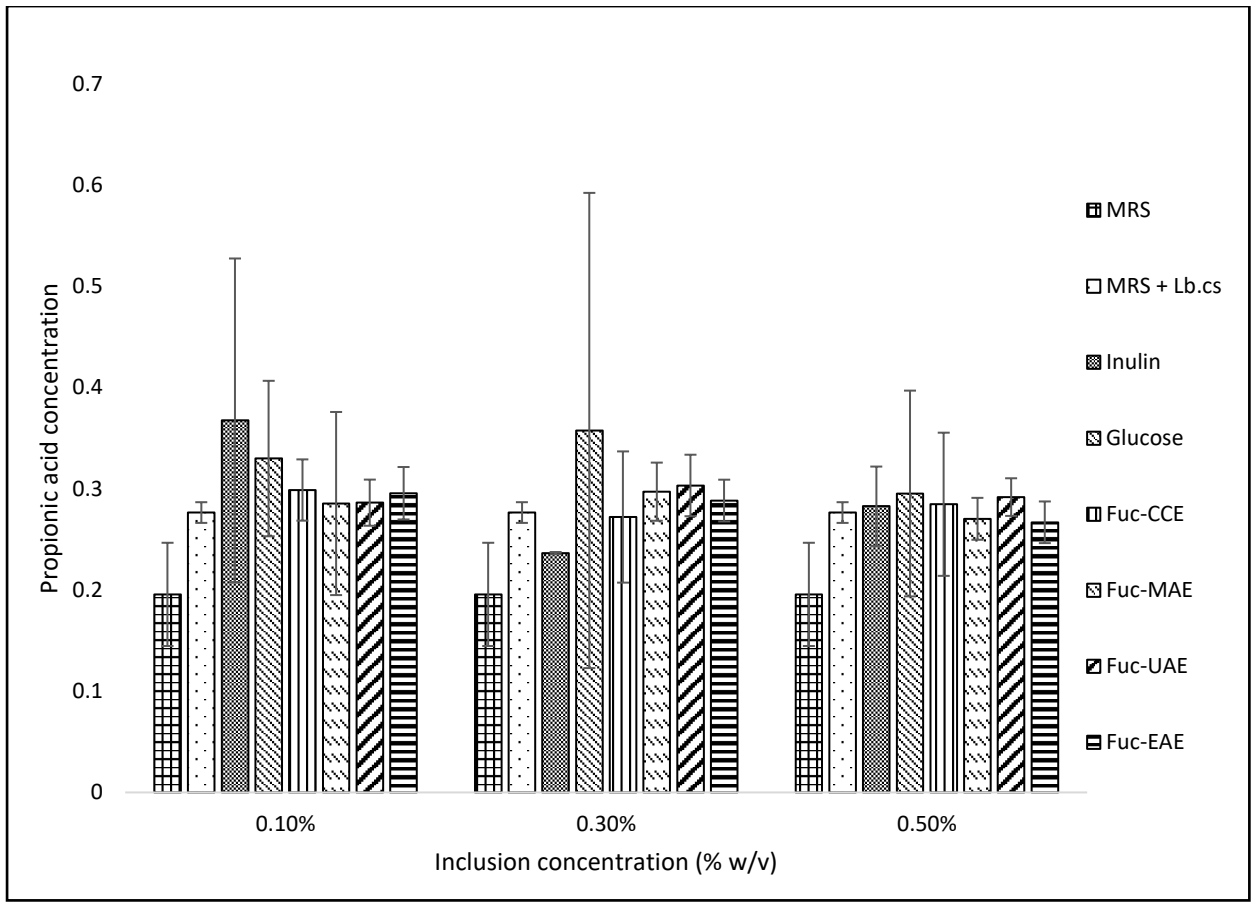


FIG. 3.6. THE PROPIONIC ACID CONCENTRATION IN *Lactobacillus casei* GROWTH MEDIA

Lb.cs – *L. casei*
MRS + Lb.cs – *L. casei* in MRS broth
Fuc-CCE – Fucoidan from conventional chemical extraction
Fuc-MAE – Fucoidan from microwave-assisted extraction
Fuc-UAE – Fucoidan from ultrasound-assisted extraction
Fuc-EAE – Fucoidan from enzyme-assisted extraction
(The results are presented as mean ± standard deviation (error bars))

CHAPTER 4

THE IMPACT OF EXTRACTION TECHNOLOGIES ON THE STRUCTURE-FUNCTION RELATIONSHIP BETWEEN SODIUM ALGINATE EXTRACTS FROM *ASCOPHYLLUM NODOSUM* AND THEIR *IN VITRO* PREBIOTIC ACTIVITY

4.1. ABSTRACT

The impact of both conventional and novel extraction technologies on the structure-prebiotic activity relationship of sodium alginate extracts of air-dried *Ascophyllum nodosum* was investigated. Sodium alginate was extracted with conventional (Alg-CCE), microwave-assisted (Alg-MAE), ultrasound assisted (Alg-UAE), and a combination of both enzyme-assisted & conventional (Alg-EAE/CCE) extraction methods. Single (1×) and triple extractions (3×) were performed for CCE, MAE and UAE, with only a triple extraction for EAE/CCE. Extract yields were significantly increased by triple than single extraction. Average molecular weight data were within the range of 65 to 215 kDa, with similar dispersity index for all extract groups. The uronic acid content was highest in the Alg-MAE (1×) extract with significantly lower amounts in MAE (3×) as compared to the other extracts. The growth rate of an *L. delbruecki* strain in supplemented growth media at 0.1, 0.3 and 0.5 %, were dose-dependently enhanced as compared to un-supplemented growth medium. The same trend was observed for the *L. casei* strain, except at the 0.5% inclusion rate where growth rate declined. No significant impacts on SCFA production were observed, though pH declined further in supplemented sodium alginate growth media. Findings from the present study indicated that, alginate extracts were able to illicit a prebiotic response in two strains of *Lactobacillus in vitro*, though extraction method did not influence this.

4.2. INTRODUCTION

Alginates are a group of commercial food hydrocolloids derived from some brown seaweeds, with a vast range of industrial and pharmaceutical applications. Currently, the global value of alginates is estimated at approximately US$ 300 million, with retail price of about 12 US$/kg (Rhein-Knudsen et al., 2017). These hydrocolloids are localized in the cell wall of some seaweeds and make up a high (16-52%) proportion of most algal polysaccharides depending on the seaweed species (Bertagnolli et al., 2014; Fertah et al., 2017; Rioux et al., 2007). In addition, some alginates can also be found in some bacteria as capsular polysaccharides (Draget & Taylor, 2011). Alginates occur as alginic acid in most seaweeds and are extracted in salt forms, such as sodium, potassium, and calcium alginate. Structurally, alginates are linear copolymers of β-D-mannuronic acids (M) and α-L-guluronic acids (G) linked together by 1→4 linkages. The arrangements of these uronic acids in alginate are either homo-polymeric (MM and GG) or hetero-polymeric (MG), and may vary in proportion depending on certain conditions such as seaweed sources, harvest season, geographical location, conditions, extraction method/conditions, and environmental condition of the ocean (Dettmar et al., 2011; Rhein-Knudsen et al., 2017; Youssouf et al., 2017). Also, the arrangement of these uronic acids in various alginate polymers relates to specific gelling properties and functions. For instance, a high guluronic acid content provides various alginates with their unique, gel-forming functionalities, for which it is used in the food and textile industries as stabilizers, thickeners, gel-formers and film-formers (Dettmar et al., 2011; Youssouf et al., 2017). On the other hand, mannuronate-rich alginates have been reported to illicit an immune response via interaction with pattern recognition receptors (PRR) (Espevik et al., 2009). Other potential biomedical applications of various alginates include drug delivery,

immobilization of cells, and modulation of appetite (Draget & Taylor, 2011). Due to the commercial relevance of some seaweeds and their components, the application of technological processes, with potentially enhanced efficiencies may significantly impact yield. Product maximization on the basis of improved yields and active compounds, and increased revenue taking into consideration, cost of raw materials/extraction process, time, and labour. Also, alginate structure and composition could be modified to best suit the targeted bioactivity or industrial use by selection of appropriate processing/extraction technologies. Technological processes may play key roles in modifying alginate composition particularly depolymerization of the complex matrix of alginate polymers.

Current technical processes that have been applied to improve yield, structural properties, and functionality of extracts include microwave-assisted extraction (MAE), ultrasound assisted extraction (UAE), and enzyme-assisted extraction (EAE). These techniques all apply different operational mechanisms in extracting active compounds from raw materials. The microwave technology uses microwave energy as a volumetrically distributed heat source generated by ionic conduction of dissolved ions and dipole rotation of polar solvents (Rodriguez-Jasso et al., 2011; Yuan & Macquarrie, 2015a). This fast internal heating process results in the splitting of the cell wall, and subsequent release of its content into the extraction medium (Yuan & Macquarrie, 2015a). Ultrasonication technique apply physical forces generated by acoustic cavitation such as shear, shockwaves, microjets and acoustic streaming in the extraction of molecules (Feng et al., 2017). Acoustic cavitation results in the rapid formation and collapse of cavitation bubbles within irradiated liquid medium, leading to intense stress and irreversible chain splitting (Yan et al., 2016). The enzymatic technique uses cell-wall degrading enzymes, such as cellulase, to rupture thick seaweed cell walls and release its contents into the liquid

medium (Charoensiddhi et al., 2017). The expectation is that these techniques would improve the conventional process and produce alginate extracts with better quantitative and qualitative properties. However, this may not always be the case, since the hydrolytic effect of these processes may influence the structural and functional properties of alginate extracts. Also, the extraction conditions may positively or negatively impact structural properties.

A prospective application of various alginate extract is their application as a prebiotic in functional foods and as nutraceuticals. Alginates are non-hydrolysable by human/gut digestive enzymes but they are often fermented by intestinal microbial populations. Alginates are carbon dense structures that can act as a carbon source for the growth and stimulation of beneficial populations of the gut microbiota particularly the genera from the *Lactobacilli* and *Bifidobacteria* genera, hence their prebiotic potential (Ramnani et al., 2012; Wang et al., 2006). *Lactobacillus casei* and *L. delbruecki* are two probiogenic bacteria that can be found in the gut and have also been used as commercial probiotics in dairy products (Cats et al., 2003).

This study therefore investigated the impact of extraction processes on the structure and prebiotic activity of sodium alginate extracts of *A. nodosum*.

4.3. MATERIALS AND METHODS

4.3.1. MATERIALS

The brown seaweed, *Ascophyllum nodosum*, in air-dried condition, was provided by Acadian Seaplants Limited (Dartmouth, Canada). The whole harvested seaweed was

chopped and ground into fine powder with a food processor (Black and Decker, China FP2500C) using a stainless-steel chopping blade (5½ inches), prior to pre-extraction. Guluronic acid standard was purchased from Carbosynth (UK); commercial food grade sodium alginate from Landor Trading Co. Limited (Canada); sulphuric acid, hydrochloric acid, and ethanol from Fisher Scientific; *Lactobacillus delbruecki ss bulgaricus* and *Lactobacillus casei* from Ward's Science (USA); sodium acetate from OmniPur® (Germany); sodium hydroxide and acetic acid from BDH VWR Analytical (USA); prebiotic standard, inulin, from BENEO GmbH (Germany). Short-chain fatty acid standards (propionic, butyric, iso-butyric, valeric and iso-valeric acids), sodium carbonate, Celluclast, sodium phosphate, mannuronic acid, and de Man, Rogosa, and Sharpe (MRS) broth were all purchased from Sigma-Aldrich (St. Louis, USA).

4.3.2. EXTRACTION OF SODIUM ALGINATE FROM ASCOPHYLLUM NODOSUM

4.3.2.1. PRE-EXTRACTION OF *ASCOPHYLLUM NODOSUM*

Prior to the extraction of sodium alginate, pulverised brown seaweeds were suspended in ethanol for the removal of alcohol-soluble impurities such as pigments and proteins. This was carried out in accordance with steps outlined by Rioux et al., (2007). In brief, 100 g of pulverised seaweed was added to 500 mL of 80 % ethanol and placed under constant mechanical stirring for 20 hours at room temperature (23 ºC), after which, the temperature of the mixture was increased to 70 ºC and stirred continuously for another 5 hours. This step was repeated two more times, after which, the mixture was filtered, and the residual seaweed used for further extraction.

4.3.2.2. CONVENTIONAL CHEMICAL EXTRACTION

As a purification step, fucoidans were first extracted to prevent interference with alginate, our component of interest. The stepwise extraction of alginate was carried out as described by Rioux et al., (2007) with some modifications. Pre-extracted brown seaweed (10g) was dissolved in 100 mL of 0.01 M hydrochloric acid (HCl) and kept under constant stirring at 70 ºC for 3 hours. Two more rounds of extraction were performed on the same residue, with 0.01M HCl, in an attempt to exhaustively extract all fucoidans from the residue (3x). At the end of each 3-hour heating period, the filtrate was separated from the residue by centrifugation (3000 ×g; 20 min). The single extraction (1×) was carried out by hydrating another 10 g of pre-extracted seaweed in 100 mL of 0.01 M HCl once. The final residues (3× and 1×) after fucoidan extraction, were re-hydrated in 100 mL of 3 % (w/v) sodium carbonate (Na_2CO_3) for three hours, with the process repeated 3× and 1×. At the end of the extraction, the filtrates were precipitated with 4 volumes of 95% ethanol and kept at room temperature for 3 hours, after which the mixtures were centrifuged, and the precipitated sodium alginates separated from the filtrates. The sodium alginate precipitates were then freeze-dried, ground with a ball mill (20 min, medium-sized spherical balls), and stored in Falcom sterile air-tight containers at room temperature, for further analysis.

4.3.2.3. MICROWAVE-ASSISTED EXTRACTION

The microwave extraction of sodium alginate from *A. nodosum* was done in line with steps described by Yuan & Macquarrie (2015), using the CEM Discover and Explorer Microwave (MARS 6 230/60 910900, USA). Hydrochloric acid (0.01M, 15 mL) was added to each of three microwave tubes containing 1 g of pre-extracted *A. nodosum*.

Microwave irradiation was performed for 15 min at 90 ºC, (3×) and (1×), for fucoidan removal. A solution of 3 % (w/v) Na_2CO_3 (15 mL) was then added to residual seaweed from (3×) and (1×) for sodium alginate extraction at 100 ºC for a period of 10 min. The filtrates obtained after separation of sodium alginate extracts from residues were precipitated with 95% ethanol (4:1) and left to stand for about 3 hours. The mixtures were centrifuged (3000 ×g, 20 min), and the precipitates freeze dried, ground with a ball mill (20 min, medium-size spherical ball), and stored in Falcom sterile air-tight containers at room temperature.

4.3.2.4. ULTRASOUND-ASSISTED EXTRACTION

The ultrasound-extraction of alginate was performed using a Sonics and Materials Inc. Ultrasonicator (USA VCX750), in accordance with steps described by Kadam et al., (2015) with some modification. Pre-extracted seaweed (10g) was suspended in 100 mL of 0.01 M HCl and sonicated (20 kHz; 35 min) continuously with a half inch (13 mm in diameter) probe with removable tip, at an amplitude of 40 %. The process was repeated 3×. Residual seaweed material from (3×) and (1×) extractions were then treated with 100 mL of 3 % (w/v) Na_2CO_3 for alginate extraction at the same conditions of 20 kHz and 40 % amplitude for 35 min. The filtrates after extraction were precipitated with ethanol (4:1) and left to stand for about 3 hours, after which the mixtures were centrifuged, and the precipitates freeze-dried, ground with a ball mill (20 min, medium-sized spherical balls), and stored in Falcom sterile air-tight containers at room temperature.

4.3.2.5. ENZYME-ASSISTED/CONVENTIONAL CHEMICAL EXTRACTION

The enzyme-assisted extraction of sodium alginate was done in accordance with steps described by Charoensiddhi et al., (2016), Borazjani et al., (2017), and Rioux et al., (2007). Pre-extracted seaweed (10g) was immersed in 100 mL of sodium acetate buffer (pH 4.5) and heated to 50 ºC in a water bath with orbital shaker. The enzyme Celluclast (pH 4.5, 50 ºC) which disrupts cellulase in the cell wall, was added to the mixture and kept at this condition for 24 hours, after which the filtrate was separated from the residue by centrifugation (3000 ×g; 20 min). The resulting residue was then mixed with 100 mL of 3 % (w/v) Na_2CO_3 and extraction was performed using the conventional method at a temperature of 70 ºC for 3 hours (3×). At the end of the extraction process, the filtrate containing alginate extract was precipitated with 4 volumes of ethanol and allowed to stand for 3 hours. The precipitate, after centrifugation, was freeze-dried and stored in air tight containers, at room temperature, for further analysis.

4.3.3. HPLC MONOSACCHARIDE ANALYSIS OF SODIUM ALGINATE EXTRACTS

4.3.3.1. HYDROLYSIS OF SODIUM ALGINATE EXTRACTS

Sodium alginate extracts were hydrolysed with steps outlined by Wu et al., (2014). Sodium alginate extracts were dissolved in 0.1 M HCl at a concentration of 20 mg/mL and hydrolysed using microwave irradiation (1600 W) at 130 ºC for 15 min, after which the solutions were neutralized to pH 7 with NaOH.

4.3.3.2. HPLC ANALYSIS OF SODIUM ALGINATE EXTRACTS

Monosaccharides present in sodium alginate extracts were analysed using the Perkin Elmer HPLC system with refractive index (RI) detector. The data were obtained using Chromera software manager provided by Perkin Elmer Company (USA). Hydrolysed sodium alginate extracts were analysed for monosaccharide composition using the Bio-Rad Aminex HPX-87H column (300 x 7.8 mm), with 0.01 M sulphuric acid as the mobile phase at a flow rate of 0.6 mL/min, column temperature of 60 °C, and an injection volume of 50 µL (Templeton et al., 2012). A second column, Bio-Rad Aminex HPX-87P (300 x 7.8 mm), with ultrapure water as the mobile phase at a flow rate of 0.6 mL/min and RI detector. Monosaccharide standards (mannuronic and guluronic acid) were prepared at concentrations of 0.5, 1.0, 1.5, 2.5 mg/mL.

4.3.4. DETERMINATION OF URONIC ACID CONTENT OF SODIUM ALGINATE EXTRACTS

The uronic acid content of the sodium alginate extracts was determined as described by Cesaretti et al., (2003). Different concentrations (250, 125, 62.5, 31.25, 15.625, 7.813 and 0 µg/L) of standards (guluronic acid, mannuronic acid, and glucuronic acid) and sodium alginate extracts (1 mg/mL) were prepared prior to the analysis. Standard solutions and samples (50 µL) were added to a 96 well plate. Subsequently, 25 mM of sodium tetraborate in concentrated sulphuric acid (200 µL) was added. The plates were then heated in an oven (100 ºC, 15 min), after which, left to cool for 15 min. After cooling, 0.125 % of carbazole in absolute ethanol was added and heated in an oven at 100 ºC for another 15 min. The microplate was then left to cool for 15 min and the absorbance readings taken at 550 nm.

Guluronic, mannuronic and glucuronic acid equivalent of the samples were obtained from the respective standard curves.

4.3.5. MOLECULAR WEIGHT DISTRIBUTION OF SODIUM ALGINATE EXTRACTS

The Agilent Technologies 1260 Infinity II Gel Permeation Chromatography (GPC) System was used to determine the molecular weight distribution of sodium alginate extracts in accordance with steps described by Lim et al., (2016). The GPC system consists of an Agilent pump, an Agilent autoinjector and a refractometer. The GPC system was connected to a multi-angle laser light scattering detector (MALLS) and a RI detector. The PL – aquagel-OH MIXED-M 8µm 300 × 7.5 mm (PL1149-6801) column was used for the molecular weight analysis. The mobile phase, 0.1M sodium acetate prepared with ultrapure water, was prefiltered through a 0.45 µm filter. The GPC/SEC system calibration and performance were verified using Agilent GPC/SEC calibration kits containing pullulan polysaccharides with molecular weights ranging from 0.18 kDa – 642 kDa. A differential refractive index increment (dn/dc) value of 0.129 was used and the data obtained were analysed using the Agilent GPC/SEC software manager. The number average molecular weight, average molecular weight, polydispersity index, and peak area data were determined from the analysis.

4.3.6. PROTON NUCLEAR MAGNETIC RESONANCE ANALYSIS (^{1}H NMR)

Sodium alginate samples were depolymerized prior to NMR analysis to reduce the viscosity of the samples as described by Rhein-knudsen et al., (2017). The samples (100 mg each) were dissolved in 300 mL of ultra-pure pure water and the pH of the mixtures adjusted to 5.6 with 0.1 M HCl and heated in a water bath at 95 ºC for 1 hour. The pH of the mixtures was re-adjusted to 3.8 and heated for another 45 min at the same temperature, after which the solutions were neutralized to pH 7 with 0.1 M NaOH. The solutions were placed in an oven at 60 ºC to evaporate the water. Subsequently, the dried samples were reconstituted in water and freeze-dried. The freeze-dried samples were dissolved in about 400 µL of D_2O for NMR analysis. The NMR spectrum was recorded at 20 ºC on a Bruker Avance 500 MHz spectrometer.

4.3.7. FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

Freeze-dried sodium alginate samples were ground and mixed with the potassium bromide (KBr) standard, 10% (w/w), in an agate mortar and pelleted prior to a FTIR analysis with a FTIR Nicolet 6700 Spectrometer (Thermo Instruments, Canada). 64 scans were taken with a resolution of 4 cm^{-1}. The spectra of the samples were recorded within the range of 450 – 4000 cm^{-1}.

4.3.8. DETERMINATION OF THE SODIUM, POTASSIUM AND CALCIUM CONTENTS OF SODIUM ALGINATE EXTRACTS

The sodium, potassium and calcium content of the various sodium alginate extracts were determined using the Thermo-Fischer M-Series Flame Atomic Absorption Spectrometer (AAS). The AAS was equipped with a deuterium lamp for background correction and an air-acetylene flame for the quantification of the minerals. The wavelength and bandwidth used for the analysis were 589 nm and 0.2 nm for sodium; 766.5 nm and 0.5 nm for potassium; 422.7 and 0.5 nm for calcium. The sodium, potassium and calcium equivalents of sodium chloride, potassium chloride and calcium chloride, prepared at different concentrations (0, 1, 3, 5, 7, and 10 ppm), were measured. The sodium alginate samples (10 and 100 ppm) were measured and the concentrations of each of the trace elements were determined from their respective standard curves. Both standards and samples were measured for 4 seconds at a flow rate of 1.2L/min.

4.3.9. PREBIOTIC ACTIVITY OF ALGINATE EXTRACTS

4.3.9.1. GROWTH RATE AND DOUBLING TIME

The influence of sodium alginate extracts on the growth rate of two lactobacilli, *Lactobacillus casei* and *L. delbruecki* ss *bulgaricus,* was studied in line with steps described by Chen et al. (2016) with some modifications. In summary, *L. casei* and *L. delbruecki* strains were purchased from Ward Science (USA) and cultured in MRS agar for about 48 – 96 hours. The MRS agar was prepared by adding 1.2% agar to a MRS broth (5 % (w/v)). The mixture was subjected to mechanical stirring until the agar was

completely dissolved in the solution, after which it was sterilized at 121 °C for 25 min. After sterilization, the MRS was allowed to cool for about 15-20 min and then poured into petri dishes and left over-night in the fume hood. Isolated strains from both bacterial cultures were inoculated in freshly prepared and sterilized MRS broth and incubated till the turbidity (optical density at 600 nm) of the culture was 1.0.

Inulin (positive control), glucose (negative control) and sodium alginate extracts were separately added to MRS broths at inclusion rates of 0.1, 0.3 and 0.5 %. Supplemented MRS broths (200 µL) were added to a falcon 96-well plate after which 7.5 µL of the *L. casei* and *L. delbruecki* cultures were added to an un-supplemented MRS broth and supplemented samples. MRS broth and supplemented samples without inoculation were also added as blank, and the 96-well plate sealed with a Falcom 96-well plate seal. A 24-hour incubation was done, with the optical density (OD) (at 600 nm) measurement taken every 0.5 hour and the temperature maintained at 37 °C throughout the experiment. At the end of the incubation period, the optical densities of the blank samples were subtracted from those of the inoculated samples, the resulting data were plotted against time to obtain a growth curve. The growth rates and doubling times were calculated from the exponential phase of the growth curve using the formula below:

$$\text{Growth rate} = \frac{\log Nt - \log No}{0.301 \times t}$$

Where, Nt = cell number or optical density at time, t

No = cell number or optical density at initial time

t = difference between final time and initial time

Doubling time = 1/growth rate (Neidhardt et al., 1990)

4.3.9.2. SHORT CHAIN FATTY ACIDS ANALYSIS

The concentrations of short chain fatty acids (SCFA) (often referred to as volatile fatty acids (VFA)) present in sodium alginate-supplemented bacterial cultures were determined in accordance with steps described by Chen et al. (2016). Cultured samples were centrifuged (3000 ×g; 5 min) to remove bacterial debris. The supernatant was collected for analysis. SCFA standards (acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, and iso-valeric acid) and samples were analysed using the Perkin Elmer HPLC system and a Bio-Rad Aminex HPX-87H column. Sulphuric acid (5 mM) was used as the mobile phase, at a flow rate of 0.6 mL/min and column temperature of 50 °C. The data were collected and analysed using Chromera software manager.

4.3.9.3. pH CHANGE DURING MRS INCUBATION

The pH of the MRS broth and all cultured samples were measured prior to a 24-hour incubation period and after the incubation period using a pH meter (Thermo Fischer Scientific, USA). The pH meter was calibrated with standard solutions of pH 4, 7, and 10, prior to sample measurement.

4.3.10. STATISTICAL ANALYSES

All experiments, except for M/G ratio, H^1 NMR, and pH measurements, were performed in triplicate. The results were expressed as mean ± standard deviation and analysed for statistical significance at $p < 0.05$, using the statistical package for social sciences (SPSS) version 20 (IBM SPSS statistics for windows, Armonk, New York, USA). The Levene's and Welch's test for homogeneity of sample distribution were performed, followed by a one-way analysis of variance (ANOVA) using the Duncan's multiple comparison test. The impact of extraction methods and number of extractions on the structural properties of sodium alginate extracts was also assessed using factorial analysis.

4.4. RESULTS AND DISCUSSION

4.4.1. CHARACTERIZATION OF THE SODIUM ALGINATE EXTRACTS

4.4.1.1. EXTRACT YIELD

Extract yield is often used as a measure of the efficiency of extraction processes. The yield of sodium alginate from the four extraction processes was compared in this study. The protocol for the extraction of sodium alginate using the conventional method (Rioux et al., 2007) recommended a three times extraction on the same residue, whereas a single extraction was carried-out for the MAE protocol (Yuan & Macquarrie, 2015b). For effective comparison and to ensure exhaustive extraction of all components present in the matrix, both single and triple extractions were conducted in this study for CCE, MAE, and UAE. The fourth extraction process was a combination of enzymatic and conventional

extraction technique. The enzyme, Celluclast, was used to disrupt the cell wall, while the conventional method was used to extract alginates (3×) (Borazjani et al., 2017).

Compared to literature reports, the alginate yield obtained in this study (21 – 90% (w/w)) appeared to be higher. Some of the reported yields include: *F. vesiculosus* – 16.2% (w/w) (Rioux et al., 2007), *Sargassum filipendula* – 17.0% (w/w) (Bertagnolli et al., 2014), *Padina pavonica* - 17.5% (w/w) (Ammar et al., 2018), *A. nodosum* – 18 and 23% (w/w) (Yuan & Macquarrie, 2015b), *Saccharina longicruris* – 20.0% (w/w) (Rioux et al., 2007), *A. nodosum* – 24.0% (w/w) (Rioux et al., 2007), *Macrocystis pyrifera* – 26% (w/w) (Rhein-Knudsen et al., 2017), *Laminaria digitata* – 29% (w/w) (Rhein-Knudsen et al., 2017), *S. vulgare* – 30.2% (w/w) (Behairy & El-Sayed, 1983), and *L. digitata* – 38–52% (w/w) (Fertah et al., 2017).

The results (Table 4.1) from this study show a significant increase in yield of sodium alginate in 3× as compared to 1× extraction for CCE, MAE and UAE. Also, alginate from enzyme-assisted/conventional extraction (Alg-EAE/CCE) 3× was significantly higher ($p < 0.05$) than alginate from microwave-assisted extraction (Alg-MAE) 3× and alginate from ultrasound-assisted extraction (Alg-UAE) 3×. These results therefore indicated that the extract yield was improved with a 3× extraction, as compared to 1×. While this may seem to reflect an increase in the bioactive components, this may not always be the case, as the extract may contain other excipients, such as minerals, which may be higher than sodium alginate content. The sodium alginate content in 3× extraction (56 – 90% w/w) appeared not to be consistent with the literature values (20 – 30 %). Some of the excipients that may be present in the extract may include unbound sodium. Several other factors may be responsible for the marked difference in the yields obtained in the present study and

literature values. They include origin of seaweed species, extraction technique, and the number of excipients in the extraction medium. An assessment of the impact of extraction methods and number of extractions, on the yield of sodium alginate was done using factorial analysis. The results (Appendix A, Table A3) indicated that the number of extractions significantly ($p < 0.05$) influenced the yield, but not the extraction methods. This implied that doing a 3× extraction significantly increased the yield and that there were no major differences amongst the yields from the different extraction methods.

4.4.1.2. MONOSACCHARIDE CONTENT OF THE SODIUM ALGINATE EXTRACTS

Alginates are linear co-polymers of mannuronic and guluronic acids and these monosaccharides exist in varying amounts, composition, and sequence, which may be influenced by certain factors such as seaweed species, geographical location and environmental condition. In an attempt to measure the amount of mannuronic and guluronic acids present in the extracts, the Bio-Rad Aminex HPX-87P column (300 x 7.8 mm) was used in HPLC. However, quantifying sodium alginate monosaccharides with the aforementioned methods proved unsuccessful, as the methods were unable to properly separate and quantify the guluronic and mannuronic acid standards. A similar observation has been noted by Amaniampong et al., (2016), who reported that they could not separate/detect guluronic acid with an ICE-COREGEL 107 H column 300 × 7.8 mm (Amaniampong et al., 2017).

4.4.1.3. DETERMINATION OF URONIC ACID CONTENT IN SODIUM ALGINATE EXTRACTS

Glucuronic, mannuronic, and guluronic acids are all stereoisomers of uronic acids and may be used as standards in the carbazole reaction for measuring total uronic acid content (Braccini et al., 1999). Both guluronic and glucuronic acid contain the same number of carbon, hydrogen and oxygen atom, but differ in spatial arrangement on the third and sixth carbon (Braccini et al., 1999). Guluronic and mannuronic acids are the two major uronic acid constituents in the sodium alginate fraction. The total uronic acid content of the extracts was analysed with two standards (guluronic and mannuronic acid) using the colorimetric uronic acid carbazole reaction method. Estimation of total uronic acid became necessary since the separation and quantification of mannuronic and guluronic acid proved unsuccessful with the hydrolysis/HPLC conditions applied in this study. The carbazole reaction operates on the principle of hydrolysing extracts (with tetraborate in concentrated sulphuric acid), prior to the reaction with carbazole, which gives off a violet colour, and the absorbance measured. The hydrolysis with a much more concentrated acid may have been aided in splitting the sodium alginate molecules as compared to the 0.1M HCl applied in the hydrolysis stage prior to HPLC analysis. Although both mannuronic and guluronic acid standards were used in this, this should not be confused with measuring the mannuronic and guluronic acid content of sodium alginates. Both standards were used to comparatively estimate the total uronic acid content given that they are the major uronic acids in alginate.

From the results presented in Table 4.2, Alg-MAE (1×) had the highest uronic acid content of all four extraction methods, for both standards. The 1× extracts of both Alg-MAE and

Alg-UAE were significantly higher than Alg-MAE (3×) and Alg-UAE (3×), for both standards. Although not significant, uronic acid were higher in CCE (1×) extracts than CCE (3×). Also, whilst Alg-MAE had the highest uronic acid content in 1×, its content was significantly low in 3× extraction when compared to Alg-EAE/CCE and Alg-UAE. It may be that, with 3×, more undesirable components, such as minerals, may have been extracted which may have resulted in the reduction of uronic acid content in the 3× extracts with respect to their overall composition. The results suggested that single extraction products may contain more uronic acids than triple extraction relative to the weight of the sodium alginate extract. A factorial analysis of the impact of extraction methods and number of extractions on the uronic acid content was also undertaken. The results (Appendix A, Table A3) indicated that there was a significant impact of both independent variables (extraction methods and number of extractions) on the guluronic and mannuronic acid equivalent of the total uronic acids. Both uronic acid contents were significantly ($p < 0.05$) lower in 3× extraction compared to 1× extraction. It also suggested that there were significant differences in the uronic acid contents from the different extraction methods. The interaction between both independent variables also significantly influenced the total uronic acid contents of both guluronic and mannuronic acid standard equivalent.

Overall, the total uronic acid contents (0.3 – 10.8% w/w) obtained using both mannuronic and guluronic acid standards in the present study appeared to be low when compared to the commercial alginate obtained from Landor trading Co. (15 – 61% w/w) and to literature values (23 – 77% w/w). Commercial alginate purchased from Sigma Aldrich, has been reported to have 58% mannuronic acid and 41% guluronic acid (Rhein-knudsen et al., 2017). Reported values for the uronic acid content of a number of seaweed species including *A. nodosum* (32.3%) (Rioux et al., 2007), *F. vesiculosus* (29.6%) (Rioux et al.,

2007), *Saccharina longicruris* (23.9%) (Rioux et al., 2007), *Padina pavonica* (74.13%) (Ammar et al., 2018), *Cystoseira compressa* (76.97%) (Ammar et al., 2018), and *Dictyopteris membranaceae* (69.81%) (Ammar et al., 2018). Low uronic acid content of about 0.3% has also been reported for *Sargassum turbinariodes* extracts (Fenoradosoa et al., 2010). The marked discrepancy in uronic acid contents in the present study and the literature values may be attributed to differences in source, species, seasonal changes, preparation methods, and seaweed composition.

4.4.1.4. MOLECULAR WEIGHT DISTRIBUTION OF SODIUM ALGINATE EXTRACTS

The weight average molecular weight (Mw), number average molecular weight (Mn), and dispersity index (Đ) of sodium alginate extract are reported in Table 4.3. The PL – aquagel-OH MIXED-M 8μm 300 × 7.5 mm (PL1149-6801) column, which analyses polymers within the range of 1 kDa to about 500 kDa was used for this study. Also, the peak area and peak area percentage for all peaks above 0.2 kDa (a minimum molecular weight cut off for lowest possible disaccharides) was also recorded. The sodium alginate extracts in this study all had two peaks corresponding to two molecular weight distributions. The higher molecular weights are most likely to be in the range of alginate, while the lower molecular weights may represent other excipients or hydrolysed alginate fractions. In the present study, the average molecular weights of sodium alginate extracts were within the range of 65 – 215 kDa while that of the commercial alginate was about 318 kDa. Comparing the trend in 3×, Alg-MAE (3×) had a lower molecular weight when compared to Alg-CCE (3×), Alg-UAE (3×), and Alg-EAE/CCE (3×). For 1× extraction products,

Alg-MAE (1×) had a higher molecular weight when compared to Alg-CCE (1×) and Alg-UAE (1×). A higher peak area was observed in Alg-CCE (3×) than Alg-CCE (1×) with no significant differences amongst the Mw for 3× and 1×. Higher Đ in Alg-CCE 3× than 1× was indicative of a broader and more heterogenous molecular weight distribution. For Alg-MAE and Alg-UAE, lower average molecular weight, peak area and peak percentage were observed in 3× rather than 1× extractions. This suggested that there was more hydrolytic activity in 3× rather than 1× in both methods, resulting in the extraction of lower molecular weights, probably due to the additional energy inputs. However, there was no significant difference in Đ. In Alg-CCE (3×), the Mn was twice as low compared to Alg-CCE (1×), which is also indicative of higher hydrolytic activity in Alg-CCE (3×). The lower peak percentages in 3× rather than 1× for MAE and UAE also suggest that 3× may have resulted in the co-extraction of higher amounts of excipients. Also, lower percentage peak areas were observed in all extracts compared to the commercial alginate, although Alg-MAE (1×) and Alg-UAE (1×) had appreciable percentage peak areas which suggests that extract may be low in sodium alginate. The peak areas (peak one) of the extracts varied with the number of extraction. For example, the peak areas were lower in 3× than 1× extraction for Alg-MAE and Alg-UAE. This data suggested that more sodium alginate may be present in 1× than 3× extracts for both methods. For Alg-CCE, the percentage peak area was more in 3× than 1× extraction and thus suggested that more sodium alginate may be present in the 3× extract.

Reported molecular weight for sodium alginates differ amongst various species within the literature: *A. nodosum* - 177.3 kDa (Rioux et al., 2007), *Sargassum Vulgare* – 514 kDa (Rhein-Knudsen et al., 2017), *S. angustifolium* - 356 kDa (Borazjani et al., 2017), *S. longicruris* – 106 kDa (Rioux et al., 2007), *L. digitata* – 114 kDa (Fertah et al., 2017), *F.*

vesiculosus – 154.9 (Rioux et al., 2007), *Marcocytis pyrifera* – 719 kDa (Rhein-Knudsen et al., 2017). The differences in molecular weight may be attributed to seaweed species, source, analytical method/conditions used, column size, and seasonal influence. A factorial analysis of the influence of extraction methods and number of extractions on the average molecular weight and the dispersity index was performed. The factorial analysis data (Appendix A, Table A3) indicated that there was a significant ($p < 0.05$) impact of extraction methods and number of extractions on the average molecular weight. The average molecular weight decreased significantly in 3× extraction compared to 1× extraction. For dispersity index, neither of the two variables had a significant ($p < 0.05$) impact.

4.4.1.5. H^1 NMR ANALYSIS OF SODIUM ALGINATE EXTRACTS

The H^1 NMR analysis of commercial alginate (Fig 4.1) and sodium alginate extracts (Fig 4.2 – 4.11) was done in this study with the aim of understanding the impact of extraction processes on the M/G ratio of extracts. Extracts from the 3× extraction was used for the NMR analysis on the basis that more alginate molecules are expected to be present in the 3× extracts, considering that higher yields were recorded in the 3× than 1×. The peaks corresponding to chemical shift of signals within the extracts were assigned as described by Rhein-knudsen et al., (2017), Borazjani et al., (2017) and Youssouf et al., (2017): the alpha anomeric reducing ends (A-red) at about 5.2 parts per million (ppm); the anomeric guluronic acid proton at around 5.07 ppm; the beta reducing ends (B-red) at about 4.9 ppm, the H-5 central guluronic acid in a GGM and MGM triad at about 4.78 and 4.75 ppm respectively; are the anomeric mannuronate proton at about 4.65 ppm (Borazjani et al.,

2017; Rhein-Knudsen et al., 2017). Peaks corresponding to guluronic and mannuronic acid protons and their positions between 3.70 and 4.0 ppm were also assigned: position 3 guluronic acid at about 4.0 ppm (G-3); position 2 mannuronic acid (M-2) at about 3.95 ppm, position 2 guluronic acid (G-2) at about 3.9 ppm; Position 4 and 3 mannuronic acids (M-4 and M-3) at about 3.8 and 3.7 ppm respectively (Youssouf et al., 2017).

The M/G ratio is often calculated from the relative peak area of the anomeric proton corresponding to both M and G with ppms of about 4.65 and 5.10 respectively (Borazjani et al., 2017; Rhein-Knudsen et al., 2017). In the present study, it was observed that the solvent peaks for D_2O interfered with the anomeric proton region of mannuronate and little or no peaks were detected around the guluronate anomeric proton region. This was for both commercial alginate and sample extracts, thus making it difficult to obtain a M/G ratio. However, different peaks corresponding to chemical shifts of guluronate and mannuronate protons confirmed the presence of alginate. Optimization of sample preparation methods, such as, increasing and decreasing the concentration of the extracts, and the use of different hydrolysis methods (Borazjani et al., 2017; Rhein-Knudsen et al., 2017; Youssouf et al., 2017), did not influence the results obtained from these strategies.

4.4.1.6. FTIR ANALYSIS OF SODIUM ALGINATE EXTRACTS

The FTIR analysis of commercial sodium alginate and sodium alginate extracts (Appendix B) from the triple extraction processes was done to confirm the presence of sodium alginate in the sample. Commercial sodium alginate was used as a standard for comparison. Peaks corresponding to various vibration stretches were identified between 450 and 4000 cm^{-1}. Band signals at around 1612-1692 and 1409-1457 cm^{-1} corresponded

to asymmetrical and symmetric vibration of carboxylate groups belonging to mannuronate and guluronate moieties present in alginate (Borazjani et al., 2017; Fertah et al., 2017; Rhein-Knudsen et al., 2017; Rostami, Tabarsa, You, & Rezaei, 2017b; Youssouf et al., 2017). These signals were found in both commercial sodium alginate and experimental sodium alginate extracts, although with lower band intensity in the sample extracts compared to the commercial alginate standard. Also present in all of the FTIR spectra, were signal bands at around 1031-1034 and 1085-1095 cm^{-1} attributed to O-H bending of guluronic acid and mannuronic acid units respectively (Rhein-Knudsen et al., 2017).

Also, a broad band signal at about 3420 cm^{-1} was attributed to stretching vibrations of hydroxyl groups were present in commercial alginate and sodium alginate extracts (Borazjani et al., 2017; Rostami et al., 2017). Peaks at 2921 - 2977 cm^{-1} in the spectra corresponded to C-H stretching vibrations (Leal et al., 2008). Signals at about 2300-2400 cm^{-1} were attributed to CO_2 and all peaks above 3600 cm^{-1} were indicative of the background peaks. Similar FTIR spectra with peaks band, as seen in this study, have been recorded for sodium alginate extracts from *Sargassum* spp. and *Padina* spp. (Rhein-knudsen et al., 2017), *Laminaria digitata* (Fertah et al., 2017), *Sargassum angustifolium* (Borazjani et al., 2017), *S. binderi* and *Colpomenia peregrina* (Rostami et al., 2017).

The FTIR spectra were also used to determine the M/G ratio (Table 4.5) of the various alginate extracts. This was calculated from the peak intensity of band signals corresponding to the OH bending of mannuronic (1031-1034 cm^{-1}) and guluronic acid (1085-1095 cm^{-1}) (Sakugawa et al., 2004). The arrangement of mannuronic (M) and guluronic acid (G) in alginate are strong determinants of some of its properties. A high G content is indicative of strong gelling property, whereas a high M content may suggest

flexibility (Dettmar et al., 2011; Draget & Taylor, 2011; Sakugawa et al., 2004). Hence, the M/G ratio index is used here to indicate the ratio of M to G. In the present study, a slightly higher expression of G than M was observed in the standard and alginate extracts except for Alg-CCE (1×) which had equal expression of both uronic acids. Also observed, the peak intensities for both uronic acids were lowest in Alg-UAE (1×), Alg-UAE (3×), and Alg-MAE (1×), suggesting lower expression of uronic acids as compared to extracts prepared by other methods. This observation contradicts the uronic acid content result, since UAE had higher uronic acid content in the colorimetric assay. The FTIR analysis is a more reliable analysis for M and G as compared to colorimetric determination of uronic acids which gives the total uronic acid content, including other interfering sugars/uronic acids. The 1× extracts of Alg-MAE and Alg-UAE both had a lower M/G ratio as compared to its 3× extracts. This data suggested that the 3× extraction, with the application of microwave and ultrasound energy, may have resulted in reduced of the gelling properties of the different alginate extracts. In the Alg-CCE extract, the reverse was the case, the M/G ratio decreased with the 3× extraction. This suggested that the gelling property of the Alg-CCE extract was improved with the triple extraction. Also, the M/G ratio was lowest in Alg-UAE (1×) (0.6610) compared to all other extracts, which suggested that a single extraction with Alg-UAE produced extracts with higher gelling properties as compared to other extraction methods.

The reported M/G ratios for a variety of brown seaweed species in the literature include: *A. nodosum* – 1.5; *A. nodosum* – 1.44 (Yuan & Macquarrie, 2015a); *A. nodosum* – 1.56 (Yuan & Macquarrie, 2015b); A. nodosum – 0.85 (Rioux et al., 2007); *F. vesiculosus* – 1.44 (Rioux et al., 2007); *Sargassum vulgare* – 0.6 (Rhein-Knudsen et al., 2017); *Laminaria digitata* – 1.9 (Rhein-Knudsen et al., 2017); *Marcocytis pyrifera* – 1.8 (Rhein-

Knudsen et al., 2017). The M/G ratio obtained in the present study were well within the reported literature values and appear to possess higher gelling properties than some of the reported brown seaweeds.

4.4.1.7. SODIUM, POTASSIUM, AND CALCIUM CONTENTS OF SODIUM ALGINATE EXTRACTS

The sodium, potassium, and calcium contents were investigated to quantify the amount of these elements within the various alginate extracts and its interference with our active ingredient of interest. From the results presented in Table 4.4, the sodium content of the sodium alginate extracts was significantly higher than potassium or calcium which were both recorded < 1 % (except for potassium Alg-MAE 1×). Extraction of the alginate fraction with 3 % (w/w) sodium carbonate may be responsible for the high amount of sodium present in the extracts. Comparing the extracts from the different extraction processes, the sodium content of Alg-CCE extracts (1× and 3×) were significantly ($p < 0.05$) higher than the rest of the extracts. Also, sodium content was increased significantly in 3× extraction compared to 1× for Alg-MAE and Alg-UAE extracts but not Alg-CCE. The role of sodium in the extraction process is to bind the carboxylic end of either of the alginate monomers, which is subsequently precipitated with ethanol. Alg-CCE (1× and 3×) both had significantly ($p < 0.05$) low uronic acid content but had the highest sodium content, which is indicative of a high amount of unbound sodium. The potassium content of the extracts was higher in the 1× than in the 3× extractions with the highest content in Alg-MAE extracts. This may be attributed to the extraction of other components with the second and third extraction which may have reduced the calcium content in the 3× extracts.

Also, the potassium content was significantly ($p < 0.05$) higher in the Alg-MAE (3×) and Alg-UAE (3×) extracts, thus suggesting that the energy applied by MAE and UAE produced better potassium contents, as compared to the other extraction methods. For calcium, its content in alginate extracts were below zero except for the Alg-CCE (3×) extract which recorded 0.034 (% w/w), thus suggesting the absence of calcium in the sodium alginate extracts. The results in this study (Appendix A, Table A4), also indicated that both extraction methods and number of extractions significantly ($p < 0.05$) influenced sodium, potassium and calcium contents.

4.4.2. PREBIOTIC ACTIVITY OF SODIUM ALGINATE EXTRACTS

Alginates are considered as potential prebiotics due to their resistance to the activities of digestive enzymes, and availability to undergo fermentation by intestinal microbes, and stimulate the growth of beneficial bacterial gut populations (Wang et al., 2006). Sodium alginate extracts may be fermented in the large intestine to varying degrees, depending on the enzymatic competence of the gut microbiome (Wells et al., 2017). The impacts of sodium alginate extracts obtained from various extraction methods on *in vitro* prebiotic activity was studied. Two *Lactobacill*i strains, *L. delbruecki* and *L. casei* were used for *in vitro* prebiotic assessment. The 3× extracts were selected for *in vitro* prebiotic activity on the basis of their performances in the characterization section. Inclusion of Alg-CCE (3×), Alg-MAE (3×), Alg UAE (3×), and Alg-EAE/CCE (3×) at 0.1% in the growth media showed a 61.3%, 75.08%, 52.1%, and 61.31%, respectively increased growth rate for *L. delbruecki* strain as compared to un-supplemented growth medium (Fig 4.6). At 0.3% supplementation, Alg-CCE (3×), Alg-MAE (3×), Alg UAE (3×), and Alg-EAE/CCE (3×)

increased the growth rates by 75.7, 69.8, 39.6, and 52.5%, respectively versus un-supplemented growth media, respectively. Finally, at 0.5% inclusion, the growth rate of *L. delbruecki* media supplemented with Alg-CCE (3×), Alg-MAE (3×), Alg-UAE (3×), and Alg-EAE (3×) was higher than un-supplemented growth medium by 105.6, 150.2, 88.5 and 94.4%, respectively. Similar growth rates were observed for inulin and glucose as compared to sodium alginate extracts at 0.1 and 0.3%. The negative control (glucose), although not a prebiotic, served as a carbon source for beneficial bacteria, like inulin and other extracts *in vitro*, hence provides nutrient for bacterial growth and stimulation. At *in vivo* and human trials, glucose would be expected to be absorbed into the blood stream and may not be present in the large intestine for bacterial growth stimulation. However, appreciably higher *L. delbruecki* growth rates were seen in extracts at 0.5% inclusion, particularly in Alg-MAE (3×) and Alg-CCE (3×) groups.

For the *L. casei* strain (Fig 4.7) at 0.1% concentration, growth rates increased by 16.2, 40.3, 23.7, and 25.9%, for Alg-CCE (3×), Alg-MAE (3×), Alg UAE (3×), and Alg-EAE (3×) extracts were added, respectively, compared to un-supplemented growth media. At 0.3%, the bacterial growth rate increased by 25.4, 34.5, 32.1, and 25.9% in Alg-CCE (3×), Alg-MAE (3×), Alg UAE (3×), and Alg-EAE (3×) supplemented growth media, respectively. At 0.5% inclusion rate, a decline in growth rate was observed. The performance of the sodium alginate extracts in stimulating bacterial growth rate was as effective as the standard commercial prebiotic, inulin on both strains.

Overall, the results implied that alginate extracts had prebiotic activity with no significant differences amongst extraction processes. The data from this study indicated that the uronic acid content, however low, was sufficient for growth of *L. delbruecki* and *L. casei*

strains in the present study. Another possibility was that some excipients such as phenolics and proteins which may not have been fully removed by the pre-extraction step could have acted as additional carbon sources to support the growth of the probiotic strains. Few studies have investigated the prebiotic prospects of alginate. Wang et al., 2006 observed that alginate oligosaccharides, obtained via enzymatic hydrolysis of commercial sodium alginate, stimulated the growth of *Bifidobacterium bifidum* and *B. longum,* even more significantly than fructo-oligosaccharide (Wang et al., 2006). Although the prebiotic potential of alginate extracts observed in this study is promising, further studies should look into quantification of extracts and more complex bacterial population with *in vivo* and human trial stages would be essential to consolidate the suggestions in this study. The doubling time data (Table 4.6), which is the inverse of growth rate, showed a similar trend in activity with decreased doubling time as prebiotic activity increased.

The production of SCFA has been linked to potential health-promoting and disease preventing effects such as antimicrobial activity, modulation of bowel inflammation, reduction of carcinogenesis and improvement in gut health through intestinal epithelial cells via increased mucin production leading to a decrease in cell permeability. Of the three most prominent SCFA (acetate, propionate, and butyrate) two were detected in this study i.e. acetate and propionate. The results (Fig 4.8 – 4.11) obtained from this study indicated that there was no significant impact of supplementing MRS broth with sodium alginate extracts on the production of SCFA (acetic acid and propionic acid). The same could be said of the control samples (inulin and glucose). Although, the differences in propionic acids are not significant, both inulin and glucose supplemented media had lower propionic acid concentration compared to the un-supplemented media which may indicate that both standards decreased propionic acid content.

An increase in SCFA production would result in a decrease in pH. The maintenance of gut pH near the acidic region is an indicator of a balance between production of beneficial gut bacteria and the non-beneficial ones. The results (Table 4.7) from this study showed a higher reduction in the pH of both inulin and glucose and supplemented alginate growth media compared to un-supplemented growth media and MRS. This result adds credence to the claim of prebiotic potential of extracts already observed in this study.

4.4.3. STRUCTURE-FUNCTION RELATIONSHIP BETWEEN SODIUM ALGINATE AND PREBIOTIC ACTIVITY

The relationship between structural properties and *in vitro* prebiotic activity of sodium alginates as produced in this study is pertinent to understanding the potency and possible mechanism of action of these extracts. Beneficial bacteria require a sufficient number of carbon-rich molecules in the gut, as a nutrient source, for their growth and activities. Alginates are carbon-dense structures that can escape the hydrolytic activity of digestive enzymes due to non-hydrolysable linkages formed within their structure. The primary carbon constituents in the alginate structure are the uronic acids – guluronate and mannuronate. Comparing *in vitro* prebiotic response, it was observed that Alg-MAE (3×) performed best of the four extracts. Alg-MAE (3×) improved the growth rate of *L. delbruecki* by 75% (at 0.1% inclusion) and 150% (at 0.5%), and by 40% (at 0.1%) and 34% (at 0.3%) for *L. casei.* It was also observed that Alg-MAE (3×) had the lowest molecular weight, peak area and percentage peak area, and significantly ($p < 0.05$) lower sodium content, as compared to Alg-CCE (3×) and Alg-UAE (3×). Alg-MAE extracts possibly contained hydrolysed low molecular weight oligosaccharides or monosaccharides

with shorter chain length which may be beneficial for bacterial growth. Although Alg-UAE (3×) had a significantly ($p < 0.05$) higher uronic acid content rather than the other methods, this did not translate into producing the best prebiotic response. Alg-UAE (3×), also had an appreciable higher molecular weight (Mw) and significantly higher sodium content than Alg-MAE and Alg-EAE/CCE (3×). Alg-CCE (3×) with significantly ($p < 0.05$) lower uronic acid content, significantly ($p < 0.05$) higher sodium content, and appears to have performed lowest of the four methods for extract supplemented *L. casei* and at *L. delbruecki* (0.1% w/v) media.

4.5. CONCLUSION

The impact of extraction processes, both conventional and novel techniques, on the structural and prebiotic activity of sodium alginate extracted from *Ascophyllum nodosum* was assessed in this study. The extract yield result showed a significant ($p < 0.05$) increase in 3×, as compared 1× for all extracts. However, the increased yield was not reflected in the uronic acid content for the Alg-MAE, Alg-CCE, and Alg-UAE extracts. The Alg-MAE extract had a significantly ($p < 0.05$) lower uronic acid content in the 3× compared to 1× extract, and there was no significant ($p < 0.05$) difference between the 1× and 3× extracts in Alg-CCE and Alg-UAE (in guluronic acid equivalents only). The molecular weight data revealed a lower molecular weight of the experimental extracts (65 – 187 kDa) compared to the purchased, commercial sodium alginate. The factorial analysis of the impact of extraction methods and number of extractions on the structural properties was also investigated. The data showed that the number of extractions significantly increased yield and decreased mannuronic acid, guluronic acid and average molecular weight when 1×

extraction was compared to 3× extraction. Also, extraction methods, as an independent variable, had a significant impact on the aforementioned properties, except for extract yield. The interaction between both independent variables also significantly influenced the above-mentioned properties except for extract yield. The sodium, potassium and calcium contents decreased significantly when compared 1× extraction was compared to 3× extraction. Extraction methods also had a significant impact on all three minerals, as well as the interaction between extraction methods and number of extractions. The M/G ratio obtained from the FT-IR spectra revealed that the 3× extraction may have resulted in the reduction of the gelling properties of Alg-MAE and Alg-UAE as indicated by changes in M/G ratio, whereas the gelling property of Alg-CCE was improved with the 3× extraction method. The NMR spectra confirmed the presence of sodium alginate through representative peaks corresponding to the position of hydrogen atoms of guluronic and mannuronic acids.

For prebiotic activity, supplemented alginate growth media at 0.1. 0.3, and 0.5% dose-dependently increased the growth rate of the *L. delbruecki* strain; highest activity was observed in Alg-MAE. For the growth rate of *L. casei*, the supplemented extracts all had higher growth rates at 0.1 and 0.3%, with a decline in growth rate observed at 0.5%. The Alg-MAE supplemented growth medium had better activity than the other extract media. However, there were no significant ($p < 0.05$) differences when compared to the other methods of extraction. The prebiotic activity of the extracts had comparable growth rates with a standard commercial prebiotic, inulin, thus suggesting prebiotic potential, similarly, doubling time (which is the inverse of growth rate). Higher probiotic activity correlated with lower doubling time. No significant ($p < 0.05$) impacts of supplemented media on

SCFA production were observed. The reduction in the pH of the supplemented growth media was observed, supporting the prebiotic claim.

Overall, our results suggested that sodium alginate extracts possess prebiotic potentials with the MAE extraction process performing the best of the four methods tested. Although the uronic acid content was low, it appeared to have been substantial for growth of the selective *Lactobacilli* strains. Also, MAE extracts had low molecular weights, peak area and percentage peak area. This suggested that the hydrolytic impact of the microwave method may have resulted in the production of shorter chain oligosaccharides. This may increase the accessibility of its carbon molecules, as nutrients for bacterial growth. Despite the evidence of some prebiotic potentials reported in this study, furthermore *in vitro*, *in vivo* and human trial testing would be required to affirm these claims. This is recommended as some *in vivo* models would aid the understanding of the influence of complex/diverse microbiota, challenges of accessibility of prebiotics, and the influence of non-beneficial bacteria, amongst others. These trials are recommended, since *in vivo* models possess a diverse microbiota population. The positive and negative influence of non-beneficial bacteria can also be investigated on *in vivo* models, as well as the accessibility of prebiotics in the gut for growth stimulation. From an economic perspective, although the MAE method performed better than the other methods, the cost of procuring and maintaining a microwave unit may be expensive. However, given its *in vitro* prebiotic potentials and if confirmed in humans, it may become advantageous to apply the MAE for large scale extraction of fucoidans from *A. nodosum*. In addition, the MAE saves time, and as such may require less labour to perform. This may translate into increased revenue for industries in the functional food business.

TABLE 4.1. THE EXTRACT YIELD OF SODIUM ALGINATE FROM VARIOUS EXTRACTION PROCESSES

Sodium alginate extracts	Extract yield % (w/w of the pre-extracted *A. nodosum*)	
	Single extraction (1×)	Triple extraction (3×)
Alg-CCE	21.73 ± 1.023	71.61 ± 4.263 [b, c]
Alg-MAE	43.33 ± 3.946[f]	56.35 ± 1.344[d]
Alg-UAE	38.32 ± 11.53[f]	70.15 ± 3.953[e]
Alg-EAE/CCE	-	90.32 ± 5.198[a]

[a] EAE/CCE (3×) was significantly higher than MAE (3×) and UAE (3×); [b] CCE (3×) was significantly higher than MAE (3×); [c] CCE (3×) was significantly higher than CCE (1×); [d] MAE (3×) was significantly higher than MAE (1×); [e] UAE (3×) was significantly higher than UAE (1×); [f] MAE (1×) and UAE (1×) were significantly higher than CCE (1×) (The results are presented as mean ± standard deviation; at $p < 0.05$)

TABLE 4.2. THE URONIC ACID CONTENT OF SODIUM ALGINATE EXTRACTS

Sodium alginate extracts	Guluronic acid equivalent % (w/w of sodium alginate extract)		Mannuronic acid equivalent % (w/w of sodium alginate extract)	
	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)
Alg-CCE	0.826 ± 0.177	0.772 ± 0.150[c]	2.431 ± 0.692	1.945 ± 0.357
Alg-MAE	4.495 ± 0.097[d,f]	0.334 ± 0.077[c]	10.78± 0.229[i,k]	0.905 ± 0.183
Alg-UAE	3.076 ± 0.271[e]	4.099 ± 0.390[a, g]	7.414 ± 0.642[j,]	9.841 ± 0.926[h,l]
Alg-EAE/CCE	-	2.503 ± 0.651[b]	-	6.053 ± 1.546[h]
Alg - STD.*	25.78 ± 3.227		61.32 ± 7.661	

*Alg- STD - Food grade sodium alginate (Landor Trading Co.); [a] UAE (3×) was significantly higher than EAE/CCE (3×), MAE (3×), and CCE (3×); [b] EAE/CCE (3×) was significantly higher than MAE (3×) and CCE (3×); [d] Alg-CCE (3×) and Alg-MAE (3×) were significantly lower than Alg-UAE and Alg-EAE/CCE; [d] MAE (1×) was significantly higher than UAE (1×) and CCE (1×); [e] UAE (1×) was significantly higher than CCE (1×); [f] MAE (1×) was significantly higher than MAE (3×); [g] UAE (3×) was significantly higher than UAE (1×); [h] UAE (3×) and EAE/CCE (3×) were significantly higher than MAE (3×) and CCE (3×) (The results are presented as mean ± standard deviation; at $p < 0.05$)

TABLE 4.3. THE MOLECULAR WEIGHT DISTRIBUTION OF SODIUM ALGINATE EXTRACTS FROM VARIOUS EXTRACTION PROCESSES

Extraction method	Peaks	Number average molecular weight, Mn (kDa)	Weight average molecular weight, Mw (kDa)	Polydispersity index (PDI)	Peak area	% Peak area
Alg-CCE (1×)	Peak 1	106.2 ± 35.4	113.6 ± 34.4	1.03 ± 0.03	17.57 ± 8.65	8.11 ± 1.98
	Peak 2	2.80 ± 0.14	2.96 ± 0.22	1.06 ± 0.02	7.089 ± 3.16	3.28 ± 1.24
Alg-CCE (3×)	Peak 1	56.8 ± 4.17	103.4 ± 2.78	1.82 ± 0.09	80.92 ± 10.1	18.8 ± 3.69
	Peak 2	4.19 ± 0.05	5.02 ± 0.1	1.20 ± 0.01	43.61 ± 24.1	10.1 ± 2.52
Alg-MAE (1×)	Peak 1	143.6 ± 27.9	187.5 ± 17.3	1.32 ± 0.15	253.2 ± 51.83	57.9 ± 2.87
	Peak 2	3.37 ± 0.15	3.99 ± 0.28	1.18 ± 0.03	33.27 ± 14.03	7.61 ± 1.93
Alg-MAE (3×)	Peak 1	46.9 ± 2.89	65.4 ± 9.29	1.40 ± 0.26	59.99 ± 23.55	8.14 ± 1.52
	Peak 2	4.41 ± 0.36	5.03 ± 0.19	1.14 ± 0.08	32.15 ± 15.9	5.51 ± 0.28
Alg-UAE (1×)	Peak 1	119.1 ± 35.5	182.0 ± 15.9	1.61 ± 0.42	242.2 ± 111.0	52.62 ± 1.11
	Peak 2	3.33 ± 0.28	3.74 ± 0.48	1.12 ± 0.05	14.5 ± 7.83	3.11 ± 0.83
Alg-UAE (3×)						
	Peak 1	121.5 ± 17.9	215.3 ± 7.88	1.18 ± 0.07	88.77 ± 70.12	29.32 ± 19.7
	Peak 2	3.55 ± 0.36	3.85 ± 0.68	1.08 ± 0.08	5.54 ± 4.91	1.81 ± 1.42
Alg-EAE/CCE (3×)	Peak 1	121.9 ± 13.5	172.4 ± 14.1	1.42 ± 0.08	147.2 ± 52.54	32.41 ± 1.43
	Peak 2	4.73 ±- 0.04	5.70 ± 0.20	1.21 ± 0.03	52.04 ± 41.12	11.07 ± 8.08
Alg-STD	Peak 1	180.4	318.80	1.77	85.485	78.79

*Alg- STD - Food grade sodium alginate, Landor Trading Co. (The results are presented as mean ± standard deviation; at $p < 0.05$)

TABLE 4.4. THE SODIUM, POTASSIUM, AND CALCIUM CONTENTS OF SODIUM ALGINATE EXTRACTS

Sodium alginate extracts	Sodium % (w/w of sodium alginate extract)		Potassium % (w/w of sodium alginate extract)		Calcium % (w/w of sodium alginate extract)	
	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)	Single extraction (1×)	Triple extraction (3×)
Alg-CCE	32.28 ± 0.119[c, e]	29.70 ± 0.081[a]	0.632 ± 0.010	0.010 ± 0.008	n.d	0.034 ± 0.027
Alg-MAE	13.18 ± 0.027	18.29 ± 0.072[f]	1.398 ± 0.000	0.311 ± 0.012[h]	n.d	n.d
Alg-UAE	18.85 ± 0.027[d]	24.08 ± 0.212[b, g]	0.889 ± 0.037	0.289 ± 0.008[h]	0.267 ± 0.027	0.289 ± 0.081
Alg-EAE/CCE	-	18.14 ± 0.178	-	0.012 ± 0.005	-	n.d

[a] CCE (3×) was significantly higher than UAE (3×), MAE (3×), and EAE/CCE (3×); [b] UAE (3×) was significantly higher than MAE (3×) and EAE/CCE (3×); [c] CCE (1×) was significantly higher than UAE (1×) and MAE (1×); [d] UAE (1×) was significantly higher than MAE (1×); [e] CCE (1×) was significantly higher than CCE (3×); [f] MAE (3×) was significantly higher than MAE (1×); [g] UAE (3×) was significantly higher than UAE (1×); [h] MAE (3×) and UAE (3×) were significantly higher than EAE/CCE (3×) and CCE (3×), n.d – not detectable (The results are presented as mean ± standard deviation; at $p < 0.05$)

TABLE 4.5. THE M/G RATIO OF SODIUM ALGINATE EXTRACTS USING FTIR

Extraction method	M-OH wave number (cm^{-1})	G-OH wave number (cm^{-1})	M-OH peak intensity	G-OH peak intensity	M/G ratio
Alg-CCE (1×)	1033	1082	68.36	67.81	1.008
Alg-CCE (3×)	1032	1085	44.54	51.44	0.866
Alg-MAE (1×)	1033	1083	22.06	30.84	0.716
Alg-MAE (3×)	1034	1095	49.16	54.73	0.898
Alg-UAE (1×)	1034	1083	20.77	31.42	0.661
Alg-UAE (3×)	1034	1085	24.65	31.36	0.786
Alg-EAE/CCE (3×)	1034	1086	41.29	46.78	0.883
Alg-STD*	1031	1095	53.93	55.84	0.966

*Alg- STD - Food grade sodium alginate (Landor Trading Co.)

TABLE 4.6. THE DOUBLING TIME OF *L. casei* AND *L. delbruecki* STRAINS

Supplemented extracts	*L. casei* doubling time (hr)				*L. delbruecki* doubling time (hr)			
	MRS + Lb.cs	0.1 %	0.3 %	0.5 %	MRS + Lb.db	0.1 %	0.3 %	0.5 %
Inulin	2.426 ± 0.239	2.171 ± 0.291	2.051 ± 0.017	2.149 ± 0.157	3.90 ± 2.19	3.031 ± 0.298	2.888 ± 0.558	2.806 ± 0.479
Glucose	2.426 ± 0.239	2.015 ± 0.087	2.005 ± 0.063	2.046 ± 0.140	3.90 ± 2.19	3.057 ± 0.339	2.863 ± 0.267	2.960 ± 0.486
Alg-CCE	2.426 ± 0.239	2.080 ± 0.036	1.927 ± 0.035	2.325 ± 0.064	3.90 ± 2.19	2.03 ± 0.019	1.877 ± 0.187	1.597 ± 0.082
Alg-MAE	2.426 ± 0.239	1.726 ± 0.129	1.807 ± 0.210	2.693 ± 0.457	3.90 ± 2.19	1.870 ± 0.091	1.934 ± 0.103	1.312 ± 0.044
Alg-UAE	2.426 ± 0.239	1.953 ± 0.066	1.830 ± 0.099	2.494 ± 0.023	3.90 ± 2.19	2.135 ± 0.066	2.349 ± 0.109	1.762 ± 0.240
	2.426 ± 0.239	2.071 ± 0.095	1.928 ± 0.162	2.724 ± 0.363	3.90 ± 2.19	2.04 ± 0.128	2.152 ± 0.033	1.686 ± 0.036

(The results are presented as mean ± standard deviation; at $p < 0.05$)

TABLE 4.7. THE CHANGES IN pH OF *L. delbruecki* AND *L. casei* GROWTH MEDIA AFTER 24 HOURS INCUBATION

Extraction Method	Inclusion concentration	Change in pH of *L. delbruecki* media	Change in pH of *L. casei* media
MRS	-	-0.036	-0.036
L. delbruecki/*L. casei*	-	-1.275	-1.839
Inulin	0.1	-2.014	-1.876
	0.3	-1.984	-1.872
	0.5	-2.003	-1.927
Glucose	0.1	-2.010	-1.854
	0.3	-1.990	-1.877
	0.5	-1.996	-1.868
Alg-CE	0.1	-1.593	-2.629
	0.3	-2.318	-3.384
	0.5	-2.929	-3.469
Alg-MAE	0.1	-1.730	-2.710
	0.3	-2.448	-3.475
	0.5	-3.178	-3.451
Alg-UAE	0.1	-1.560	-2.613
	0.3	-2.172	-3.294
	0.5	-3.029	-3.405
Alg-EAE/CE	0.1	-1.410	-2.637
	0.3	-2.143	-3.175
	0.5	-3.037	-3.452

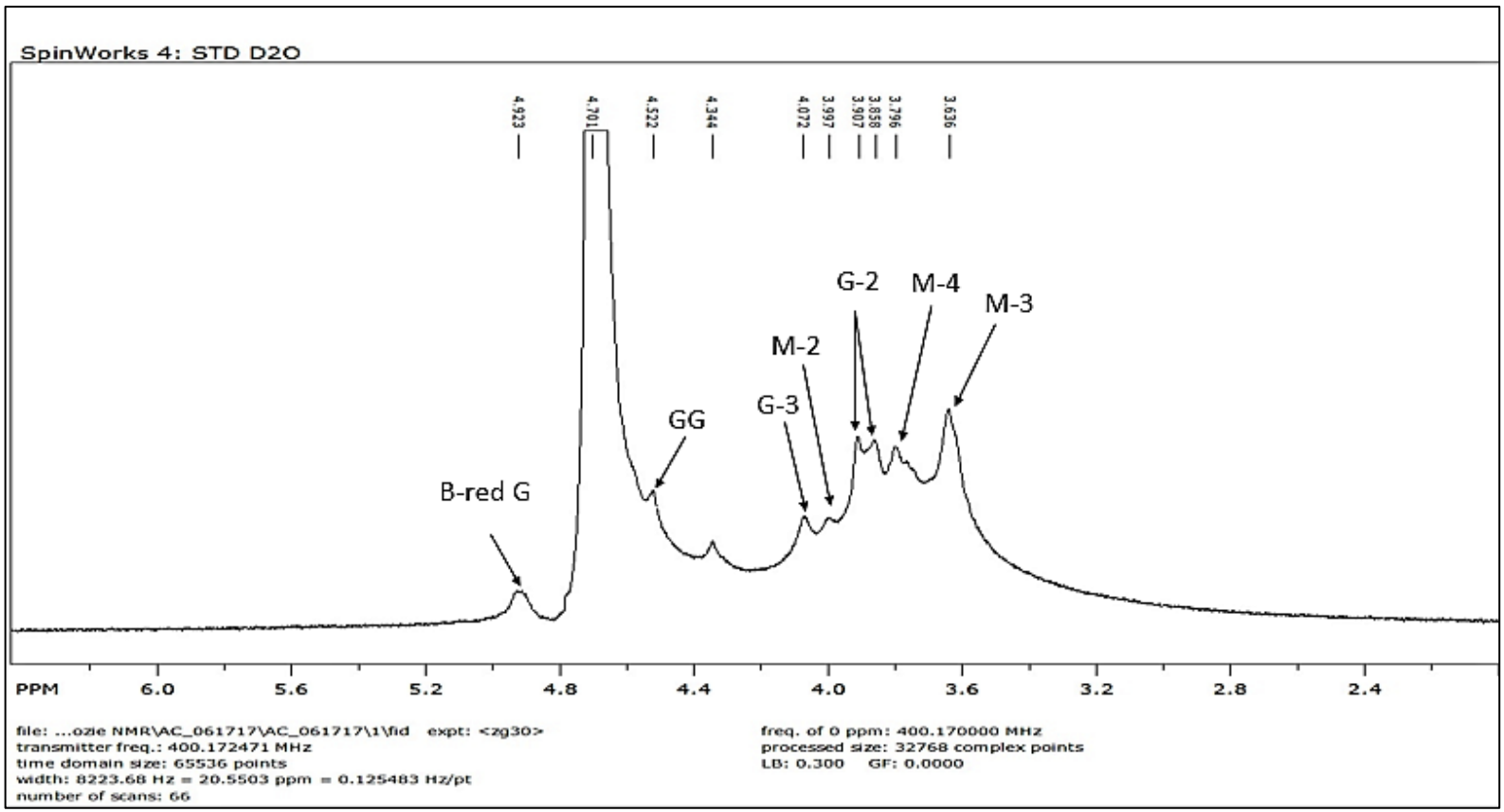


FIG 4.1. THE H^1 NMR SPECTRUM OF COMMERCIAL SODIUM ALGINATE

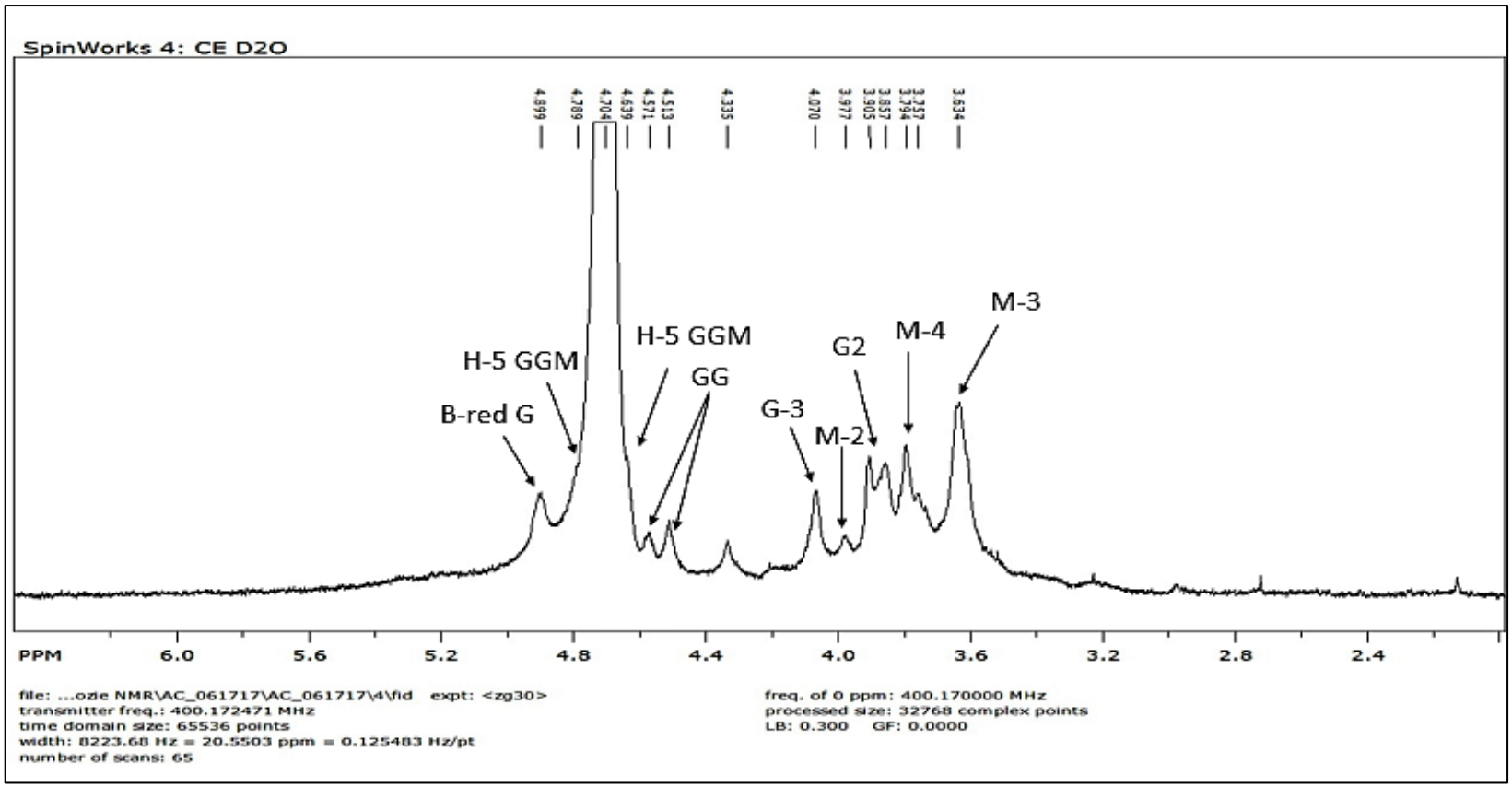
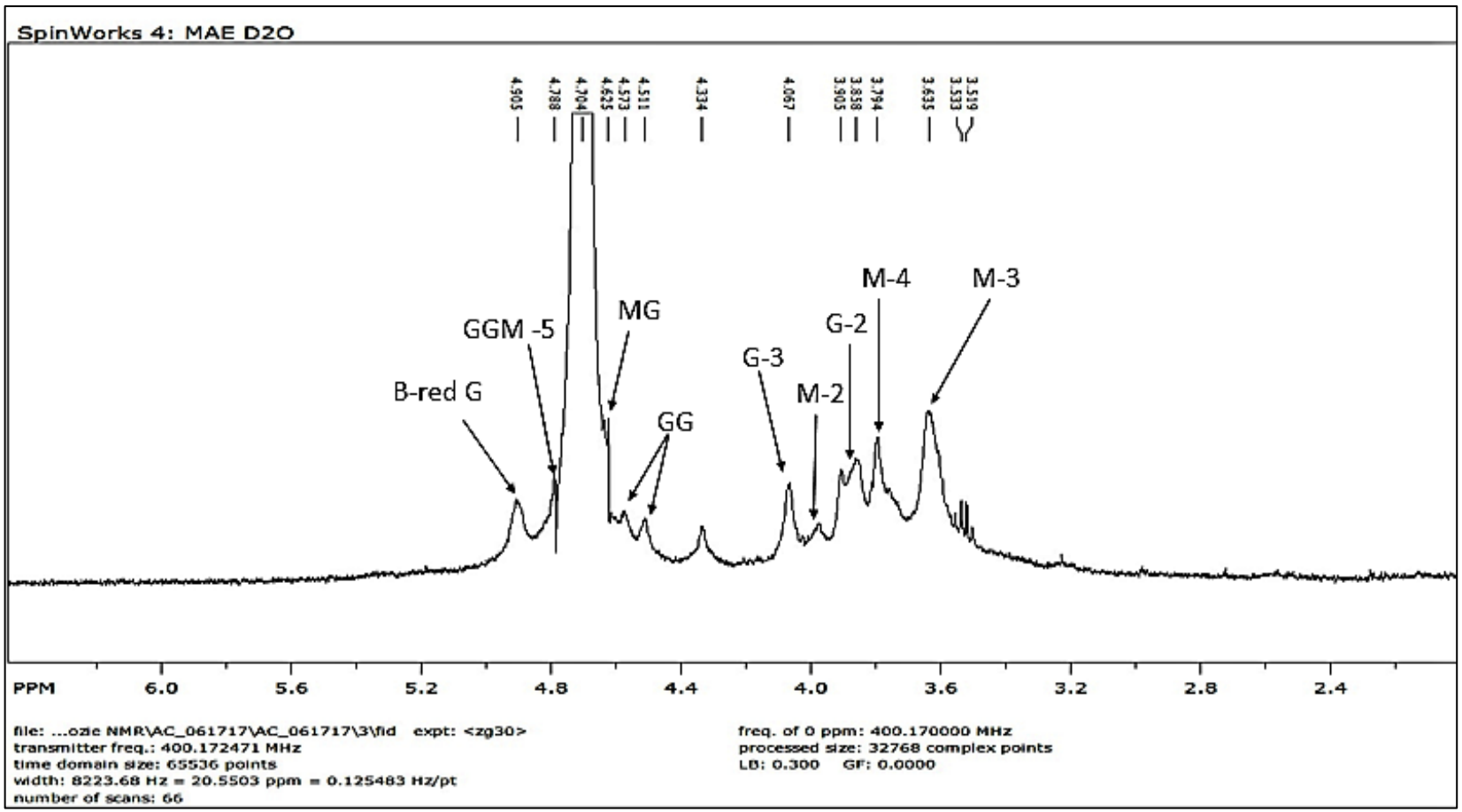
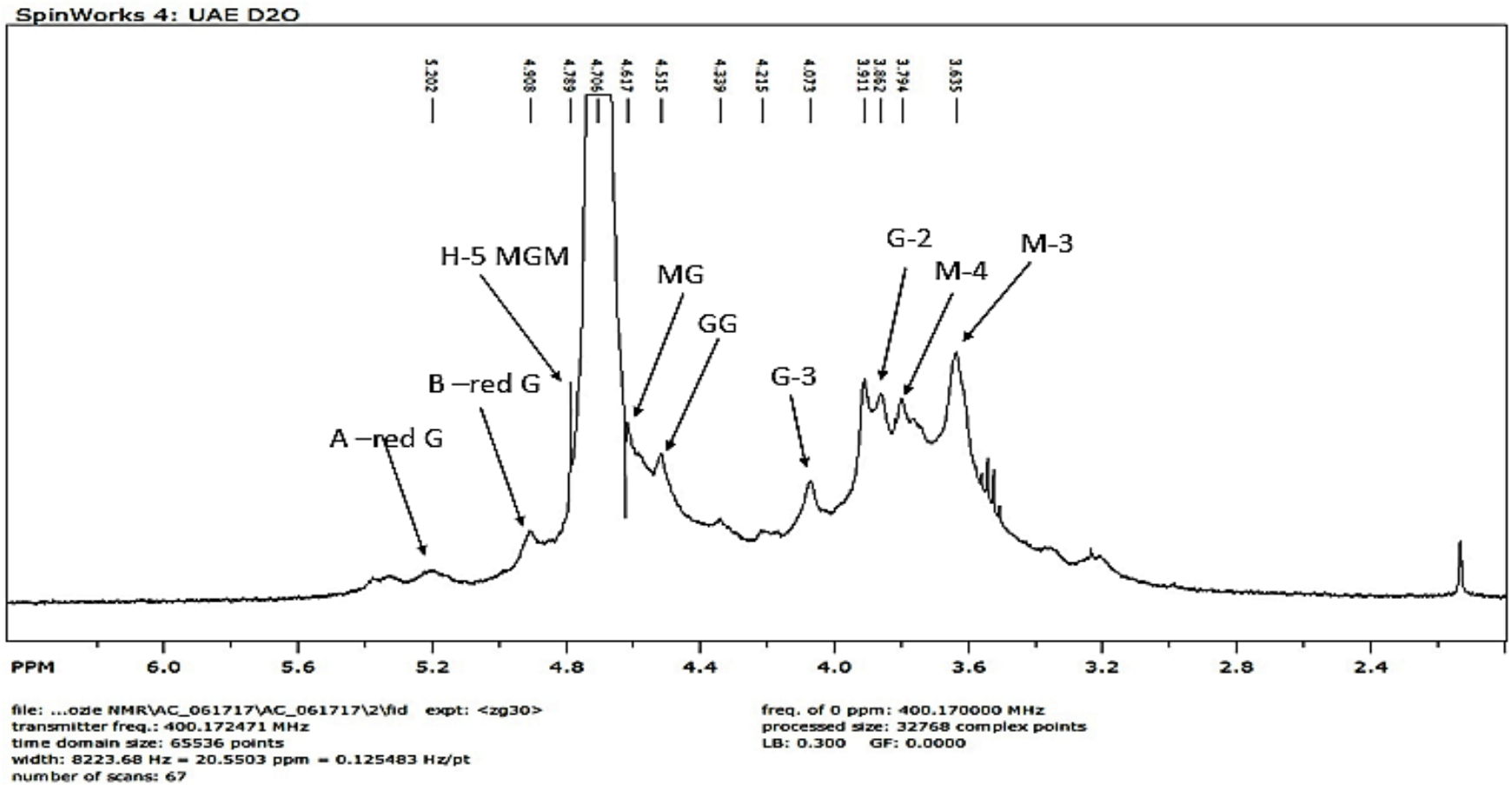


FIG 4.2.THE H^1 NMR SPECTRUM OF SODIUM ALGINATE FROM CONVENTIONAL EXTRACTION (Alg-CE) (3×)



123

FIG 4.3. THE H^1 NMR SPECTRUM OF SODIUM ALGINATE FROM MICROWAVE-ASSISTED EXTRACTION (Alg-MAE) (3×)



124

FIG 4.4. THE H^1 NMR SPECTRUM OF SODIUM ALGINATE FROM ULTRASOUND-ASSISTED EXTRACTION (Alg-UAE) (3×)

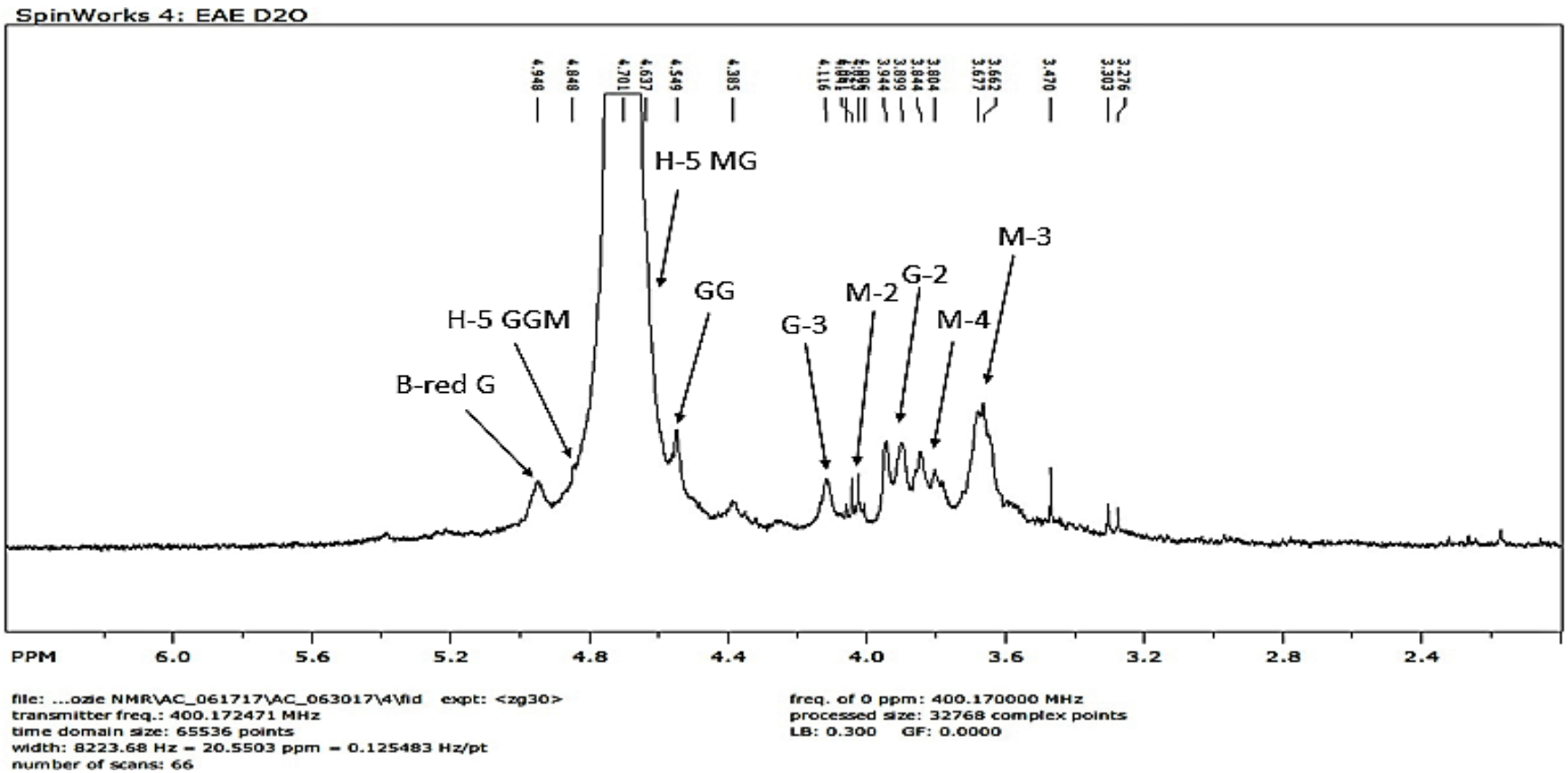


FIG 4.5. THE H^1 NMR SPECTRUM OF SODIUM ALGINATE FROM ENZYME-ASSISTED EXTRACTION (3×)

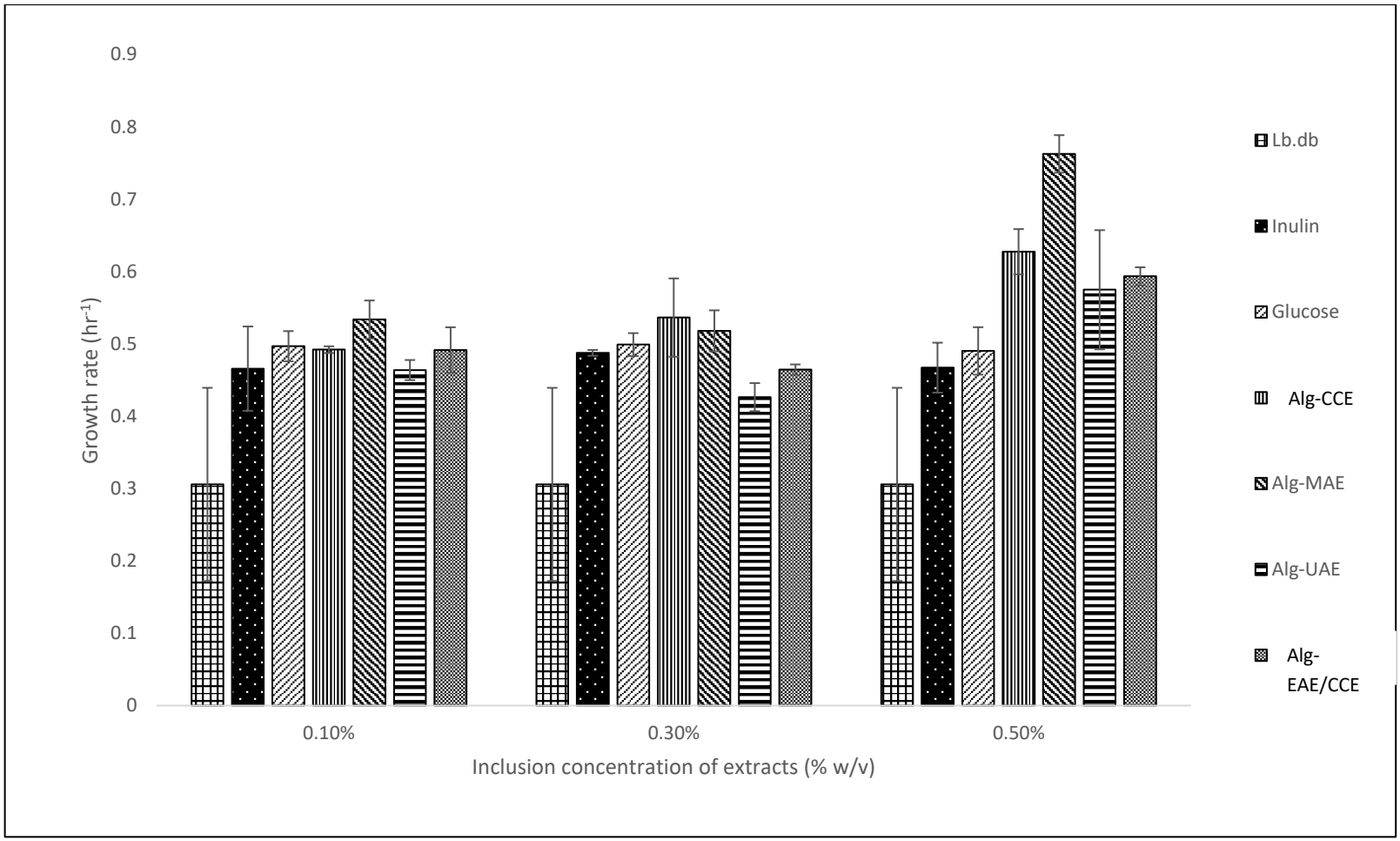


FIG 4.6. THE *Lactobacillus delbruecki* GROWTH RATE

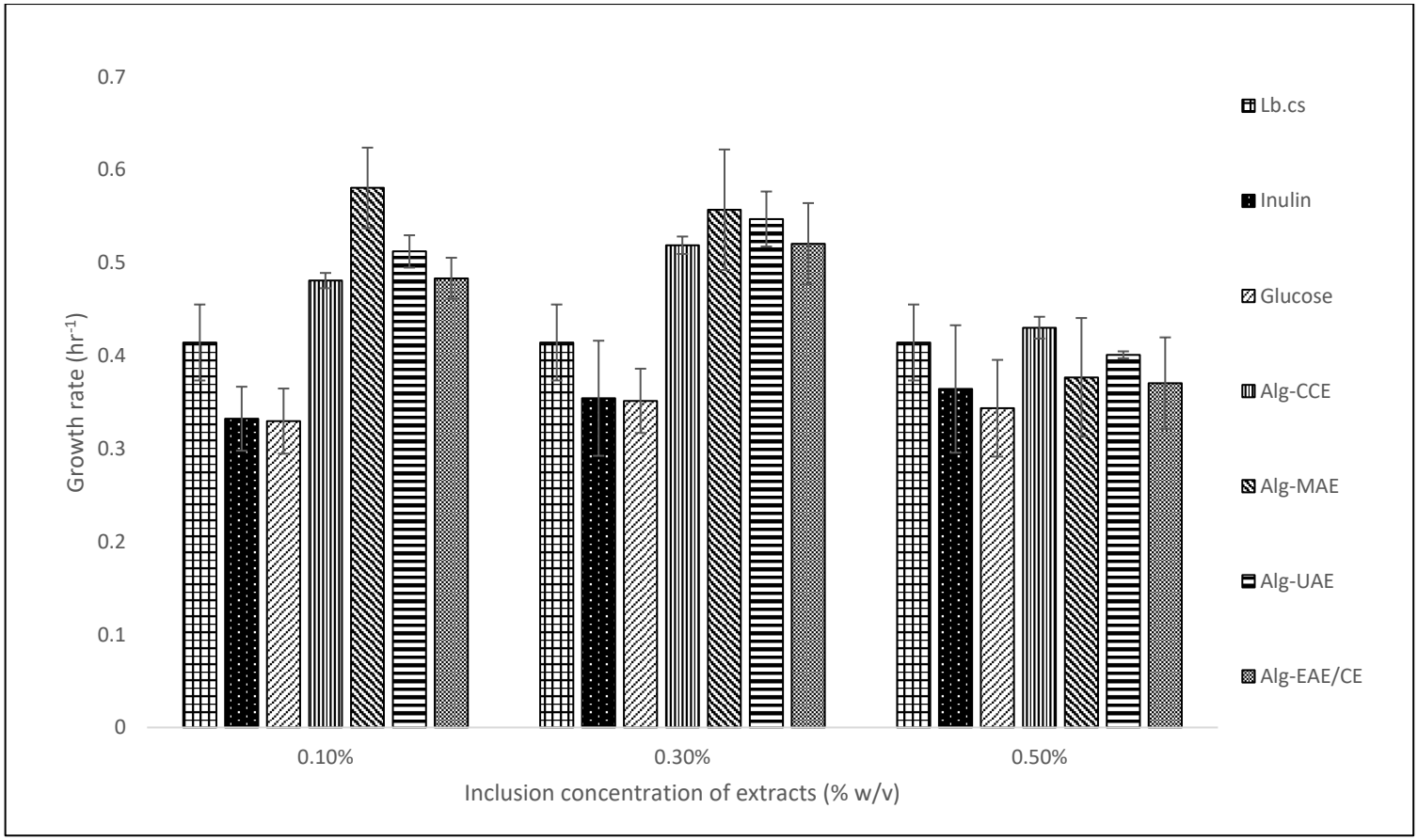


FIG 4.7. THE *Lactobacillus casei* GROWTH RATE

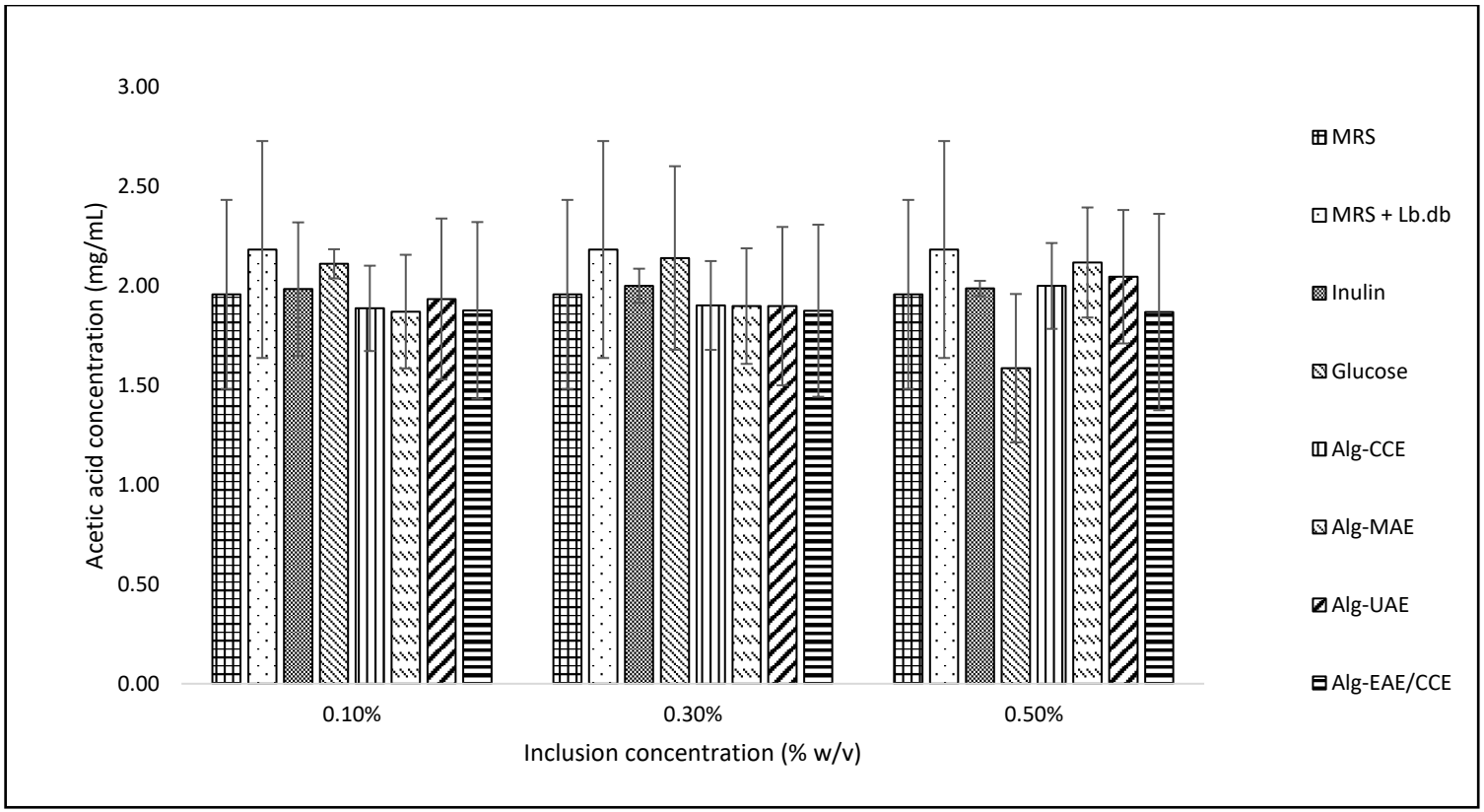


FIG 4.8. THE ACETIC ACID CONCENTRATION IN *Lactobacillus delbruecki* GROWTH MEDIA

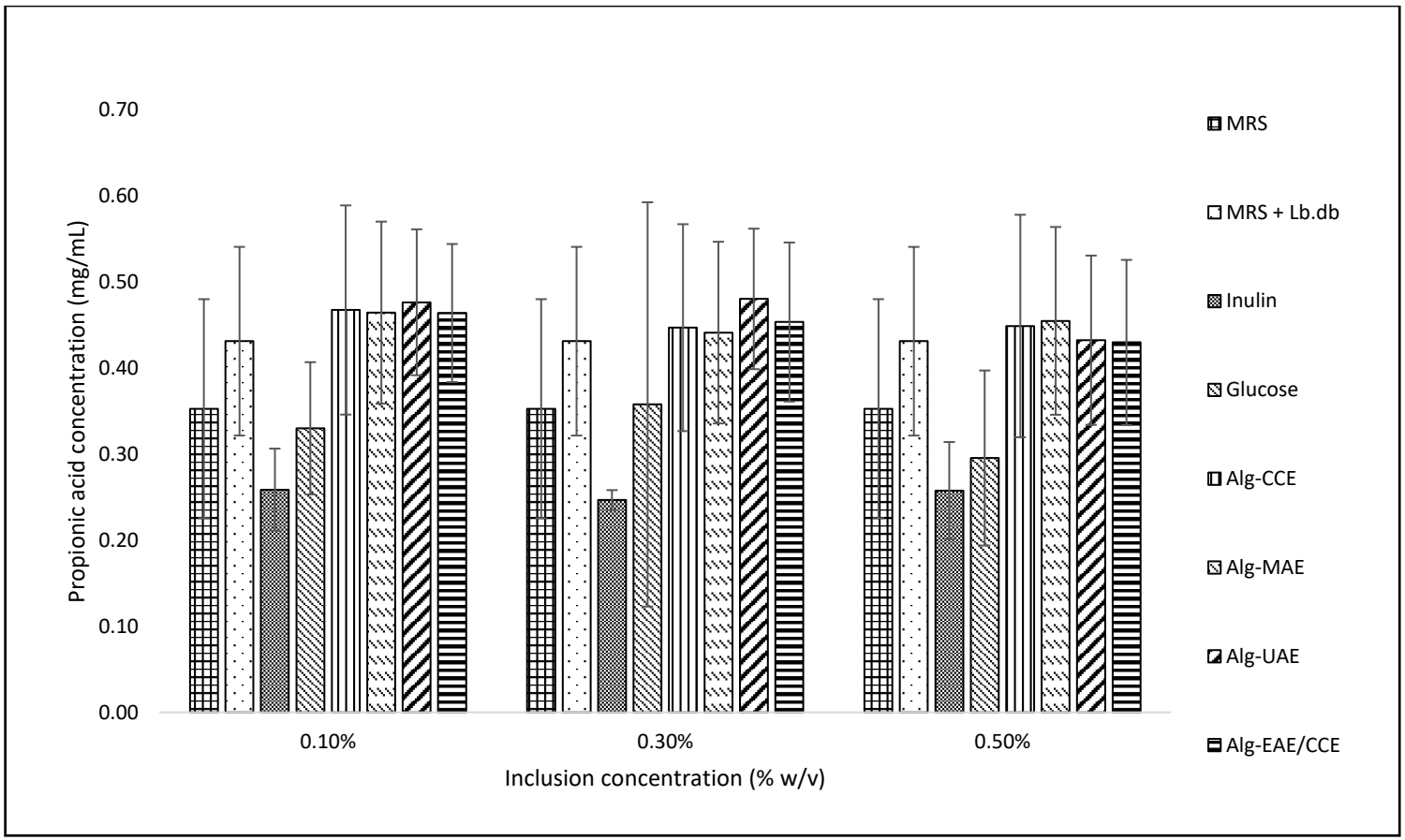
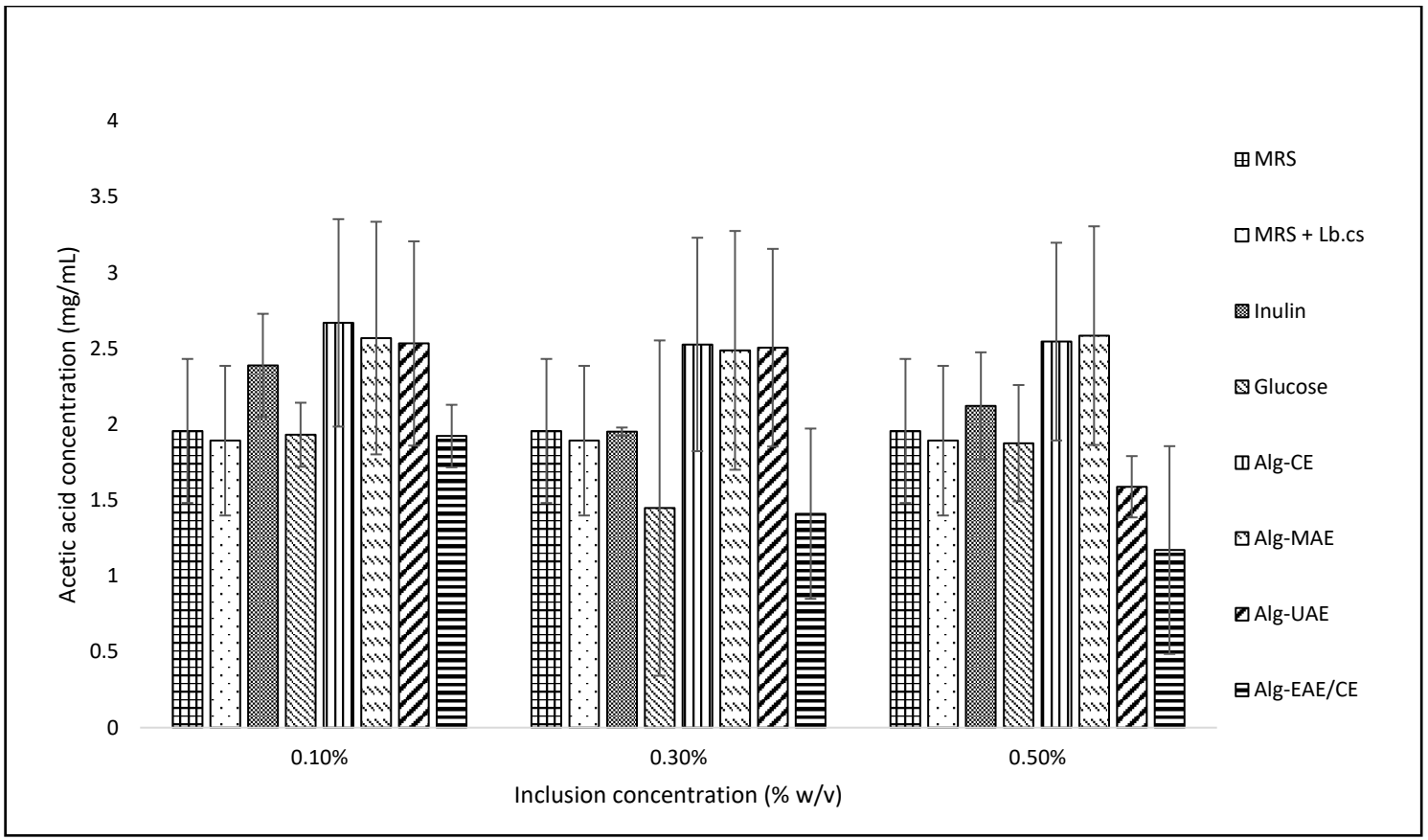
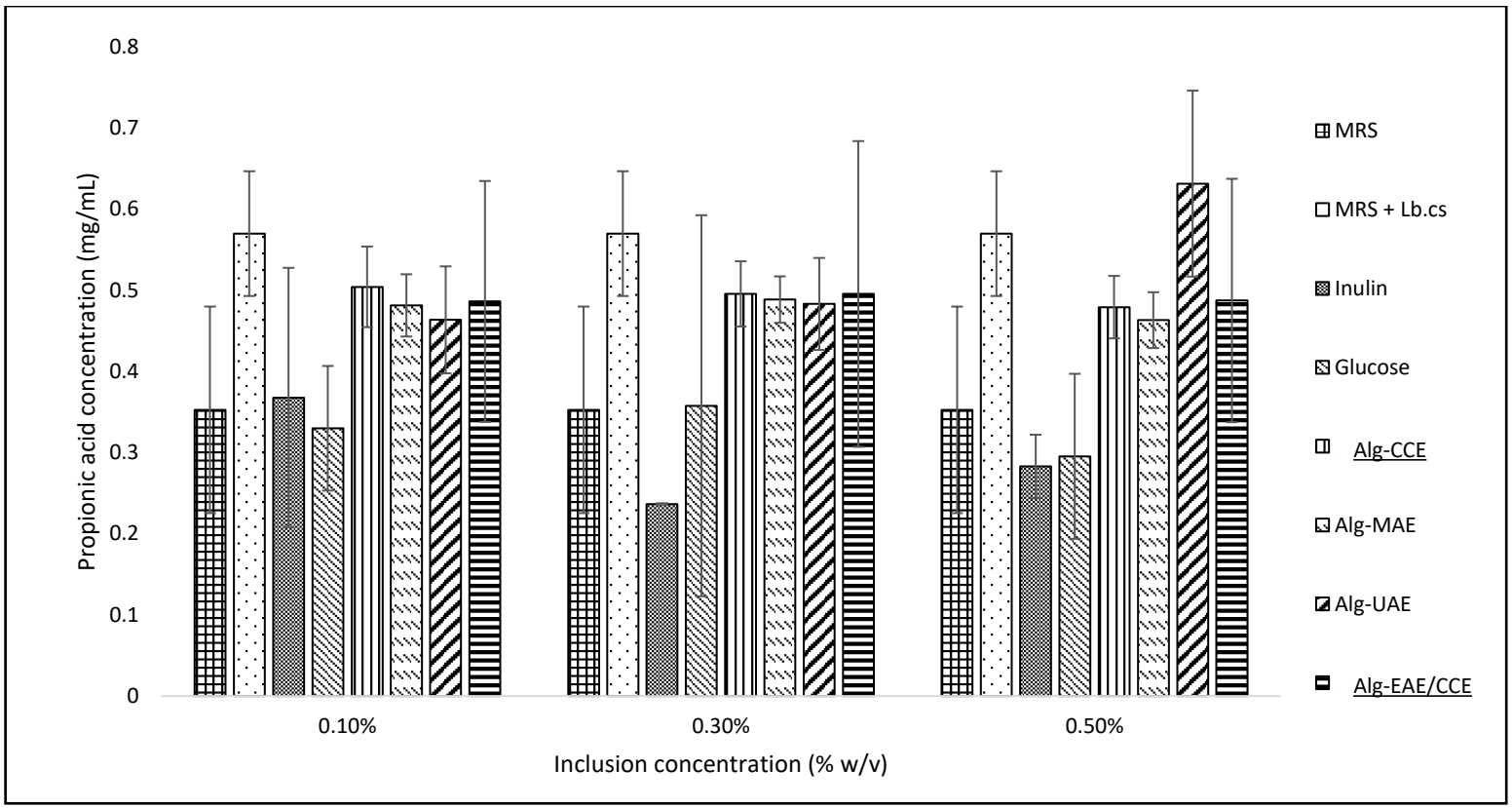


FIG 4.9. THE PROPIONIC ACID CONCENTRATION IN *Lactobacillus delbruecki* GROWTH MEDIA



130

FIG 4.10. THE ACETIC ACID CONCENTRATION IN *Lactobacillus casei* GROWTH MEDIA



131

FIG 4.11. THE PROPIONIC ACID CONCENTRATION IN *Lactobacillus casei* GROWTH MEDIA

CHAPTER FIVE

CONCLUSION

The potential application of air dried *Ascophyllum nodosum* polysaccharides (fucoidan and alginate) from Nova Scotia, Canada, as functional food ingredients with prebiotic prospects was explored in regard to the investigation into its structure-function relationship. Also, with the recent interest in sustainable alternatives to conventional sources of nutrients/functional ingredients, seaweeds appear to be a potentially viable source of prebiotics for industries in the functional food business. Additionally, the impact of conventional and novel extraction technologies on structural properties were examined as it pertains to a possible prebiotic activity.

The present study was divided into three objectives, to help attain the overall goal. The first objective was to extract specific polysaccharide fractions from the pre-extracted *A. nodosum* brown seaweed with four different extraction processes. This was done to investigate the contribution of more recent extraction processes (Microwave-assisted extraction and ultrasound-assisted extraction) to existing conventional methods towards qualitative and quantitative extraction. The second and third objectives focused on characterizing the fucoidan and alginate extracts from the four extraction processes, respectively, explaining possible relationship to prebiotic activity.

For the first objective, both fucoidan and alginate were successfully extracted, and their yields compared. Also, single and triple extractions were performed with CCE, MAE, and UAE, to balance discrepancies between the MAE and UAE protocols with the conventional method. Overall, more yield was obtained in the triple extraction process than the single extraction process for all methods. Depending on the purity of the extract,

a higher yield may not be representative of a high yield of the targeted compound, as extracts may contain higher amounts of excipients. In future, direct extraction of sodium alginate from pre-extracted brown seaweed may be attempted, rather than a sequential extraction and removal of fucoidan prior to sodium alginate extraction. Perhaps more sodium alginates may be recovered, although there may be interference from fucoidan molecules.

For the second objective, fucose and galactose were detected in the fucoidan fraction. The Fuc-MAE performed best of all four methods in the characterization section of this study, with significantly higher fucose and sulphate contents, high dispersity index and lower average molecular weight. These are desirable structural properties that may influence biological functions. Extracts obtained from all four extraction methods significantly improved the growth rate of *L. delbruecki*. There was no significant difference between the extraction methods in improving prebiotic function for either strains. Also, the efficacy of the extracts in improving prebiotic functioning were comparable to that of inulin, the commercial standard. From these findings, it could be inferred that the application of newer extraction technologies had no significant impact on the prebiotic activity, regardless of small differences in desirable structural properties. Another possible application, is the use of *A. nodosum* polysaccharides to maintain the viability of probiotics in dairy products *via* a synbiotic relationship.

With respect to the third and final objective, the separation of alginate monomers, guluronic and mannuronic acid, proved unsuccessful with the HPLC system and the methods used in this study. However, the GPC, FT-IR, and NMR analysis confirmed the presence of alginate in low amounts. In an attempt to estimate the quantity of sodium

alginate monomers present in the extract, the total uronic acid content was investigated. The uronic acid content of sodium alginate extracts were generally lower when compared to the commercial sodium alginate (Landor Trading Co.). Amongst the various extraction groups, Alg-MAE (1×) had significantly ($p < 0.05$) higher amounts of uronic acid rather than other extraction methods. The molecular weight of the extracts was also lower when compared to the commercial alginate. The M/G ratio data suggested that there might be a reduction in gelling properties from 3× to 1× extraction for the MAE and UAE processes. It was also observed that extracts were high in sodium suggesting higher amounts of excipients or additives. Despite the low content of uronic acid (the main carbon source), extracts from the four extraction processes improved the growth rate of *L. delbruecki* strain, at 0.1, 0.3, and 0.5% (w/v) at the inclusion concentrations. The growth rate of extract supplemented *L. casei* strain also showed some improvement at 0.1 and 0.3% (w/v) inclusion concentrations as compared to the un-supplemented strain. Sodium alginate extracts from the MAE had promoted greater growth in both *Lactobacilli* strains studied. The efficiency of the extracts in stimulating the growth rate, at *in vitro* level, was as effective as the commercial prebiotic, inulin.

Of the six SCFA studied (acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids) only two were detected by the HPLC method. SCFA are volatile and there may be loss/reduction of its content with the slightest delay. The delay between sample preparation and HPLC analysis, however small, may have influenced the concentration of some of the SCFA in this study. To circumvent this challenge, future studies may apply vacuum transfer prior to HPLC or gel liquid chromatography (Scheppach et al., 1987). The vacuum transfer allows the transfer of volatile materials from flask to tube under vacuum and a

temperature gradient. This method has proven to be effective in the recovery of acetate (97%), propionate (100%), and *n*-butyrate (92%) (Scheppach et al., 1987).

An evaluation of the impact of extraction methods and number of extractions, as independent variables, on the structural properties was also assessed using factorial analysis. For fucoidan, the extract yields, dispersity index values, and sulphate levels were significantly increased when 1× extraction was compared to 3× extraction, whereas uronic acid, sodium, potassium, and calcium decreased significantly. The different extraction methods, as a collective independent variable, significantly influenced all the structural properties undertaken in the fucoidan section. Also, the interaction between both extraction methods and number of extractions significantly influenced galactose content, average molecular weight, dispersity index, sodium, potassium and calcium contents. For alginate, extract yield was significantly increased, whereas mannuronic acid, guluronic acid, average molecular weight, sodium, potassium and calcium contents decreased significantly when 1× extraction was compared to 3× extraction. Also, extraction methods significantly impacted on all the aforementioned properties, except for extract yield. In addition, the interaction between both independent variables significantly influenced the above-mentioned properties, except for extract yield.

Overall, the results from this study suggested that the MAE, at the extraction conditions applied in this study, was the most effective in the extraction of polysaccharides with prebiotic potentials in regard to producing extracts with desirable structural properties and improving growth rate. Although the findings from this study suggested some prebiotic activity, future studies could be expanded to explore the prebiotic efficacy of *A. nodosum* extracts to clearly understand the influence on diverse bacterial population and strains with

methods such quantitative polymerase chain reaction and fluorescent *in situ* hybridization. This could later be extended to using faecal and intestinal gut samples from experimental animals or humans for prebiotic investigations at *in vivo* and clinical trial stages. Findings from the aforementioned suggestions are likely consolidate preliminary prebiotic claims already confirmed in this study.

From an economic perspective, the MAE method is most favoured given that it produced extracts with better structural properties than other methods. Also, the process required lesser time to execute and was not labour intensive, and as such, would save cost on time and labour. On functionality basis, the MAE also produced better prebiotic response, which makes it even more attractive to the industry for large-scale production. However, the cost of setting up the microwave unit for large scale production should be considered and may require skilled operators to run and maintain. Notwithstanding, the MAE method for *A. nodosum* polysaccharide extractions may still be profitable, provided that these claims of prebiotic potential confirmed at clinical trials. Product maximization for application as functional food ingredients/food supplement with the MAE may require less time, less labour, increased efficiency and better quality of product, which will amount to increased revenue for industries.

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APPENDIX A: FACTORIAL ANALYSIS OF THE STRUCTURAL PROPERTIES OF POLYSACCHARIDES FROM *ASCOPHYLLUM NODOSUM*

TABLE A1: THE FACTORIAL ANALYSIS OF THE STRUCTURAL PROPERTIES OF FUCOIDAN EXTRACTS FROM *ASCOPHYLLUM NODOSUM*

	Extract yield (% w/w of pre-extracted *A. nodosum*)	Fucose (% w/w of fucoidan extract)	Galactose (% w/w of fucoidan extract)	Uronic acid (% w/w of fucoidan extract)	Sulphate levels (% w/w of fucoidan extract)	Average molecular weight (kDa)	Dispersity index (Đ)
Number of extractions							
1× Fuc-CCE	5.58	26.1	13.6	3.35	15.3	90.1	1.42
Fuc-MAE	3.76	34.6	11.0	4.14	11.9	116	1.22
Fuc-UAE	0.88	14.9	1.84	1.55	12.3	105	1.08
Fuc-EAE	3.89	29.1	10.6	0.39	15.4	115	1.16
3× Fuc-CCE	11.9	27.4	6.56	0.59	21.7	97.5	2.47
Fuc-MAE	5.71	37.0	13.0	3.59	18.8	81.2	2.64
Fuc-UAE	4.56	27.1	8.53	0.49	17.3	105	1.13
Extract type							
Fuc-CCE	10.4	26.7	10.8	1.97	20.1	93.8	1.94
Fuc-MAE	4.93	35.8	12.0	3.81	16.0	94.9	2.07
Fuc-UAE	2.72	21.0	5.86	0.91	14.8	121	1.11
Fuc-EAE	3.89	29.1	8.53	0.39	15.4	115	1.16
P values							
No. of extractions	0.001	0.056	0.728	0.002	0.000	0.790	0.000
Extract type	0.001	0.002	0.023	0.000	0.046	0.004	0.000
No. of extractions and extract type	0.208	0.194	0.015	0.056	0.764	0.001	0.000

TABLE A2: THE FACTORIAL ANALYSIS OF THE SODIUM, POTASSIUM, AND CALCIUM CONTENTS OF FUCOIDAN EXTRACTS FROM *ASCOPHYLLUM NODOSUM*

		Sodium (% w/w of fucoidan extract)	Potassium (% w/w of fucoidan extract)	Calcium (% w/w of fucoidan extract)
Number of extractions				
1×	Fuc-CCE	0.15	0.78	4.92
	Fuc-MAE	0.06	0.82	3.08
	Fuc-UAE	0.13	0.34	6.69
	Fuc-EAE	0.36	0.87	4.58
3×	Fuc-CCE	0.07	0.48	2.40
	Fuc-MAE	0.08	0.72	3.18
	Fuc-UAE	0.13	0.54	3.74
Extract type				
	Fuc-CCE	0.11	0.63	3.66
	Fuc-MAE	0.07	0.77	3.13
	Fuc-UAE	0.13	0.44	5.22
	Fuc-EAE	0.36	0.87	4.58
P values				
No. of extractions		0.000	0.000	0.000
Extract type		0.000	0.000	0.000
No. of extractions and extract type		0.000	0.000	0.000

TABLE A3: THE FACTORIAL ANALYSIS OF THE STRUCTURAL PROPERTIES OF SODIUM ALGINATE EXTRACTS FROM *ASCOPHYLLUM NODOSUM*

		Extract yield (% w/w of pre-extracted *A. nodosum*)	Guluronic acid equivalent (% w/w of fucoidan extract)	Mannuronic acid equivalent (% w/w of fucoidan extract)	Average molecular weight (kDa)	Dispersity index (Đ)
Number of extractions						
1×	Alg-CCE	21.7	0.83	2.43	114	1.03
	Alg-MAE	43.3	4.50	10.8	188	1.32
	Alg-UAE	38.3	3.08	7.41	182	1.61
3×	Alg-CCE	71.6	0.77	1.95	103	1.82
	Alg-MAE	56.4	0.33	0.91	65.4	1.40
	Alg-UAE	70.2	4.10	9.84	215	1.08
	Alg-EAE/CCE	90.3	2.50	6.05	172	1.42
Extract type						
	Alg-CCE	59.4	0.79	2.19	107	1.51
	Alg-MAE	56.5	2.83	6.83	126	1.36
	Alg-UAE	51.0	3.49	8.39	195	1.44
	Alg-EAE	90.3	2.50	8.11	172	1.42
P values						
No. of extractions		0.001	0.000	0.002	0.001	0.221
Extract type		0.246	0.000	0.000	0.000	0.946
No. of extractions and extract type		0.652	0.000	0.000	0.000	0.002

TABLE A4: THE FACTORIAL ANALYSIS OF THE SODIUM, POTASSIUM, AND CALCIUM CONTENTS OF SODIUM ALGINATE EXTRACTS FROM *ASCOPHYLLUM NODOSUM*

		Sodium (% w/w of fucoidan extract)	Potassium (% w/w of fucoidan extract)	Calcium (% w/w of fucoidan extract)
Number of extractions				
1×	Alg-CCE	32.28	0.63	0.00
	Alg-MAE	13.18	1.40	0.00
	Alg-UAE	18.85	0.89	0.27
3×	Alg-CCE	29.7	0.01	0.03
	Alg-MAE	18.3	0.31	0.00
	Alg-UAE	24.1	0.29	0.29
	Alg-EAE/CCE	18.1	0.01	0.00
Extract type				
	Alg-CCE	0.11	0.32	0.02
	Alg-MAE	0.07	0.86	0.00
	Alg-UAE	0.13	0.59	0.28
	Alg-EAE	0.36	0.01	0.00
P values				
No. of extractions		0.000	0.000	0.000
Extract type		0.000	0.000	0.000
No. of extractions and extract type		0.000	0.000	0.000

APPENDIX B: THE FT-IR SPECTRA OF COMMERCIAL SODIUM ALGINATE AND SODIUM ALGINATE EXTRACTS

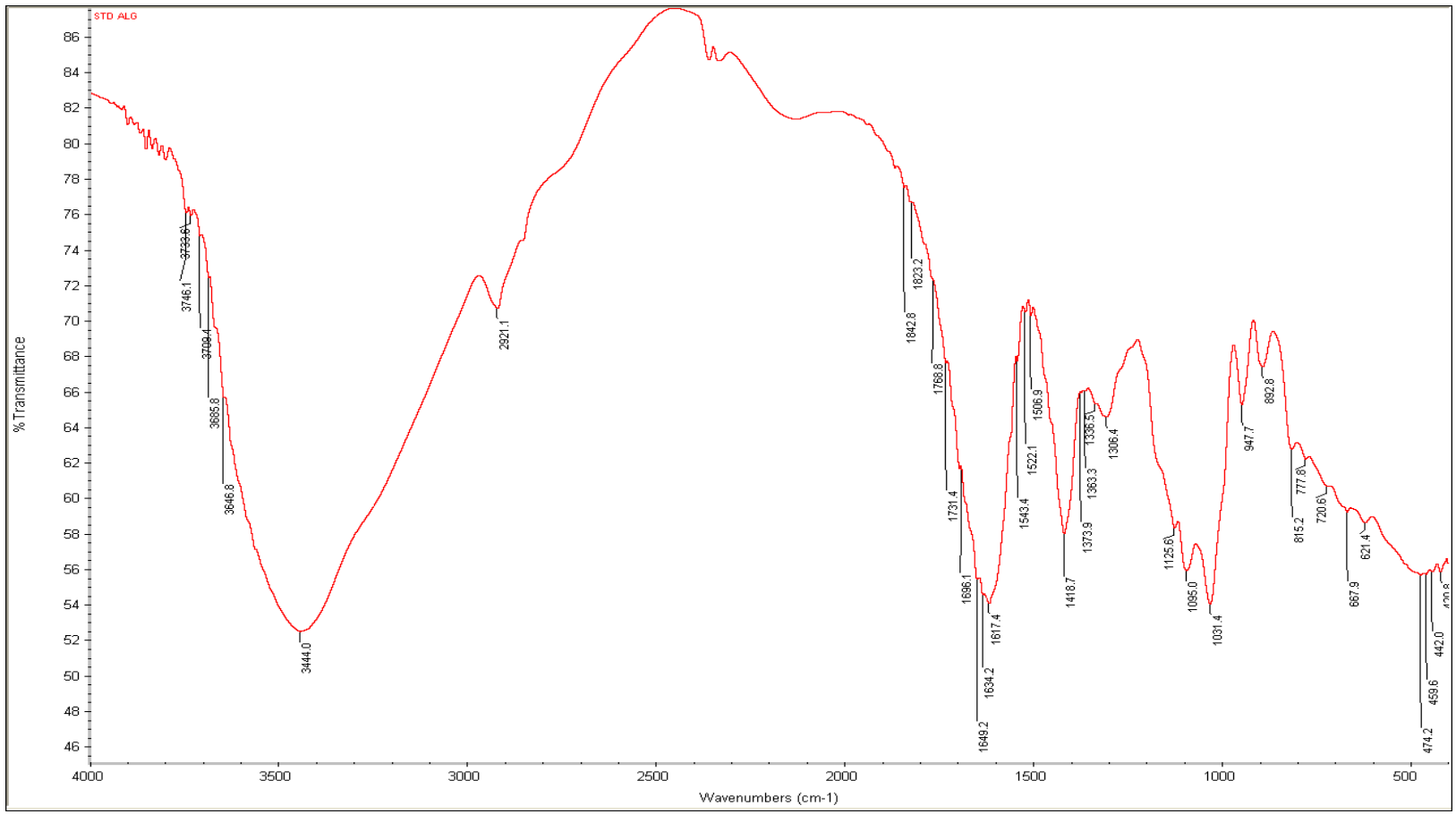


FIG B1. THE FT-IR SPECTRUM OF COMMERCIAL SODIUM ALGINATE

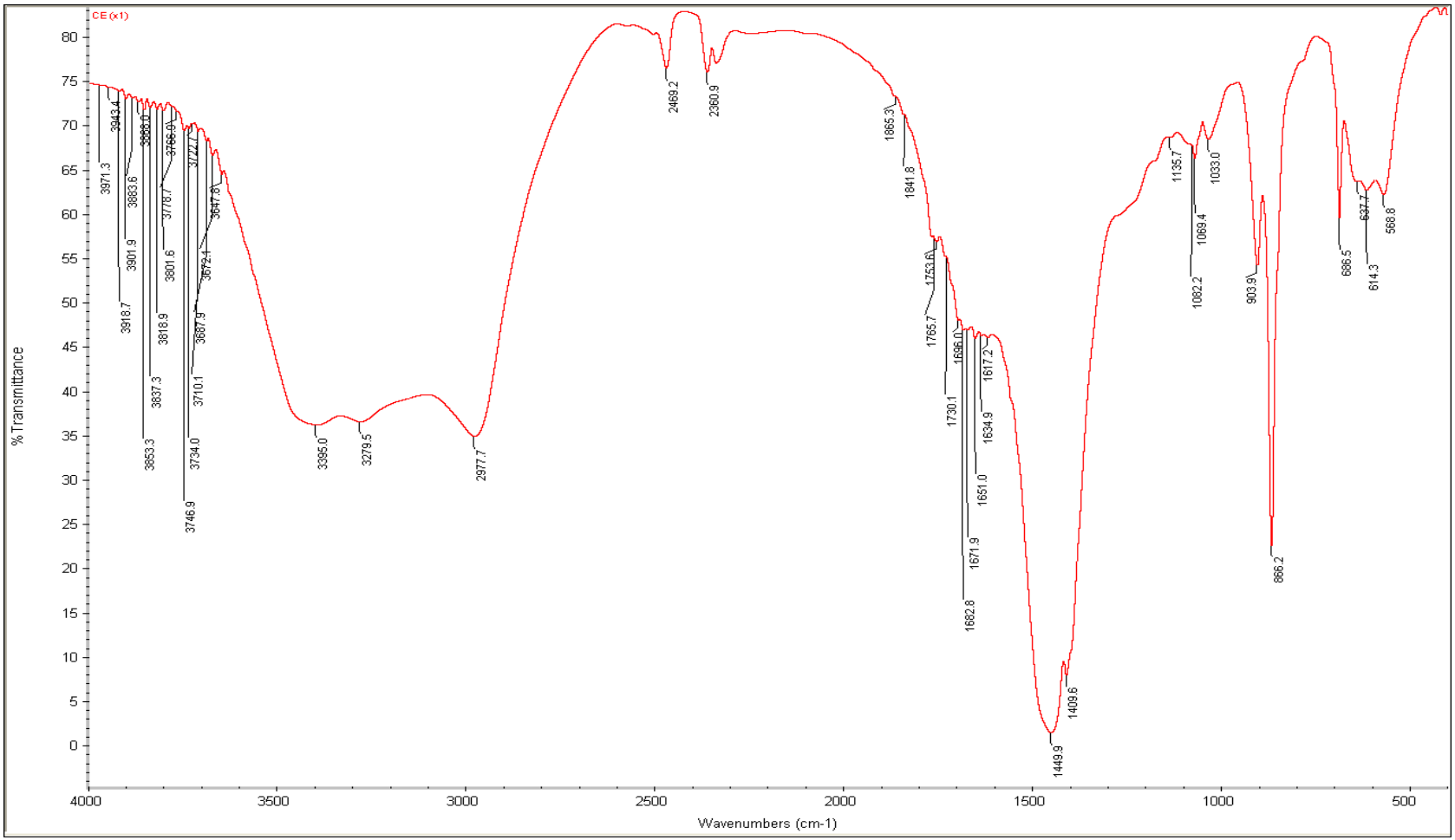


FIG. B2. THE FTIR SPECTRUM OF SODIUM ALGINATE EXTRACT FROM CONVENTIONAL CHEMICAL EXTRACTION (Alg-CCE) (1×)

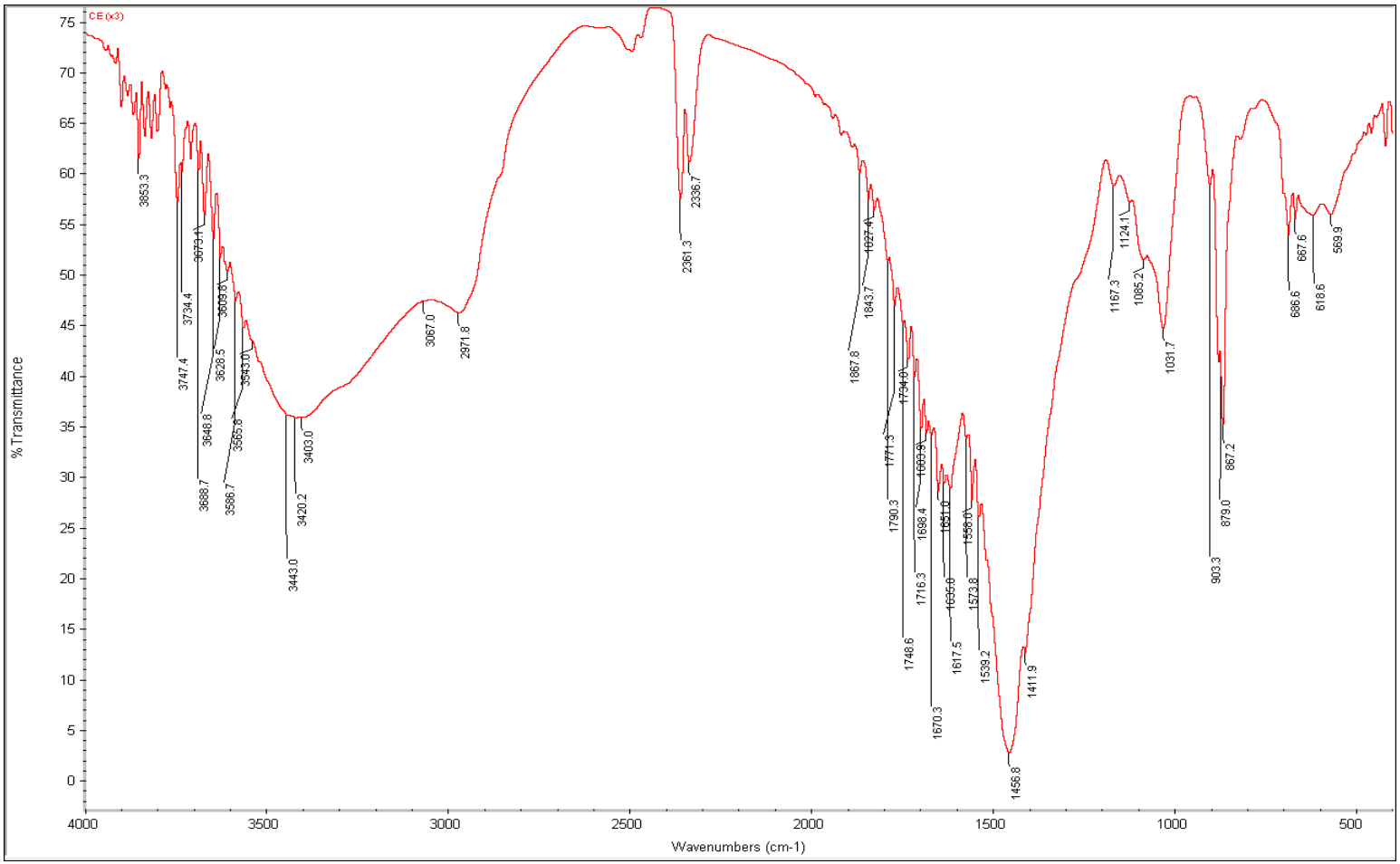


FIG. B3. THE FTIR SPECTRUM OF SODIUM ALGINATE EXTRACT FROM CONVENTIONAL CHEMICAL EXTRACTION (Alg-CCE) (3×)

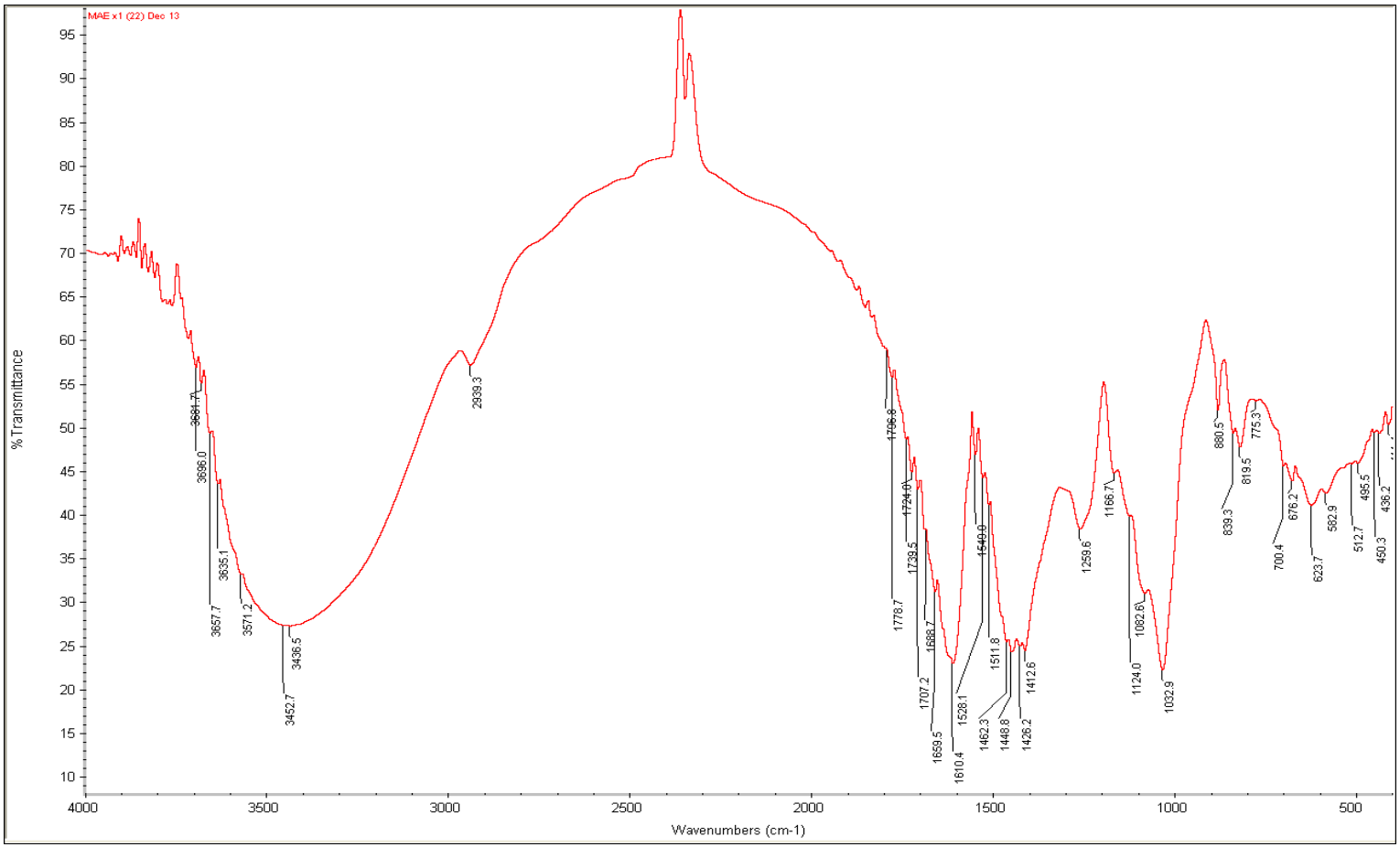


FIG. B4. THE FTIR SPECTRUM OF SODIUM ALGINATE EXTRACT FROM MICROWAVE-ASSISTED EXTRACTION (Alg-MAE) (1×)

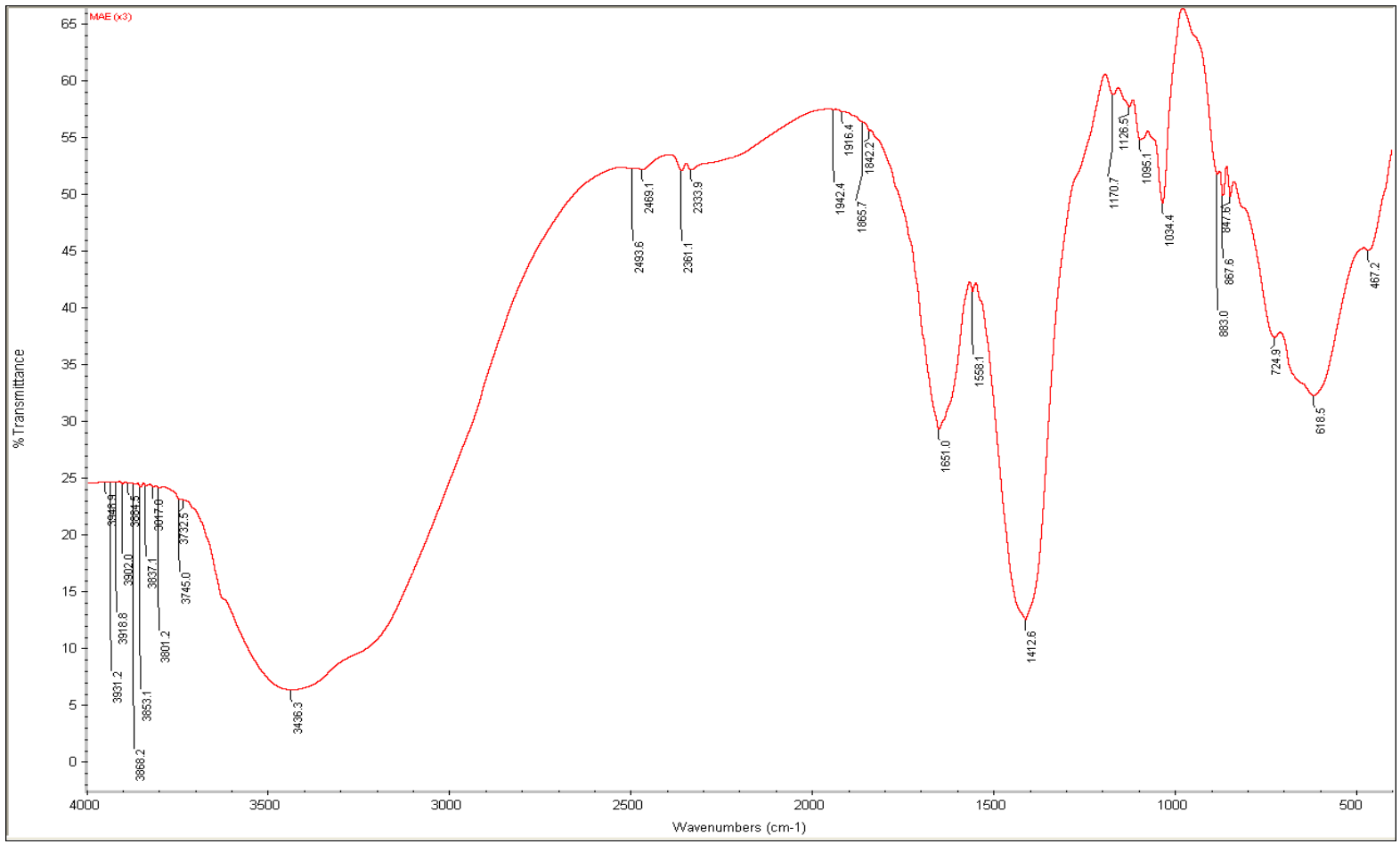


FIG. B5. THE FT-IR SPECTRUM OF SODIUM ALGINATE EXTRACT FROM MICROWAVE-ASSISTED EXTRACTION (Alg-MAE) (3×)

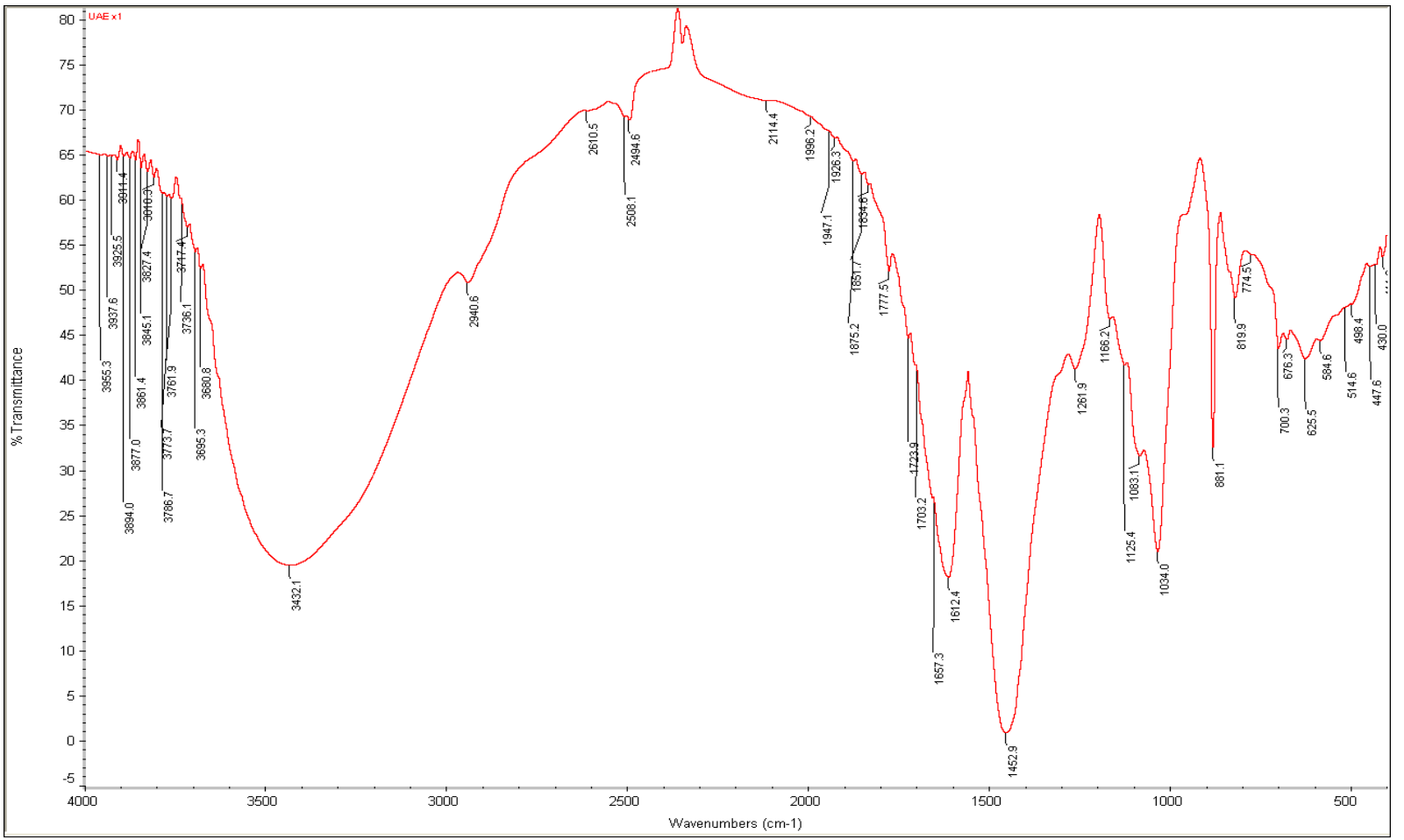


FIG. B6. THE FT-IR SPECTRUM OF SODIUM ALGINATE EXTRACT FROM ULTRASOUND-ASSISTED EXTRACTION (Alg-UAE) (1×)

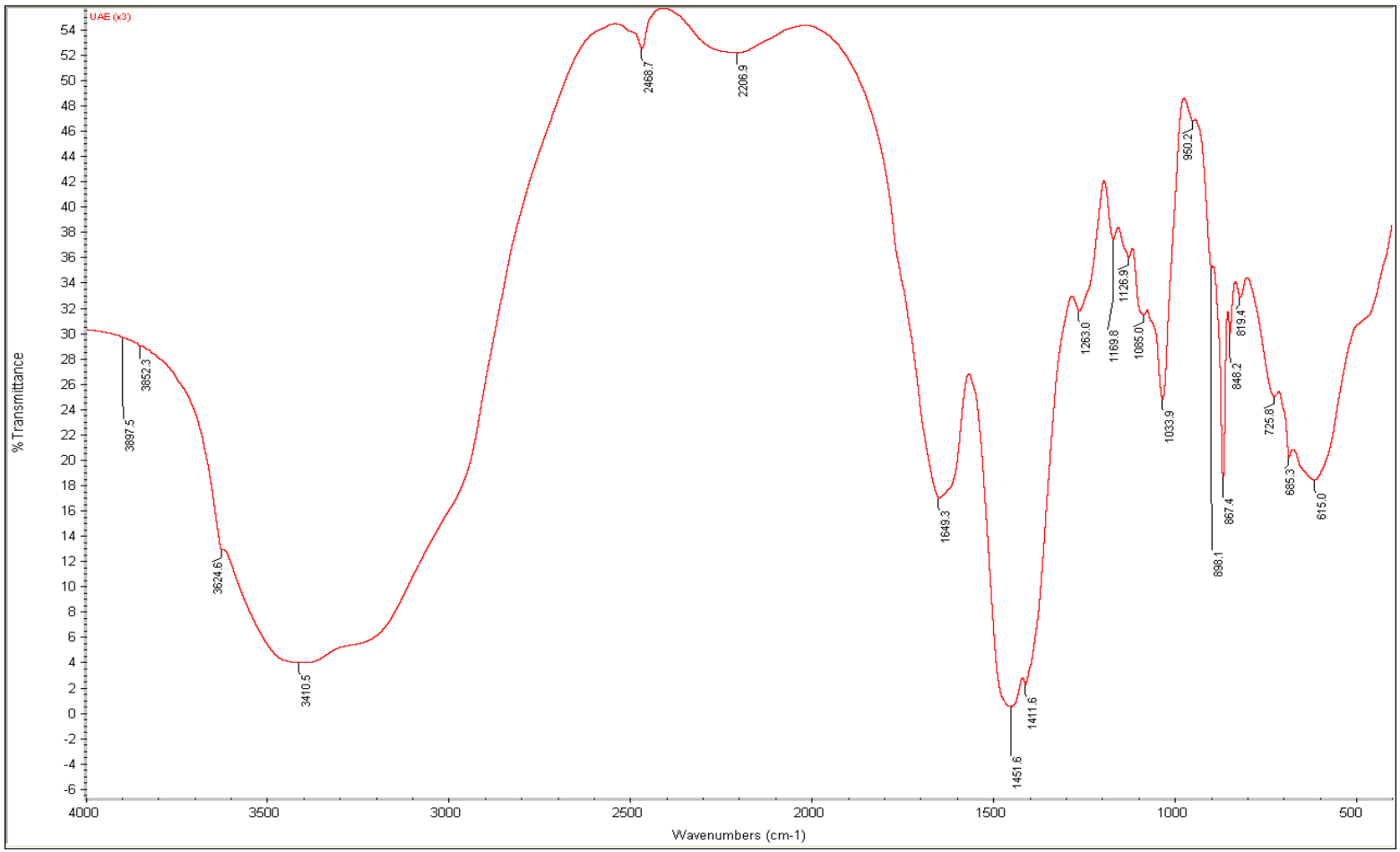


FIG. B7. THE FT-IR SPECTRUM OF SODIUM ALGINATE EXTRACT FROM ULTRASOUND-ASSISTED EXTRACTION (Alg-UAE) (3×)

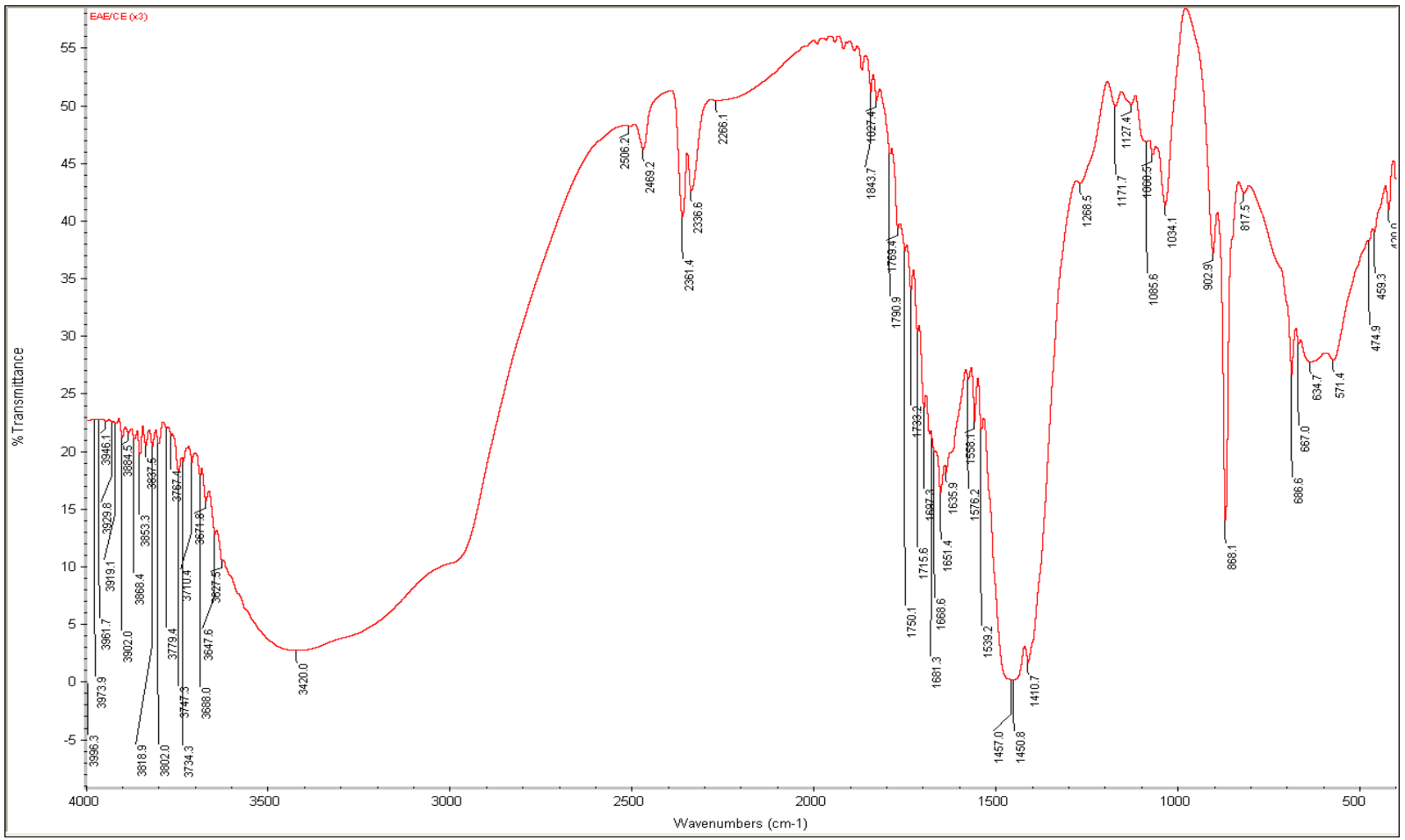


FIG. B8. THE FT-IR SPECTRUM OF SODIUM ALGINATE EXTRACTS FROM ENZYME-ASSISTED EXTRACTION (Alg-EAE) (3×)

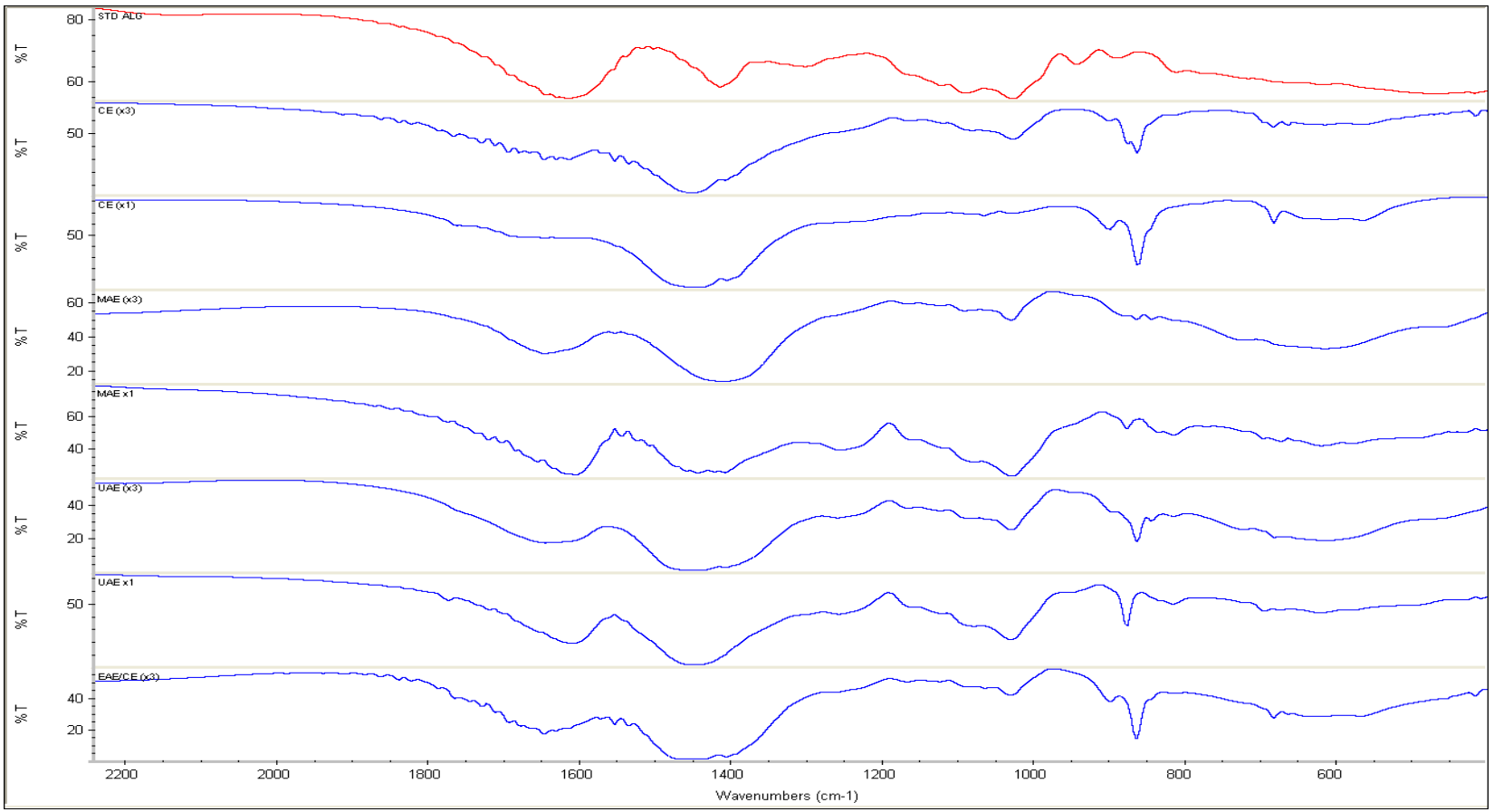


FIG. B9. THE COMBINED FT-IR SPECTRA OF COMMERCIAL ALGINATE AND SODIUM ALGINATE EXTRACTS

APPENDIX C: THE *Lactobacillus delbruecki* ss *bulgaricus* AND *Lactobacillus casei* GROWTH CURVES

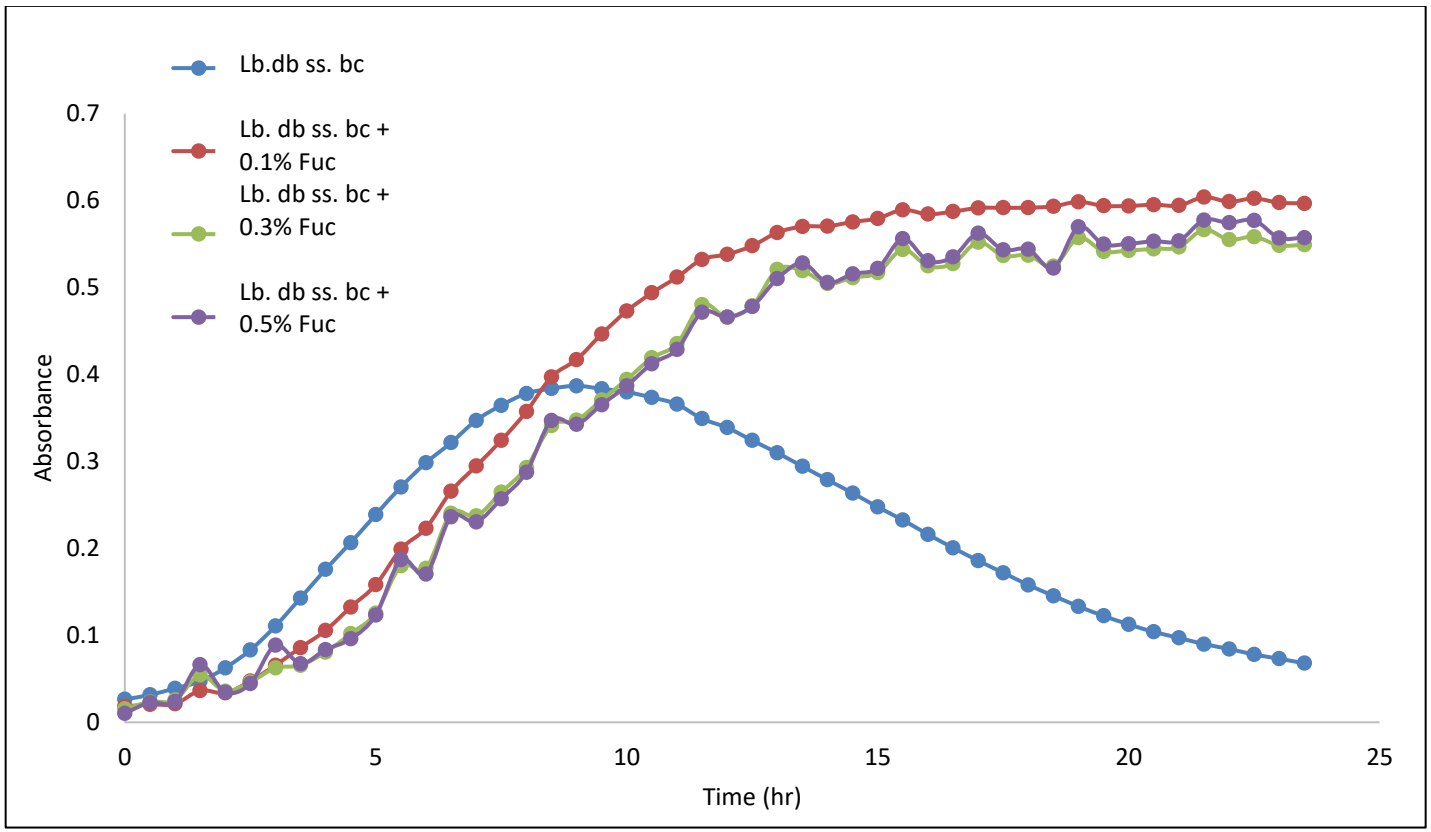


FIG. C1. FUCOIDAN FROM CONVENTIONAL CHEMICAL EXTRACTION (Fuc-CCE) (3×) SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES

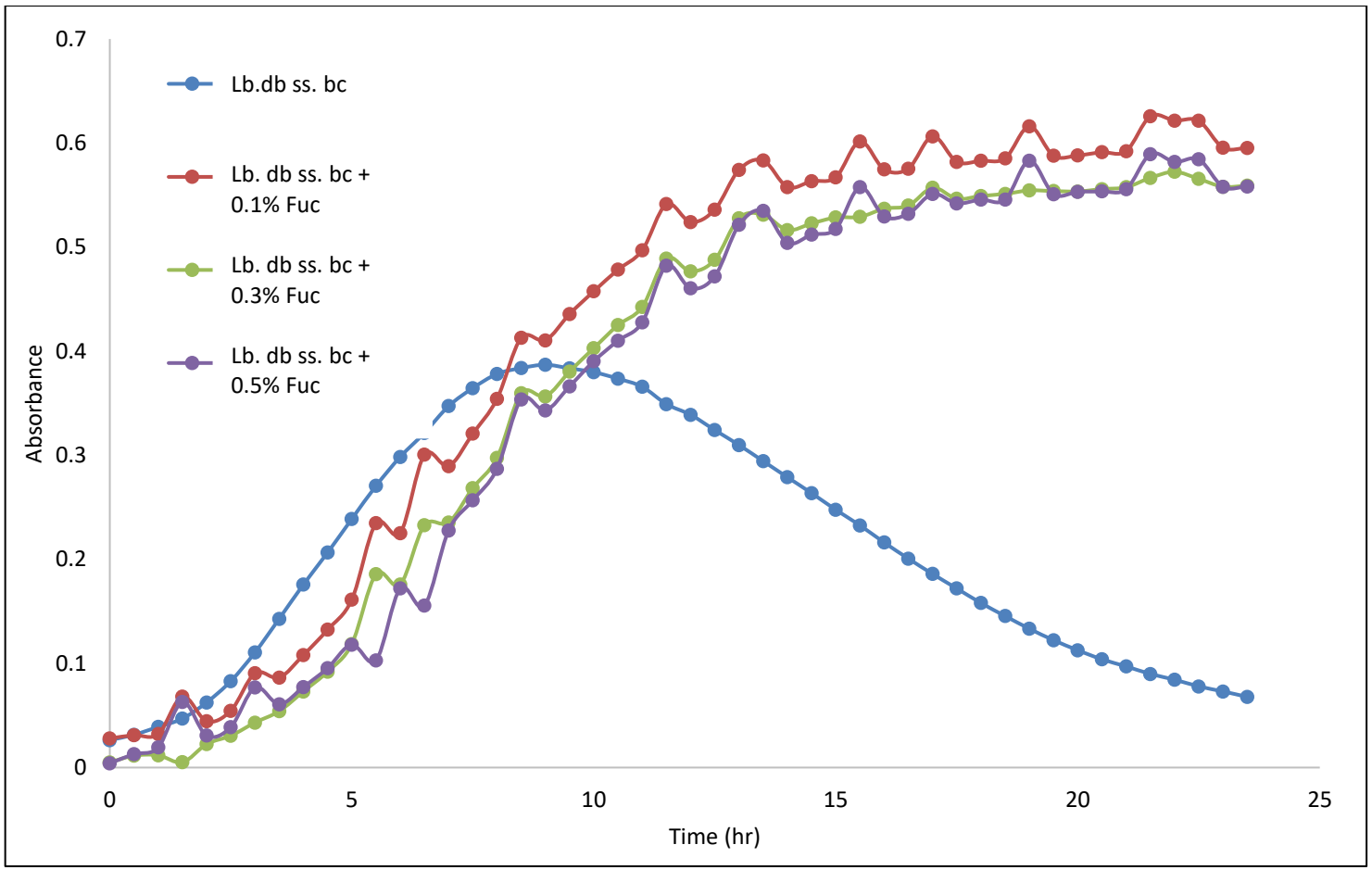


FIG. C2. FUCOIDAN FROM MICROWAVE-ASSISTED EXTRACTION (Fuc-MAE) (3×) SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES

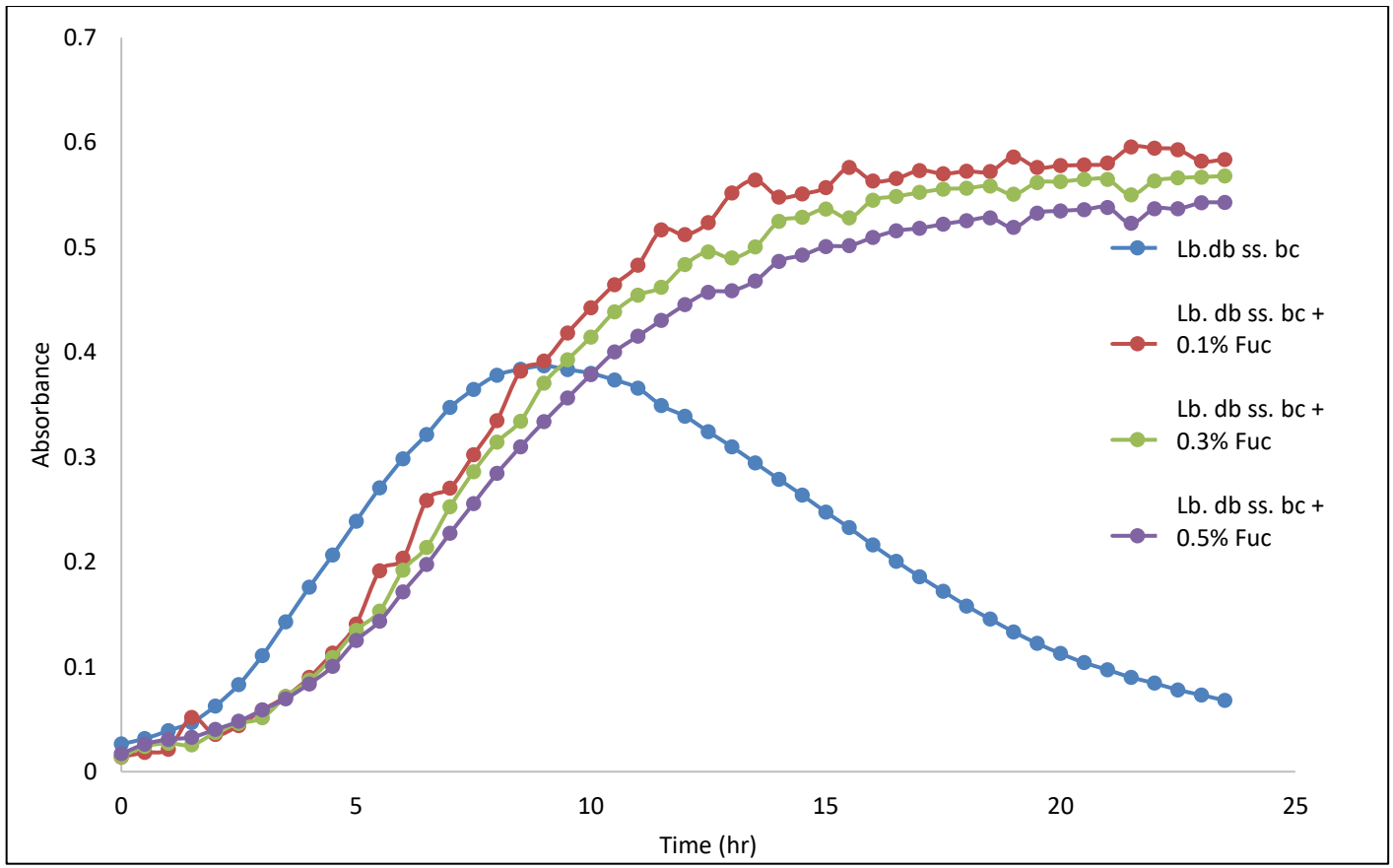
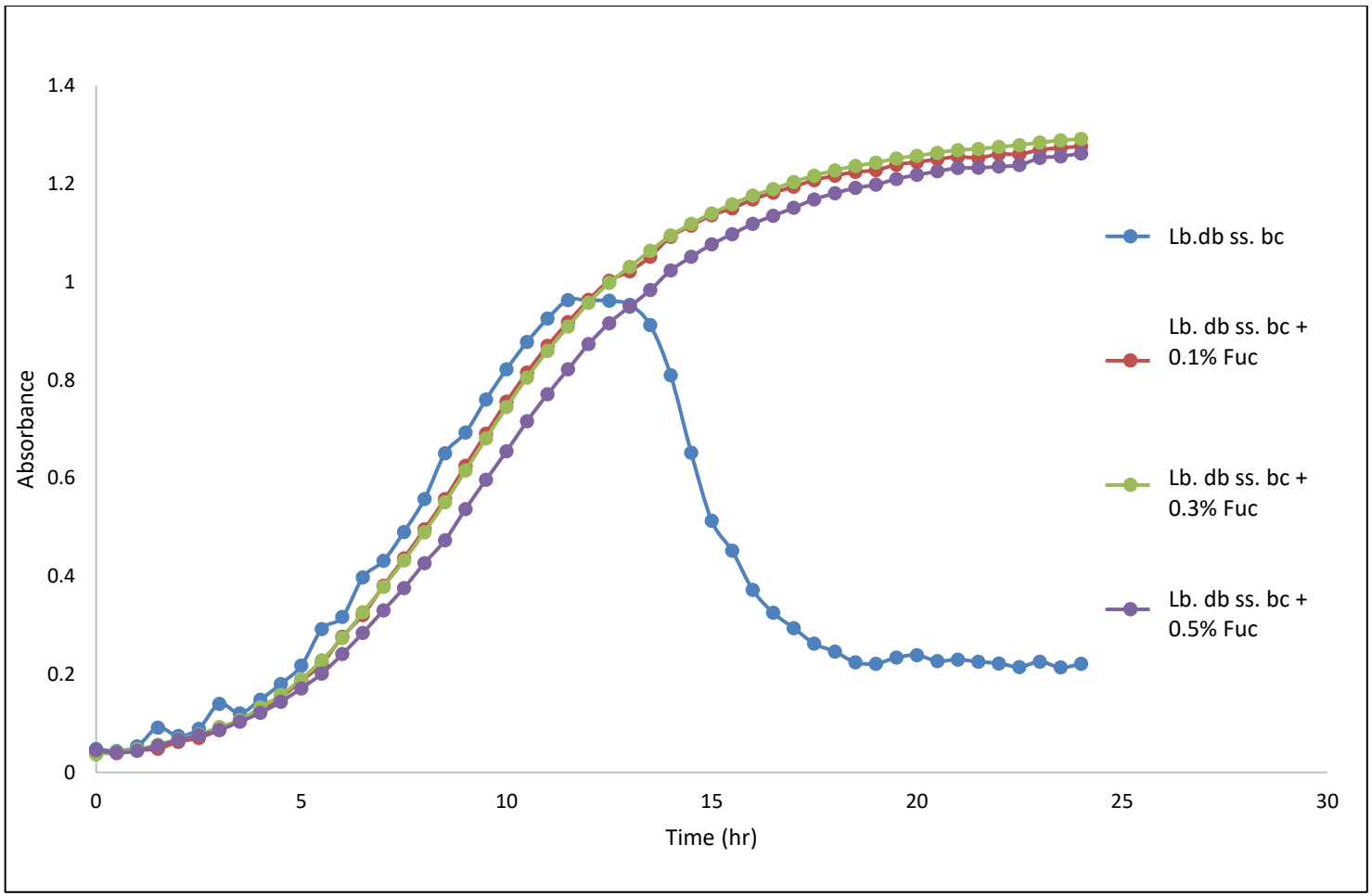


FIG. C3. FUCOIDAN FROM ULTRASOUND-ASSISTED EXTRACTION (Fuc-UAE) (3×) SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES



165

FIG. C4. FUCOIDAN FROM ENZYME-ASSISTED EXTRACTION (Fuc-EAE) (3×) SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES

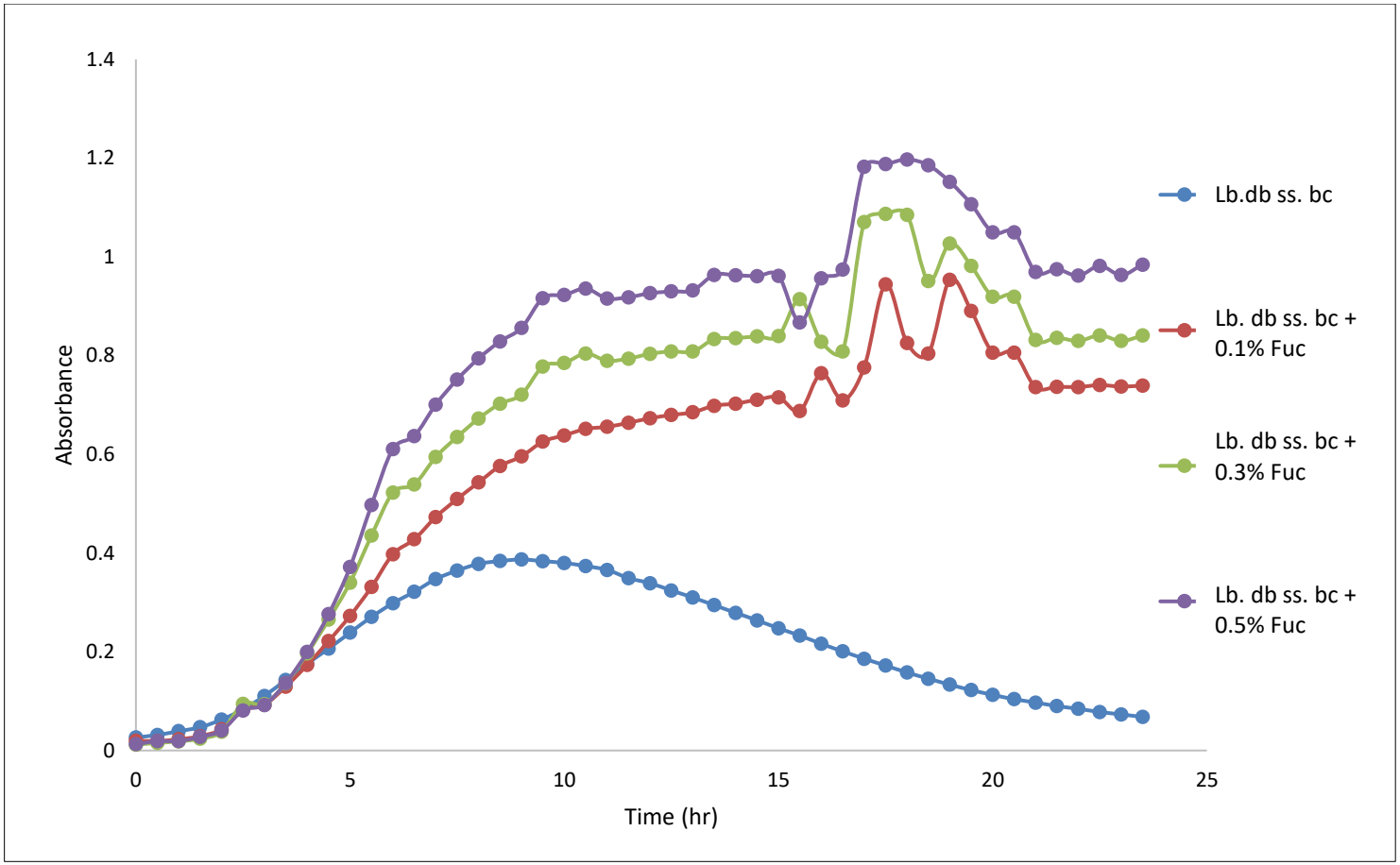


FIG. C5. SODIUM ALGINATE FROM CONVENTIONAL CHEMICAL EXTRACTION (Alg-CCE) (3×) SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES

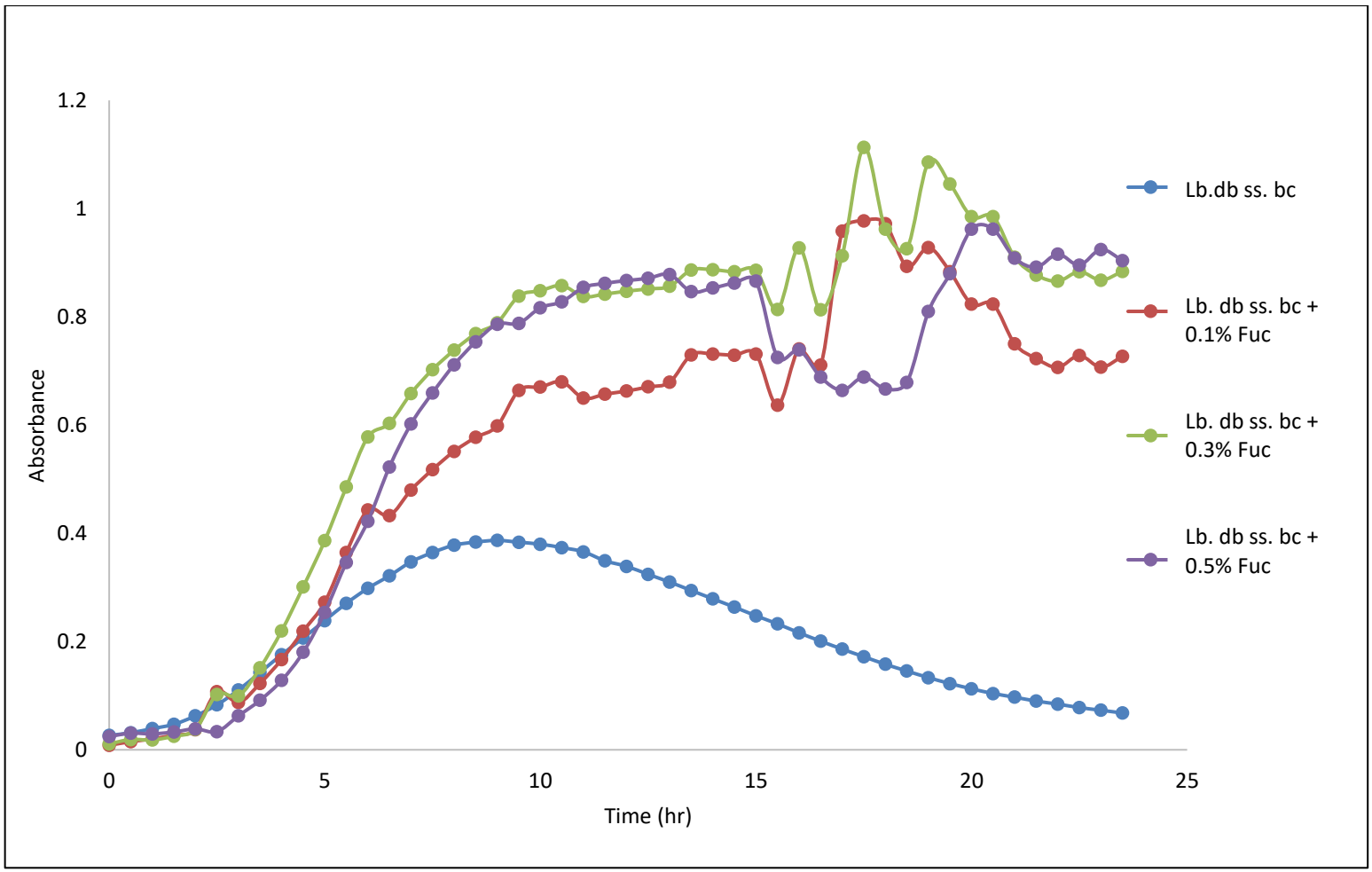


FIG. C6. SODIUM ALGINATE FROM MICROWAVE-ASSISTED EXTRACTION (Alg-MAE) (3×) SUPPLEMENTED *L. delbruecki* GROWTH CURVES

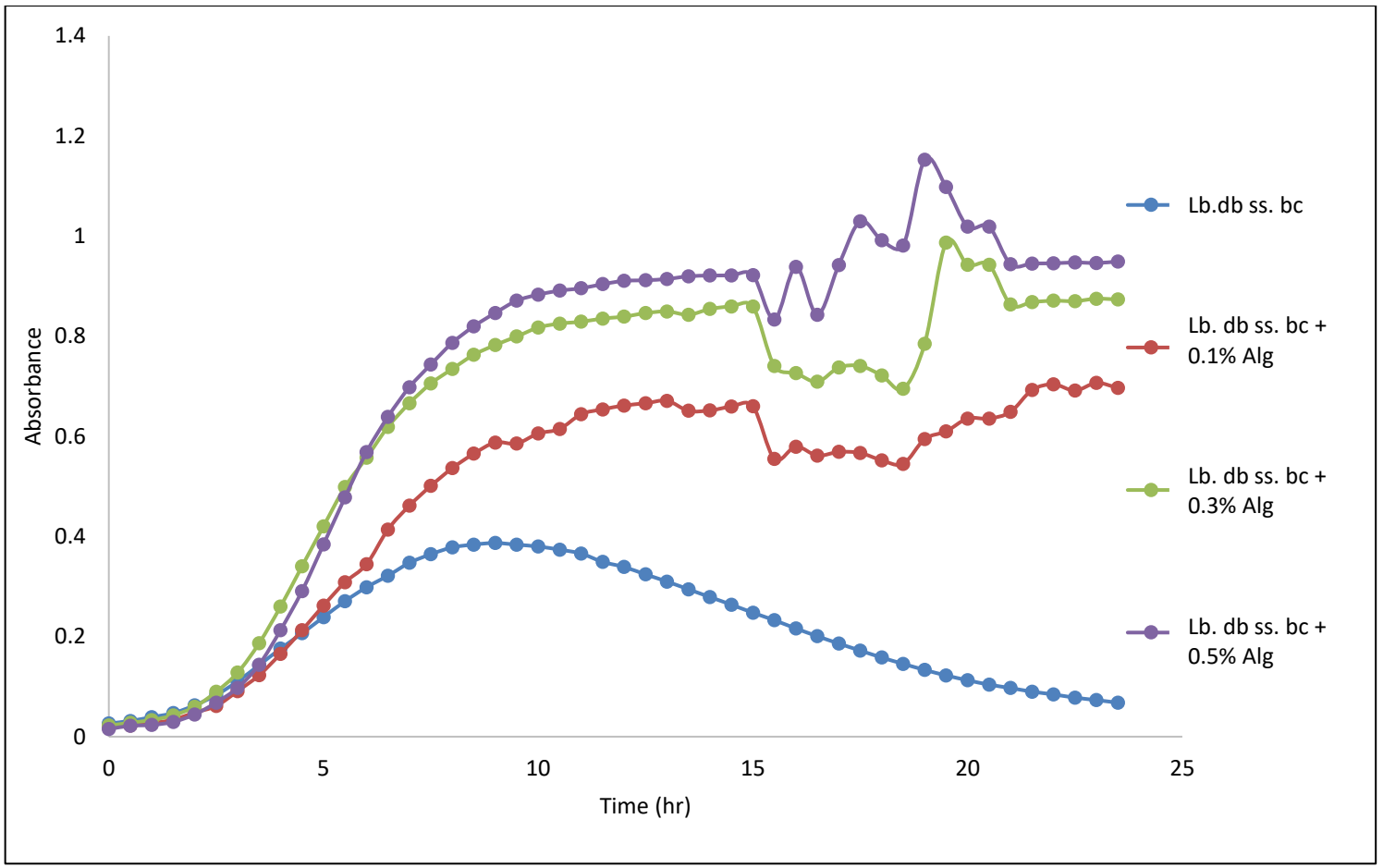


FIG. C7. SODIUM ALGINATE FROM ULTRASOUND-ASSISTED EXTRACTION (Alg-UAE) (3×) SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES

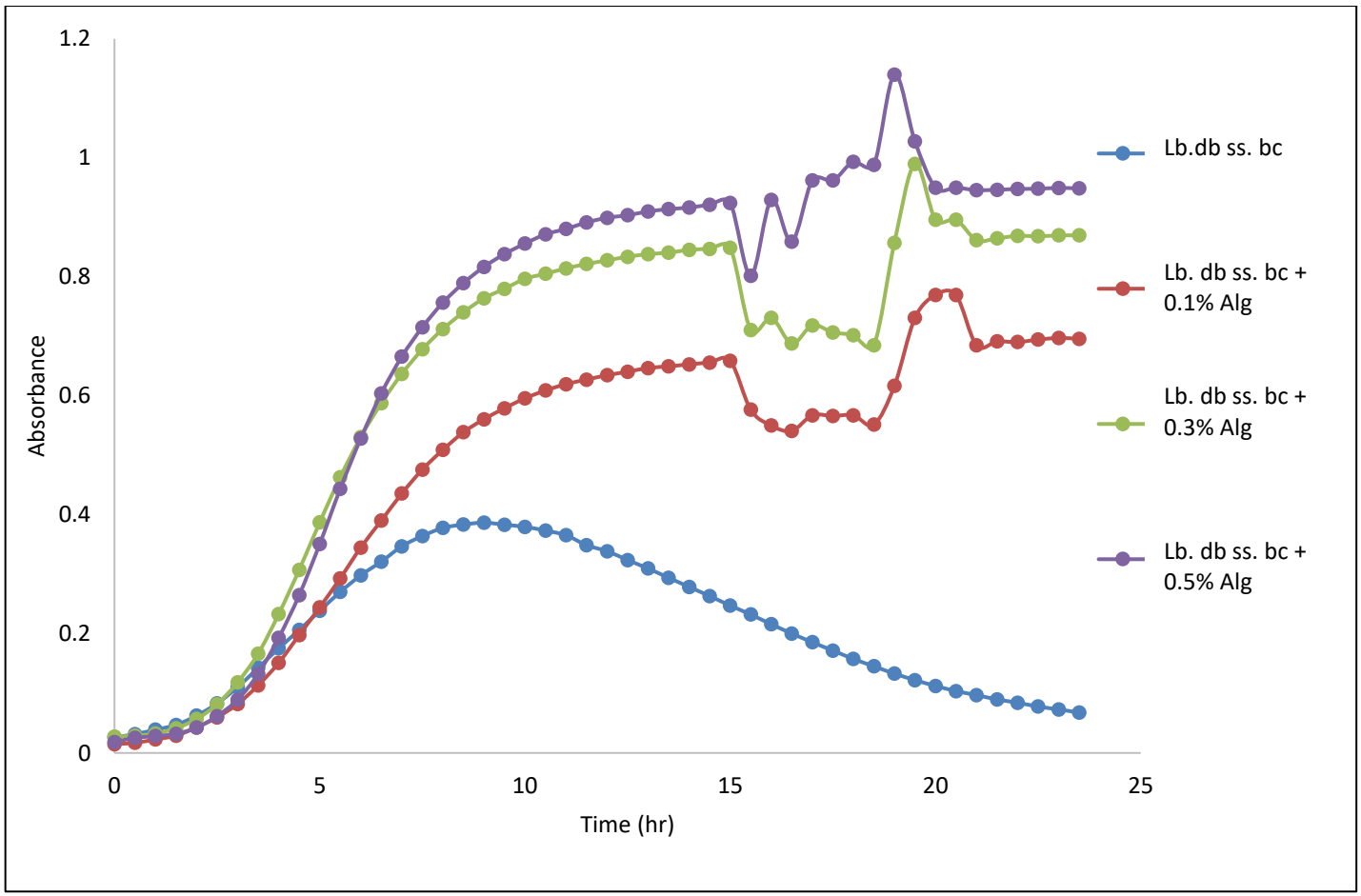
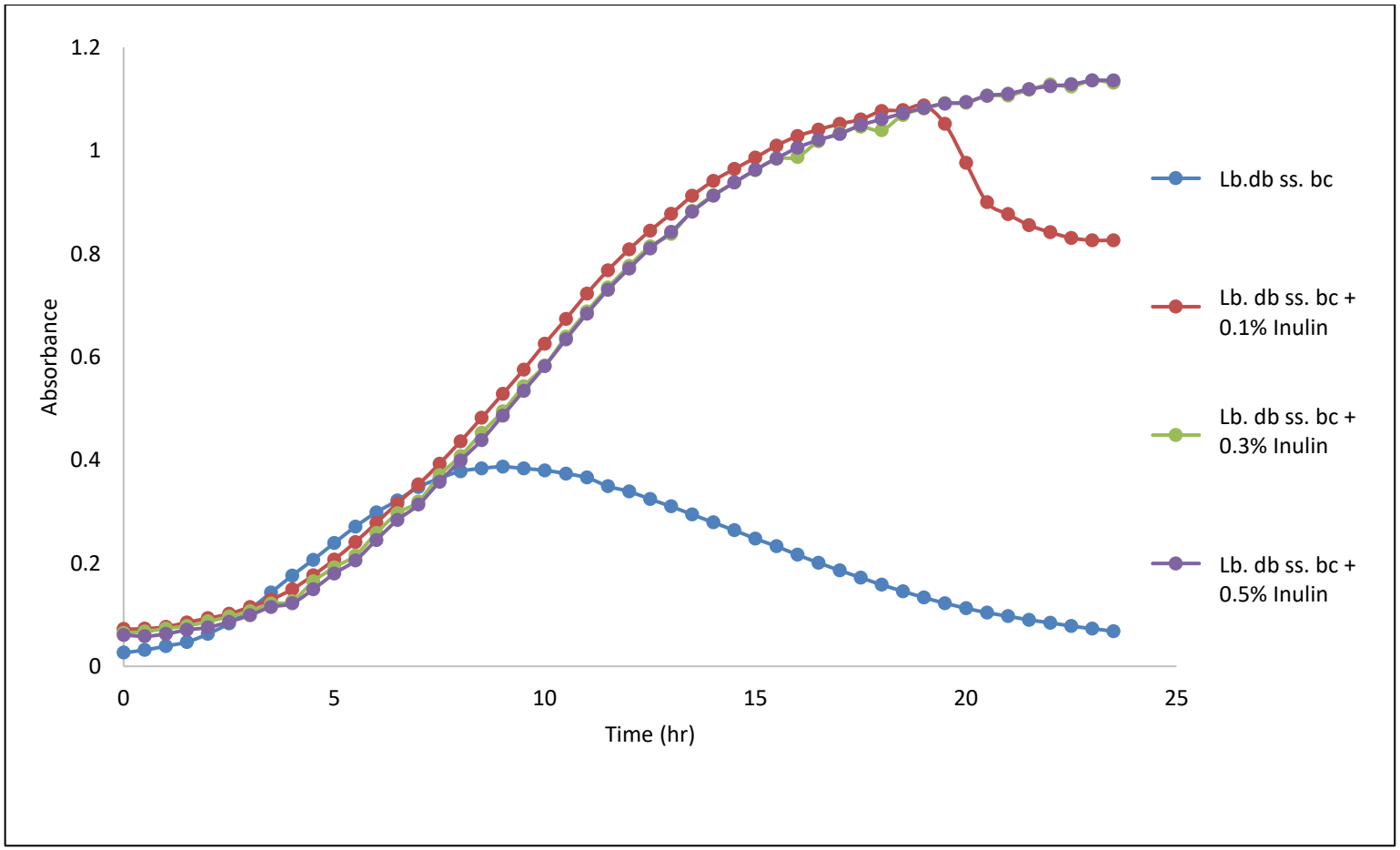


FIG. C8. SODIUM ALGINATE FROM ENZYME-ASSISTED EXTRACTION/CONVENTIONAL CHEMICAL EXTRACTION (Alg-EAE/CCE) (1×) SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES



170

FIG. C9. INULIN SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES

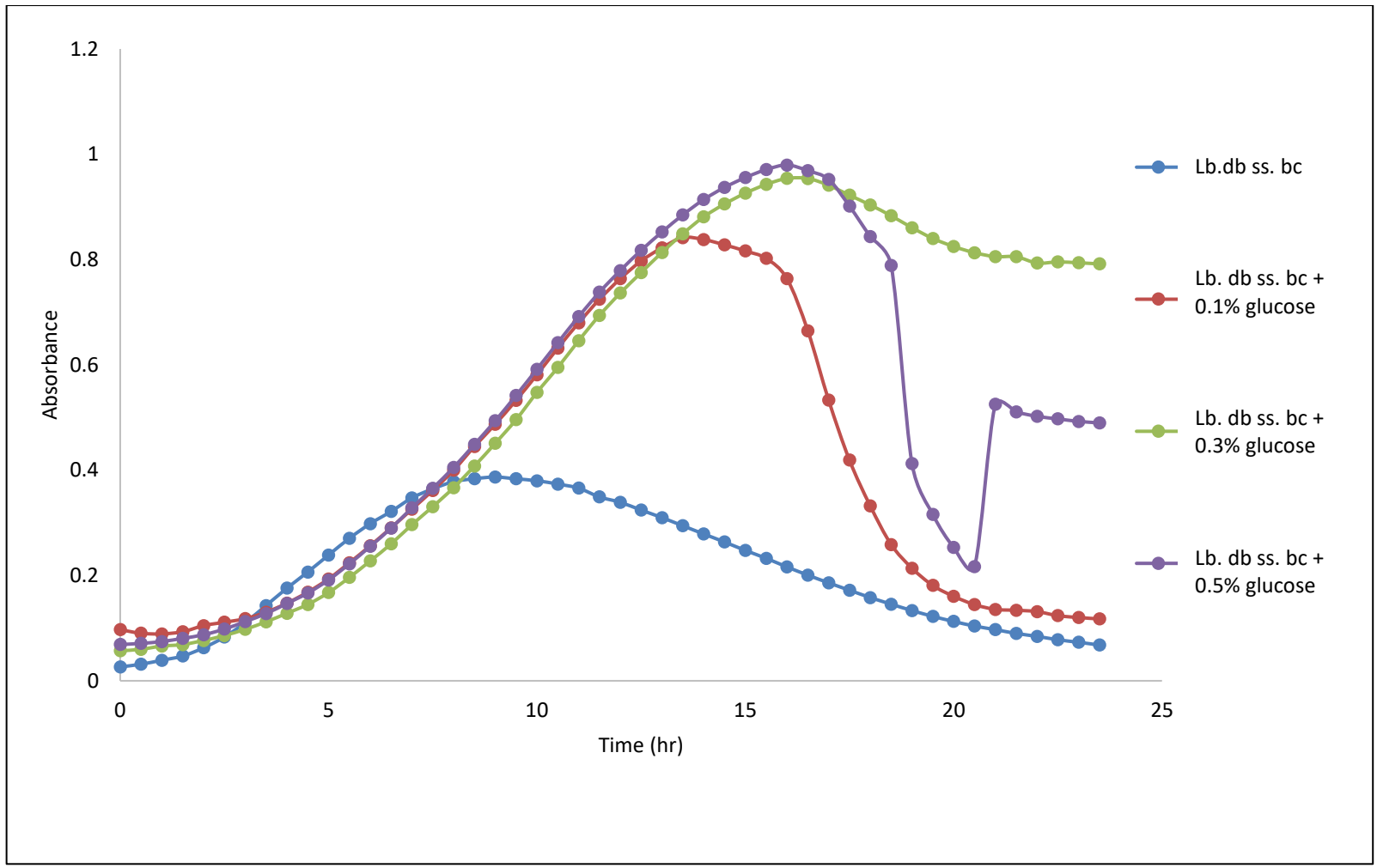


FIG. C10. GLUCOSE SUPPLEMENTED *Lactobacillus delbruecki* ss *bulgaricus* GROWTH CURVES

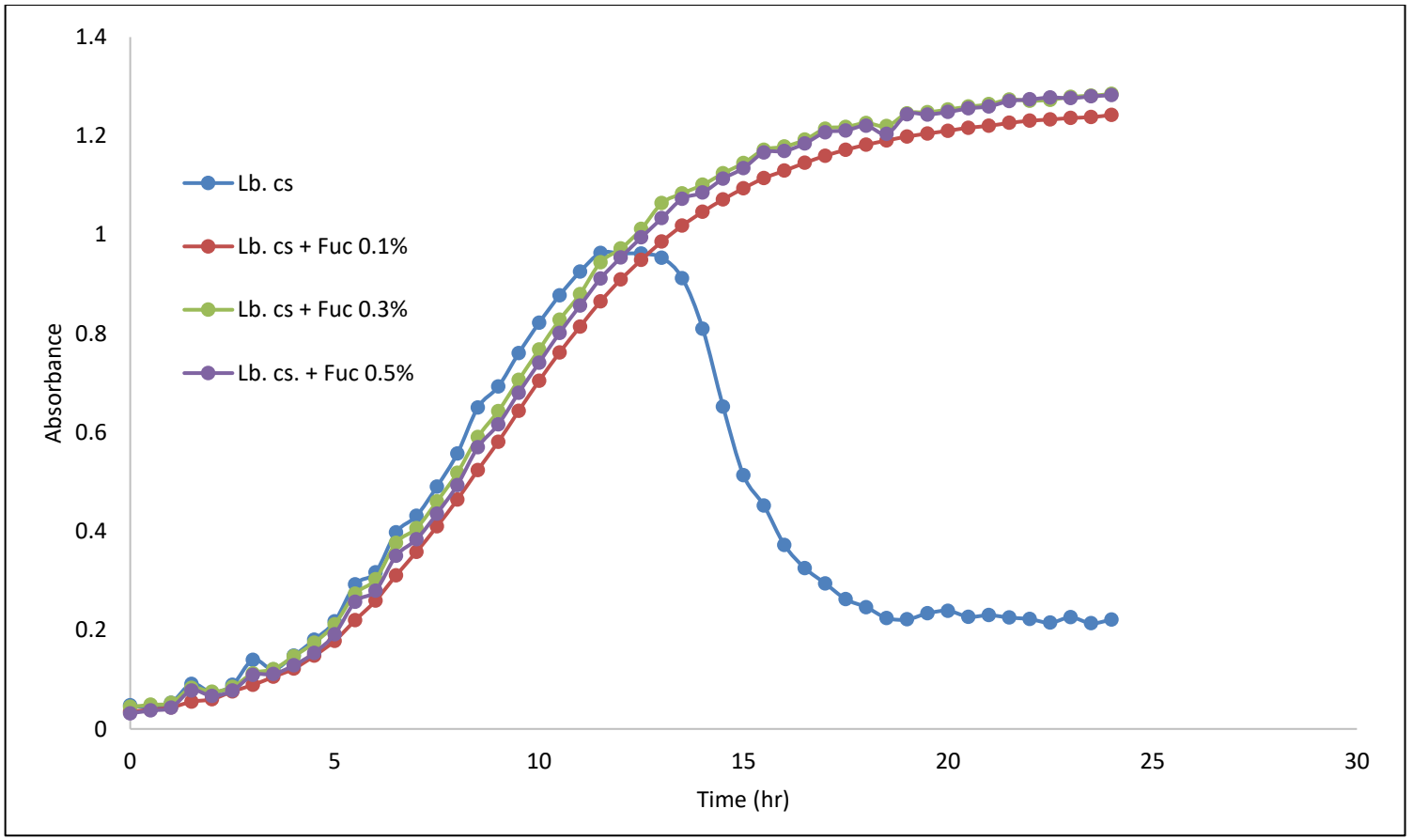


FIG. C11. FUCOIDAN FROM CONVENTIONAL CHEMICAL EXTRACTION (Fuc-CCE) (3×) SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES

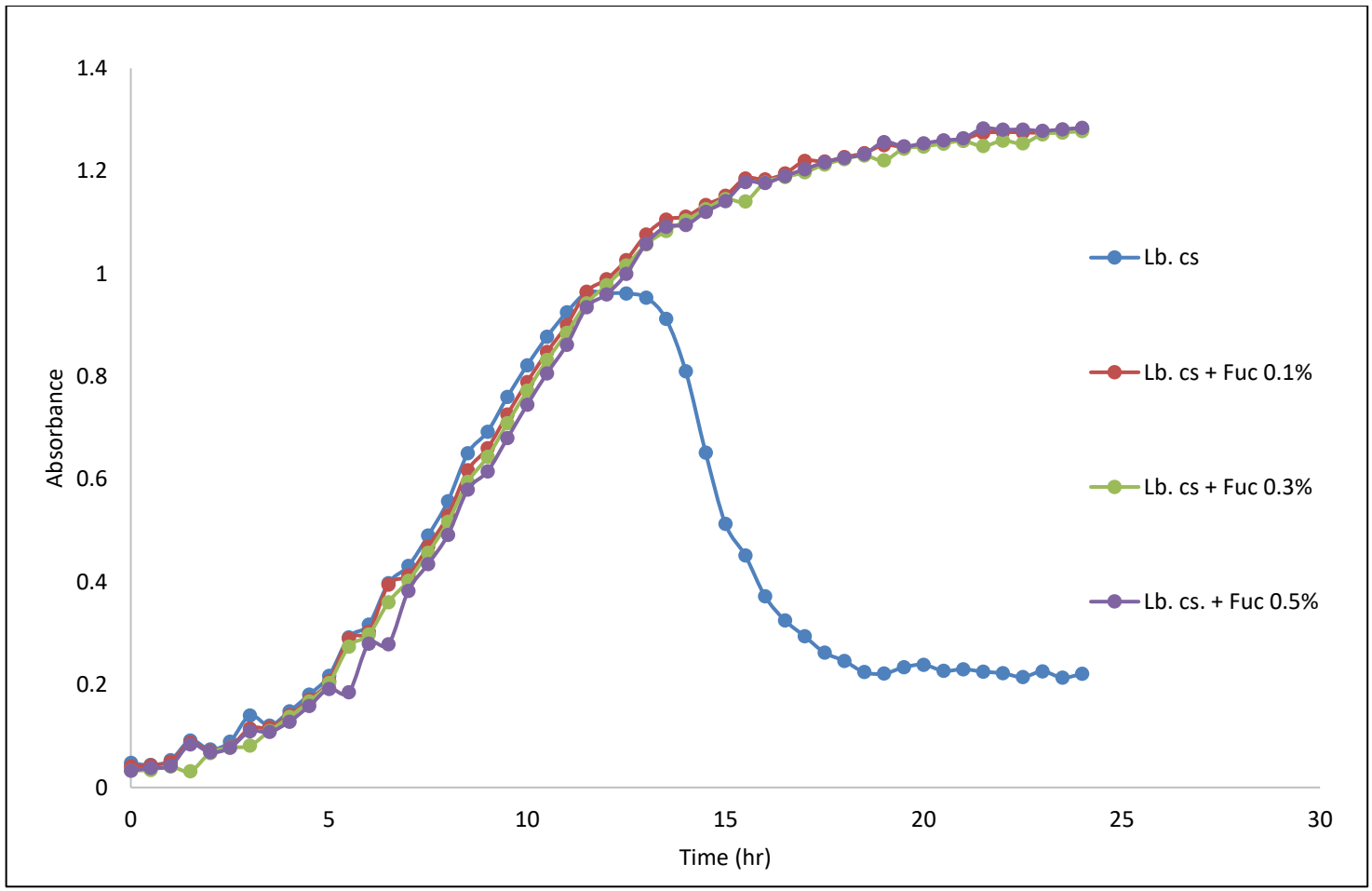


FIG. C12. FUCODIAN FROM MICROWAVE-ASSISTED EXTRACTION (Fuc-MAE) (3×) SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES

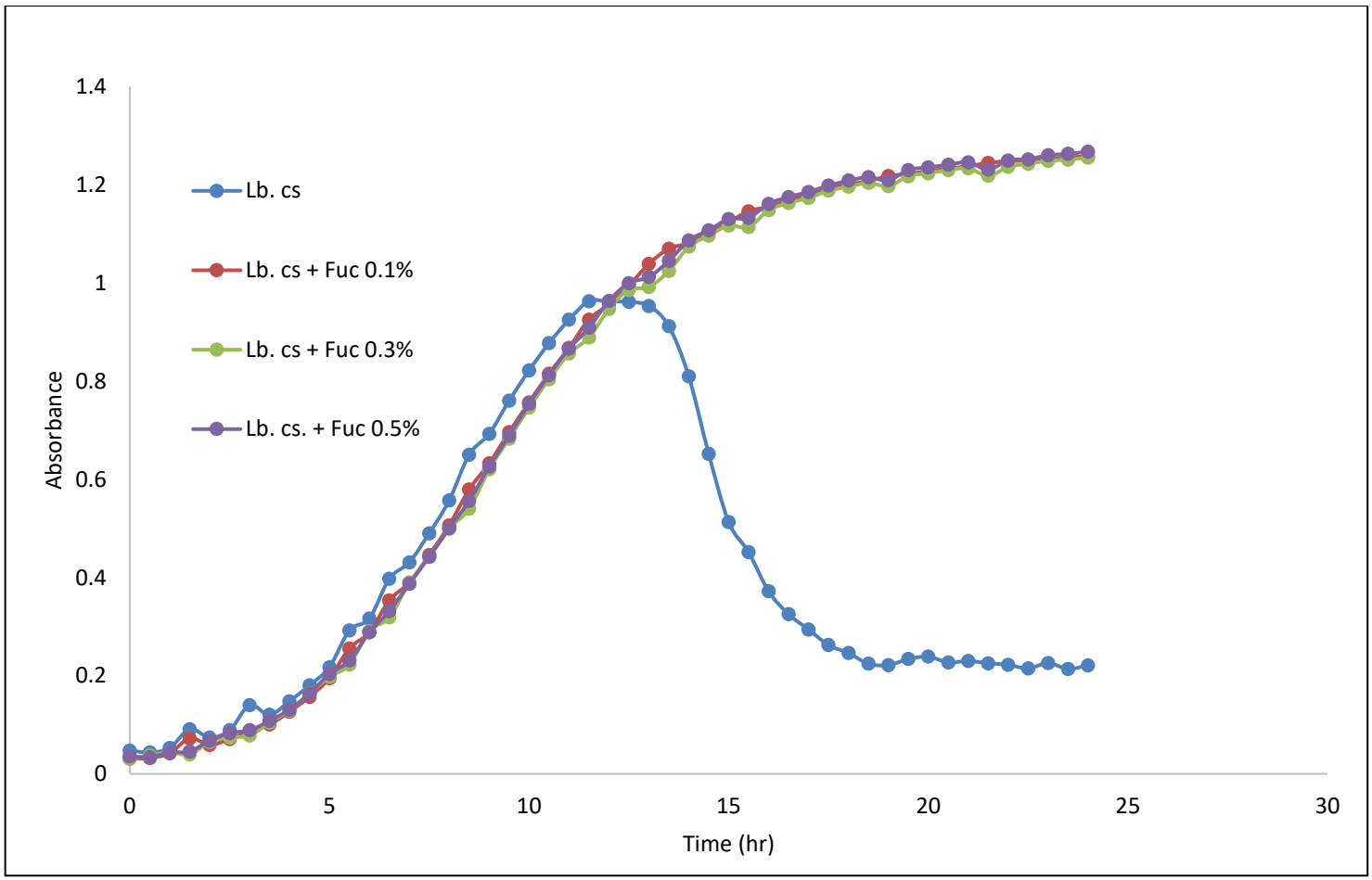
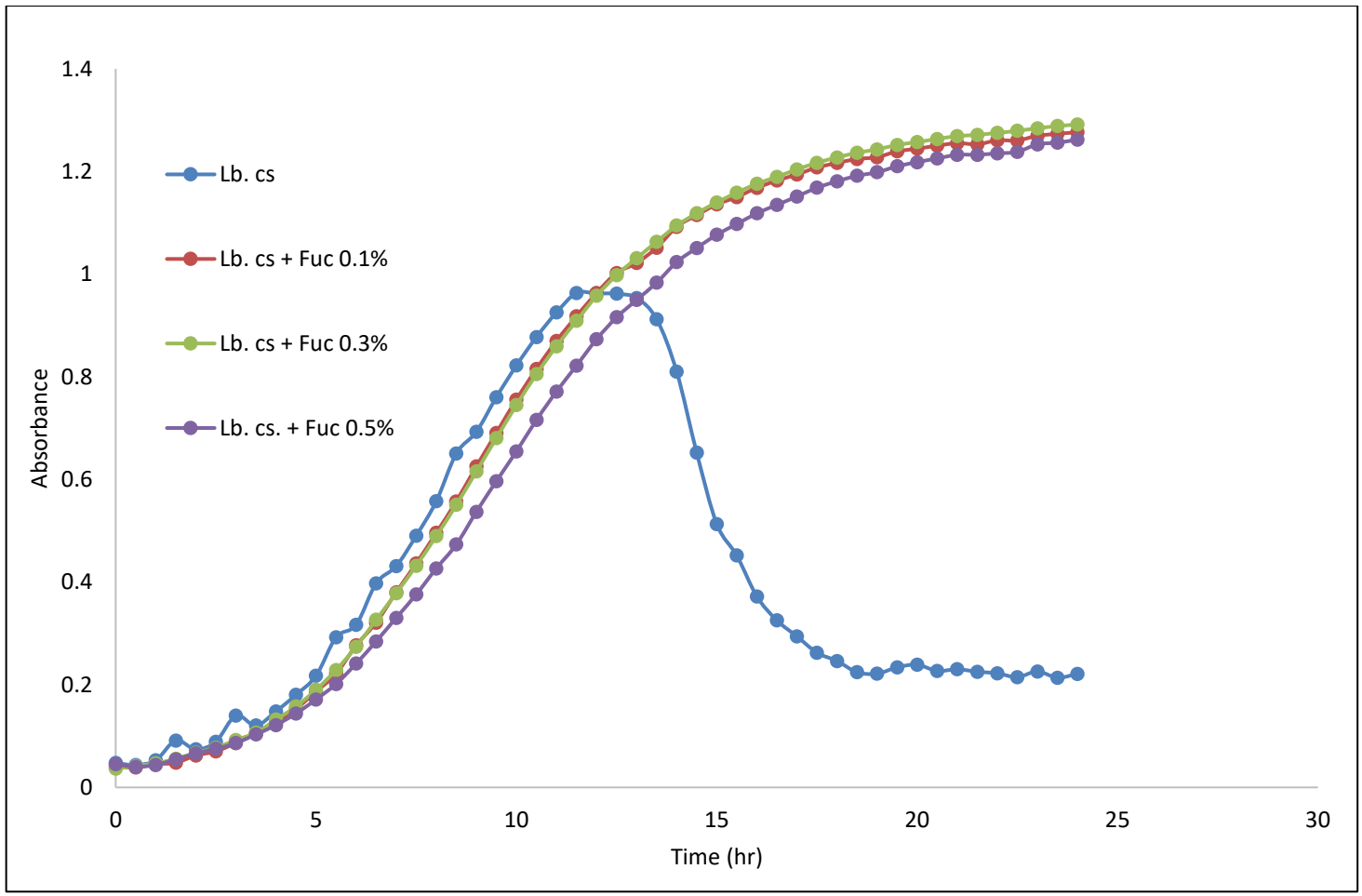


FIG. C13. FUCOIDAN FROM ULTRASOUND-ASSISTED EXTRACTION (Fuc-UAE) (3×) SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES



175

FIG. C14. FUCOIDAN FROM ENZYME-ASSISTED EXTRACTION (Fuc-EAE) (3×) SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES

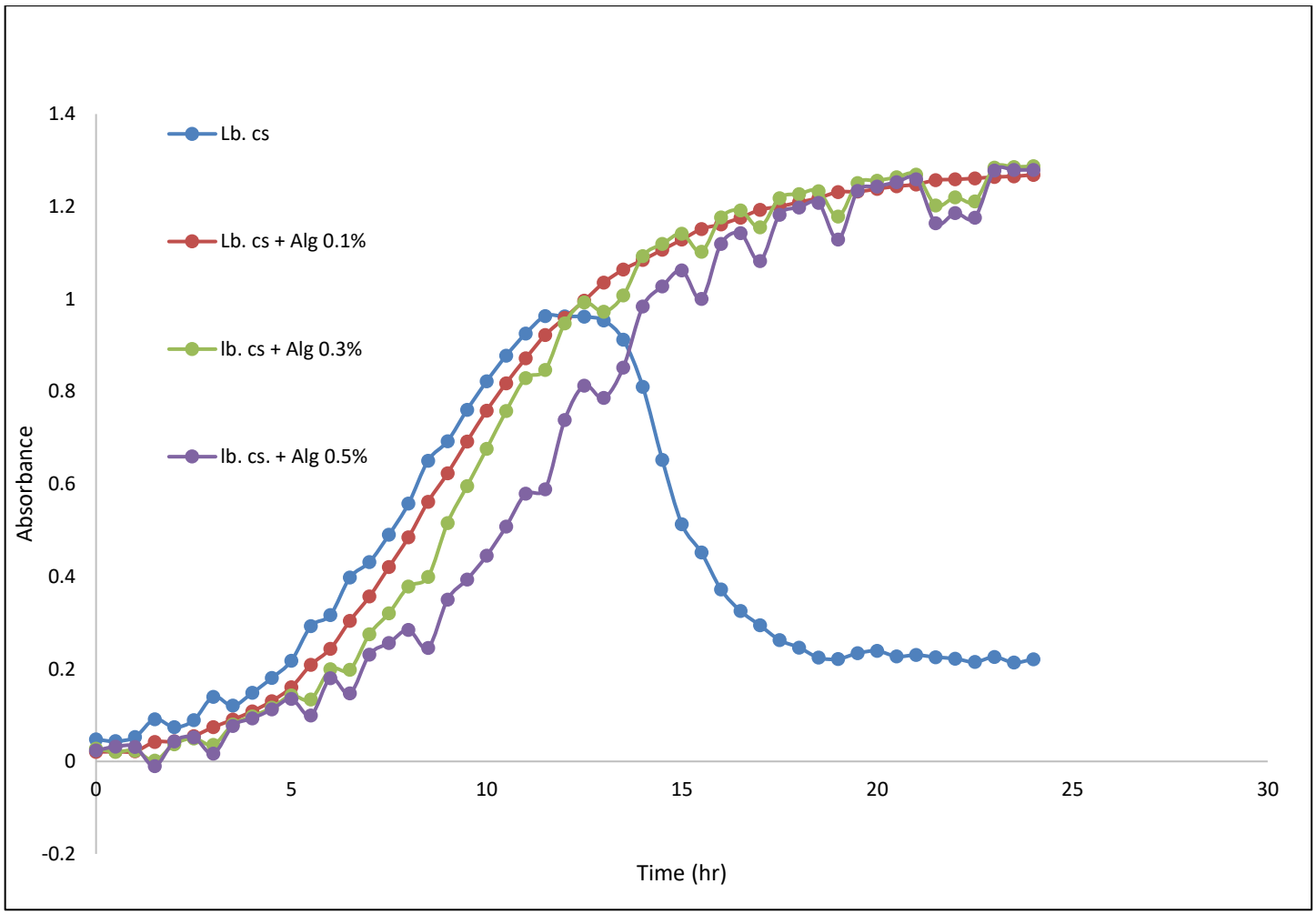


FIG. C15. SODIUM ALGINATE FROM CONVENTIONAL CHEMICAL EXTRACTION (Alg-CCE) SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES

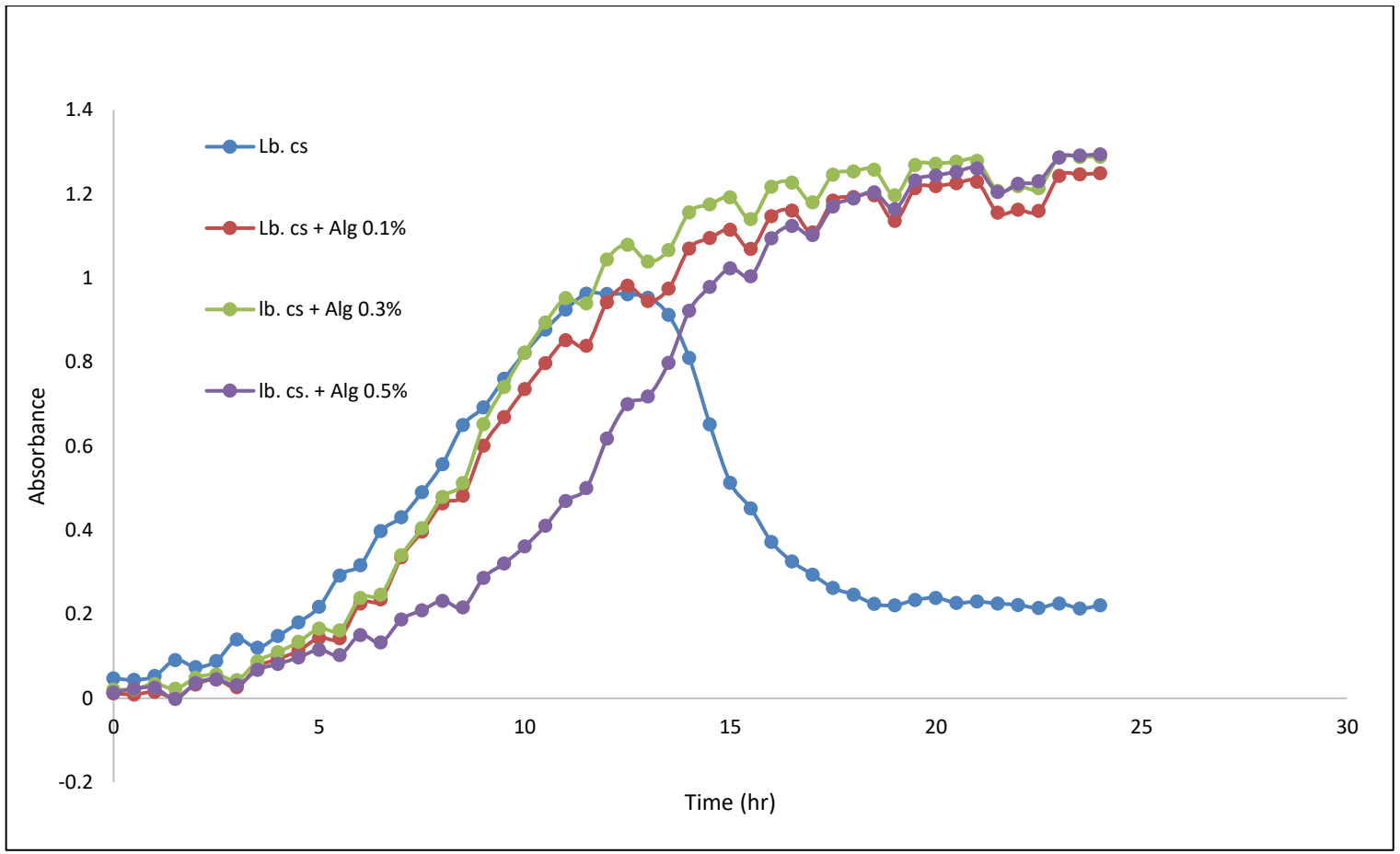


FIG. C16. SODIUM ALGINATE FROM MICROWAVE-ASSISTED EXTRACTION (Alg-MAE) (3×) SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES

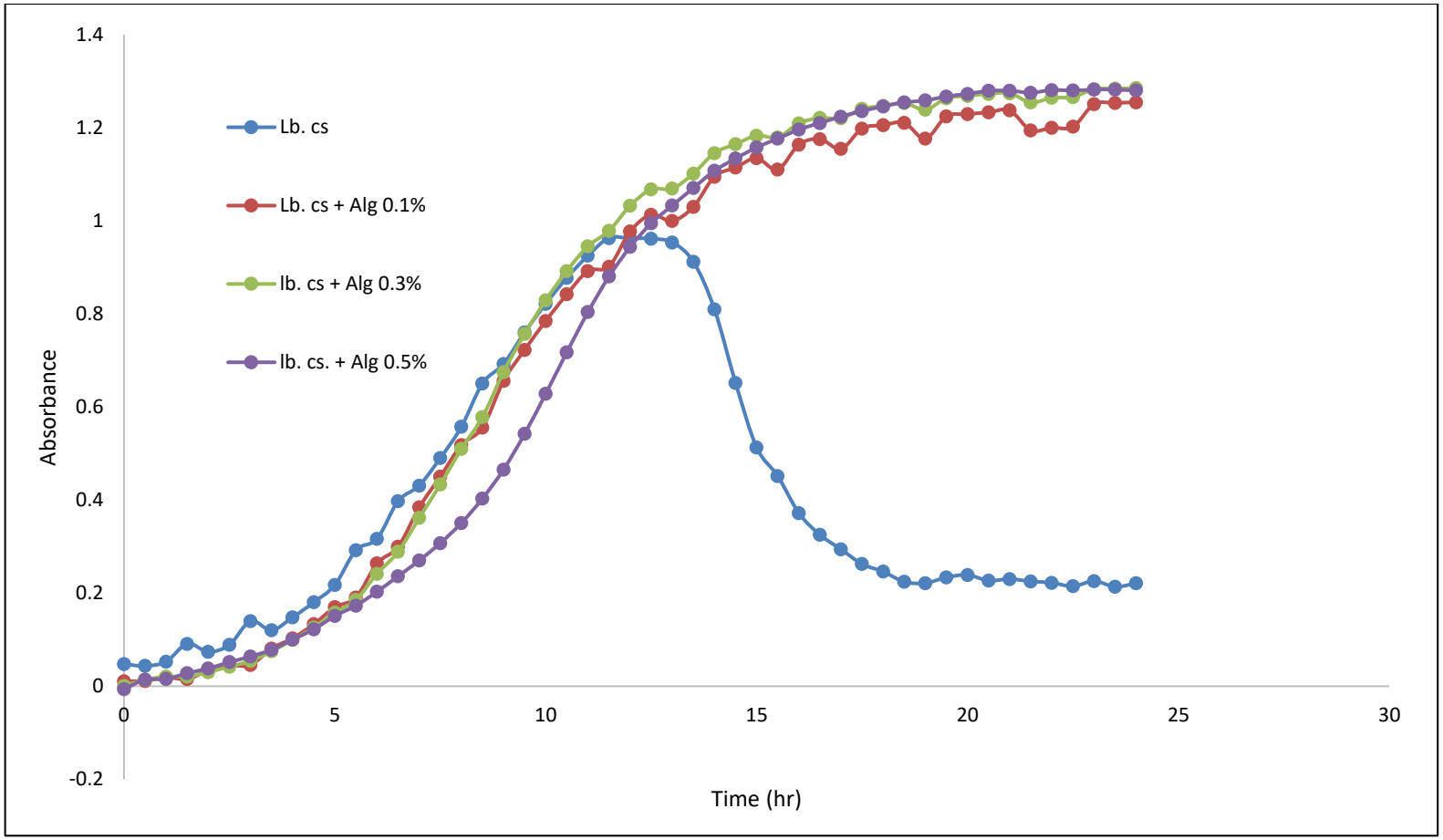


FIG. C17. SODIUM ALGINATE FROM ULTRASOUND-ASSISTED EXTRACTION (Alg-UAE) (3×) SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES

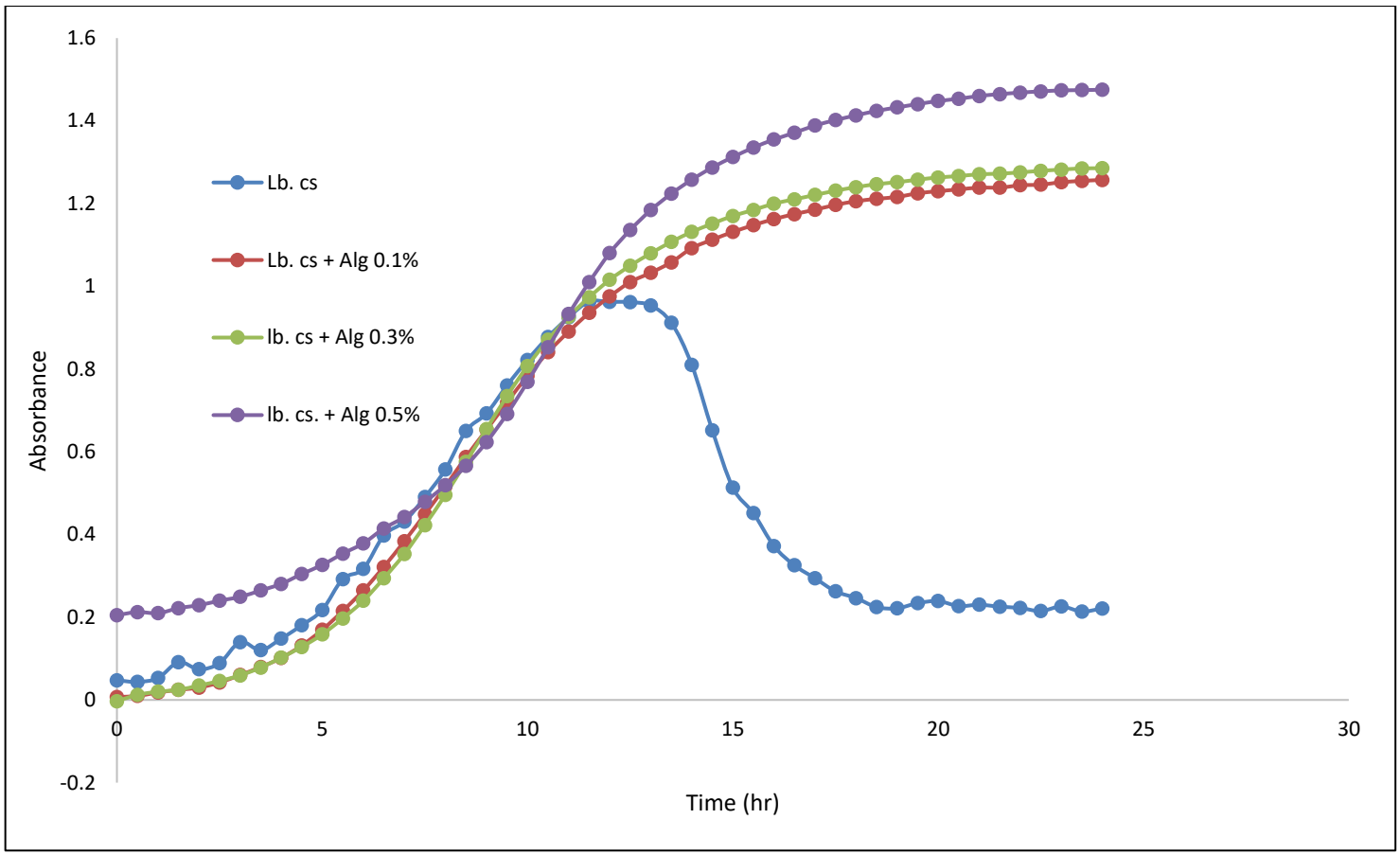


FIG. C18. SODIUM ALGINATE FROM ENZYME ASSISTED EXTRACTION/CONVENTIONAL CHEMICAL EXTRACTION (Alg-EAE/CCE) SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES

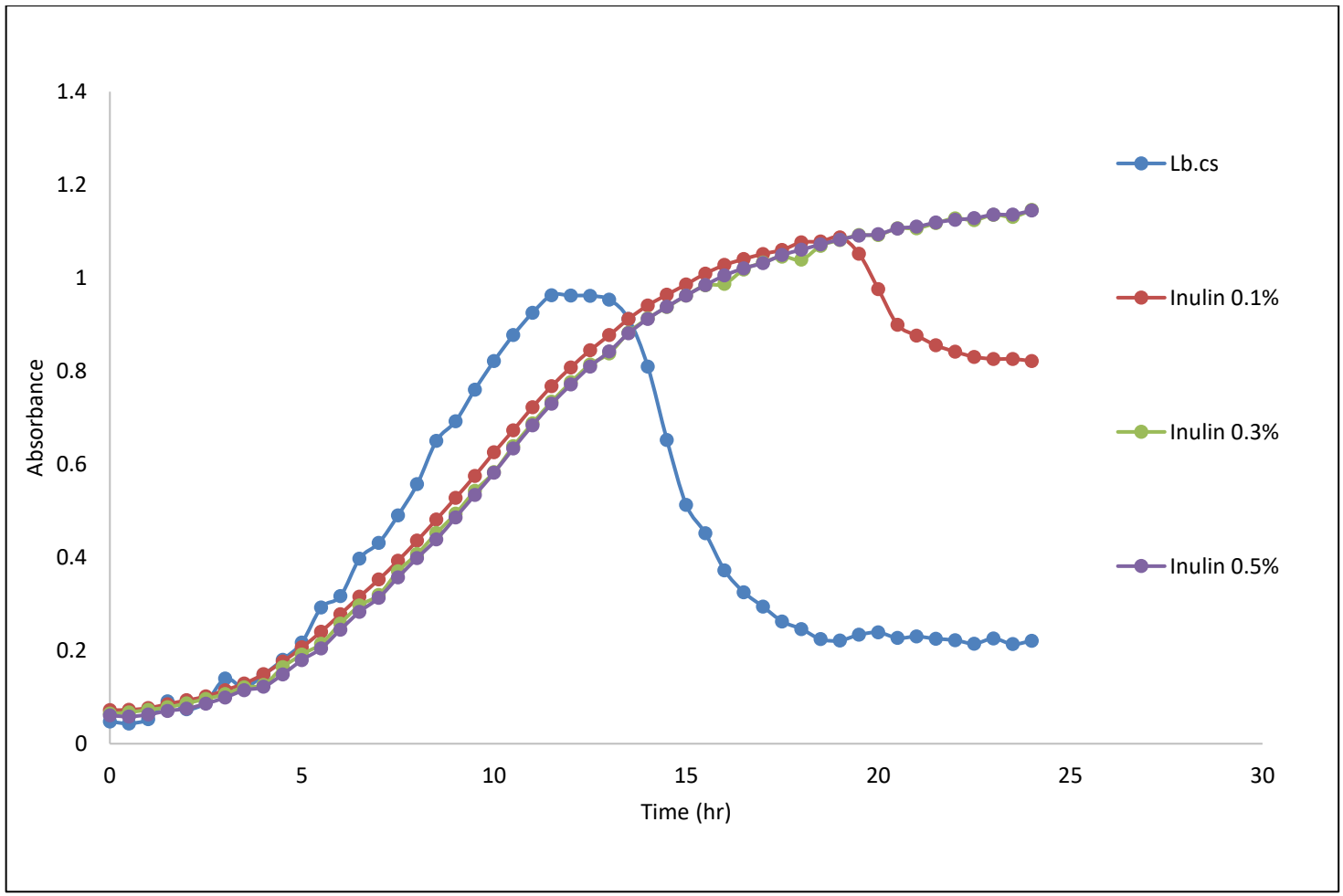


FIG. C19. INULIN SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES

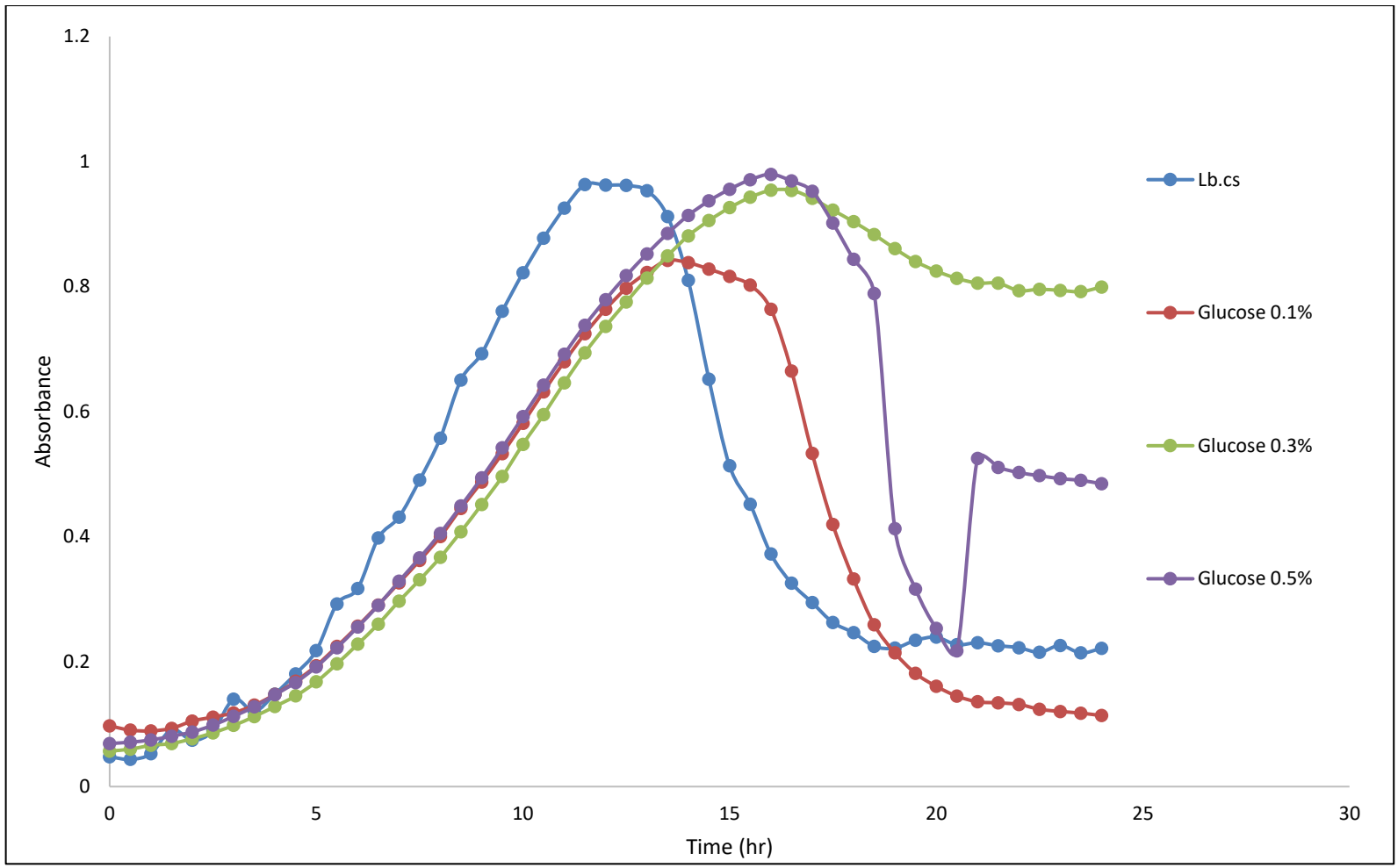


FIG. C20. GLUCOSE SUPPLEMENTED *Lactobacillus casei* GROWTH CURVES