Production and characterisation of yeast cell wall preparations with binding activity against *Salmonella* and *Escherichia coli*.

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Statement of original authorship

This thesis has not been submitted in whole or in part, to this or any other university, for any degree and is, except where otherwise stated the original work of the author.

Signed: _____

Steven Griffin

Date _____

Dedication

To Honor and Alexander

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Abstract

A rise in food safety and production standards (e.g. the ban on antibiotics) has led to a search for alternative natural products to achieve a high yielding, safe method of meat production. Mannan oligosaccharides (MOS) are one such class of product that can potentially provide this. Abundant in the cell wall of *Saccharomyces cerevisiae* as well as other yeast species, MOS have the capacity to inhibit type 1 fimbriae containing pathogenic bacteria from binding to host tissue and thus prevent infection.

Alterations in the growing conditions of several yeast strains, including *Saccharomyces*, *Kluyveromyces*, *Candida*, *Schizosaccharomyces*, *Pichia*, and *Rhodotorula* were examined to determine if by varying simple fermentation constituents it was possible to change yeast cell wall composition and increase its MOS content. The cell wall monosaccharide content (e.g. glucose and mannose) of a number of samples was significantly altered depending on the sole carbon source in the growth medium. However, the effect of carbon source on the cell wall monosaccharide content was strain specific.

A quantitative assay to assess the efficacy of MOS type products to bind bacteria *in vitro* was developed. Initially, agglutination of bacteria to MOS was observed on a microscope slide for a number of type 1 fimbriae containing strains of *Salmonella* and *Escherichia coli*. The method was transferred to a 96 well plate format and optimised. The binding activity assay was shown to be repeatable and reproducible for *Salmonella* and *E. coli* strains.

Yeast cell wall samples generated in this study were screened using the developed assay. *S. cerevisiae* 695, *K. lactis* 752, and *Schiz. pombe* 70572 cell wall samples had significantly higher binding activity than the control MOS used to develop the assay. The relationship between the yeast cell wall saccharide content and binding activity varied between strains indicating that the structure and not necessarily the quantity of the saccharides were more important in binding type 1 fimbriae containing bacteria. Analysis of the N-glycan profile of the samples with high binding activity revealed the presence of both common and unique mannan oligosaccharides. These unique mannan oligosaccharides may be responsible for the increased binding activity of these samples.

Abbreviations

2-AB	2-aminobenzamide
ABTS	2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ADG	Average daily gain
AGPs	Antibiotic growth promoters
Asn	Asparagine
ELISA	Enzyme linked immunosorbent assay
EU	European Union
FCR	Feed conversion ratio
GlcNAc	N-acetylglucosamine
GRAS	Generally regarded as safe
HILIC	Hydrophilic interaction chromatography
HPLC	High pressure liquid chromatography
Man-6-P	Mannose 6-phosphate
NSP	Non starch polysaccharides
SNPs	Single nucleotide polymorphisms
spp.	Species
UPLC	Ultra performance liquid chromatography
USDA	United States department of agriculture
USFDA	US food and drug administration
Var.	Serovar
w/v	Weight per volume
w/w	Weight per weight

YCWM	Yeast cell wall material
YP	Yeast extract (1 %, w/v) and peptone (2 %, w/v) medium

Section 1

Introduction

1. Introduction

1.1 Agricultural biotechnology

Just as society has evolved over time, our food system has also evolved over centuries into a global system of immense size and complexity. Contemporary food science and technology contributed greatly to the success of this modern food system by integrating many disciplines to solve difficult problems, such as resolving nutritional deficiencies, disease, and enhancing food safety. During the 2009 World Summit on Food Security, it was recognized that by 2050 food production must increase by about 70 % to feed the anticipated 9 billion global population, 34 % higher than the population of 2009 (FAO, 2009a). This projected population increase is expected to involve an additional annual consumption of nearly 1 billion metric tons of cereals for food and feed and 200 million metric tons of meat. As a consequence, science based improvements in agricultural production, food science and technology, and food distribution systems are critically important in decreasing this gap. Further advancements are needed to resolve the challenges of sustainably feeding the growing future population (Floros et al., 2010).

Livestock production is a dynamic and integral part of the food system today, contributing 40 % of the global value of agricultural output, 15 % of total food energy, and 25 % of dietary protein and supporting the livelihoods and food security of almost a billion people (Floros et al., 2010, FAO, 2009c). The current livestock sector has shifted from pasture-based ruminant species (cattle, sheep, goats) to feed dependent monogastric species (swine, poultry) and is marked by intensification and increasing globalization (Floros et al., 2010). The rapidly increasing demand for meat and dairy products has led, during the past 50 years, to an approximate 1.5 fold increase in the global numbers of cattle, sheep, and goats, 2.5 fold increase in pigs, and 4.5 fold increase in chickens (Godfray et al., 2010). The rates of conversion of grains to meat, milk, and eggs from food animals have improved significantly globally (Floros et al., 2010). In addition to these productivity gains, bird health, and product quality and safety have improved through applications of breeding, feeding, disease control, housing, and processing technologies (Floros et al., 2010, FAO, 2009c). Poultry meat can be produced in a very efficient way which makes it one of the lowest cost animal

proteins with a low environmental footprint. In addition, poultry does not carry any religious restrictions and is consumed almost all over the world.

1.1.1 Poultry industry

The poultry sector continues to grow and industrialize in many parts of the world, poultry meat will soon supplant pork as the world's most eaten meat (AVEC, 2014). An increasing population, greater purchasing power and urbanization have been strong drivers of growth (FAO, 2014). Poultry constitutes many different domesticated species of bird that are subdivided into different types mainly broilers, breeders, and layers, depending on their purpose. The domestic chicken, *Gallus gallus domesticus*, dominates the poultry market accounting for over 85 % of all poultry meat produced worldwide with a global population exceeding 21 billion birds per year (FAO, 2015, www.thepoultrysite.com, 2013, Muir et al., 2008) and comprise an economically valuable global protein industry (Oakley et al., 2014). Turkeys are second to chickens in the poultry market. Advances in breeding have given rise to birds that meet specialized purposes and are increasingly productive, but need expert management (FAO, 2014, Wolc, 2014). Broilers form the largest group of poultry farmed commercially. These birds have been genetically selected for rapid growth, efficient feed conversion and greater production of meat. The aim of a breeder is to maximise reproductive performance in terms of egg numbers and hatchability, in a bird whose genetic background is strongly slanted towards meat production. Layers are birds that are selected for increased egg production with egg quality being of primary importance. The main goals of commercial poultry producers worldwide are in achieving the genetic potential for growth, maintaining health, and food safety at the lowest input costs (Pattison et al., 2008).

The poultry industry has grown remarkably in the last 50 years and the production of poultry meat is expanding to meet higher demand for low cost, healthy, and convenient products. In 2012, world poultry output looked likely to approach 91 million tonnes and 93 million tonnes by 2013. In the following years to 2020, projections point to growth of around two percent a year for both Europe and the world (www.thepoultrysite.com, 2012a). Growth in meat consumption will be driven by consumer preferences together with disposable income and population growth. Since the early 1960s, broiler growth

rates have doubled and feed conversion ratios have halved (Floros et al., 2010). Modern broilers weigh about 2.5 kg at 39 days, with a feed conversion ratio of 1.6 kg of feed per kilogram of bodyweight gain. Modern commercial layers typically produce about 330 eggs per year with a feed conversion ratio of 2 kg of feed per kilogram of eggs produced. Poultry meat and eggs have food nutritional values with high amounts of protein and low fat content. As a consequence the price for consumers is reasonable which is important for meeting the challenge of feeding the growing world population (AVEC, 2014).

Since the 1990s, the production of poultry meat in low and middle income countries has increased, with chicken meat accounting for 80 percent, and duck and goose meat production also increasing. China and Brazil, in particular, have emerged as major poultry meat producers (Figure 1.1). Meanwhile North American and European producers have lost their global market shares. Over the last 30 years, egg production has also increased enormously in East and Southeast Asia. In 2007, about 45 % of the eggs consumed worldwide were produced in China (www.thepoultrysite.com, 2012a).

World poultrymeat production

Region	2000	2005	2006	2007	2008	2009	2010	2011E	2012F
Africa	3.0	3.6	3.6	4.0	4.2	4.4	4.6	4.8	4.9
Americas	30.1	35.9	37.0	38.9	41.1	40.1	41.8	42.8	43.0
Asia	22.9	27.3	28.3	30.1	31.8	32.9	34.5	36.1	37.4
Europe	11.9	13.2	13.1	14.0	14.4	15.7	16.1	16.6	16.9
Oceania	0.8	1.0	1.0	1.0	1.0	1.0	1.1	1.3	1.3
WORLD	**68.6**	**80.9**	**83.0**	**87.9**	**92.5**	**94.2**	**98.1**	**101.6**	**103.5**

World chickenmeat production (million tonnes)

Region	2000	2005	2006	2007	2008	2009	2010	2011E	2012F
Africa	2.8	3.4	3.4	3.7	4.0	4.2	4.4	4.6	4.7
Americas	27.2	32.7	33.7	35.3	37.4	36.7	38.4	39.2	39.4
Asia	18.7	22.5	23.5	24.9	26.4	27.2	28.6	29.9	31.0
Europe	9.4	10.7	10.8	11.7	12.1	13.4	13.8	14.2	14.5
Oceania	0.7	0.9	1.0	1.0	1.0	1.0	1.1	1.3	1.3
WORLD	**58.7**	**70.2**	**72.3**	**76.7**	**80.8**	**82.5**	**86.2**	**89.2**	**90.9**

E=estimate; F=forecast

Figure 1.1. World poultry meat and chicken meat production by region (million tonnes).

Source: (www.thepoultrysite.com, 2012b)

1.1.2 Pork Industry

Pork is the most commonly consumed meat in the world (Baer et al., 2013) and is the most popular meat in East and South East Asia. In 2014, swine production in China was projected to reach 723 million head, that's more than twice the U.S. population and half the Chinese population (Brasch, 2014). In China, consumption of pork is close to saturation levels which will benefit alternative sources of proteins, such as poultry. The larger consumers of poultry meat per capita (Malaysia, Israel and Saudi Arabia), are located in countries where pork is not eaten due to religious restrictions (AVEC, 2014).

In the United States, per capita consumption of pork has remained steady over the past 20 years at about 27 kg/year and in the European Union (EU) people eat more pork than any other meat (Baer et al., 2013). With 150 million pigs and a yearly production of about 22 million tonnes carcass weight the EU is the world's second biggest producer of pigmeat after China and also the biggest exporter of pigmeat and pigmeat products. The EU's main producer countries are Germany, Spain and France. Together, they represent half of the EU's total slaughter. The EU has a self sufficiency of about 111 % and exports about 13 % of its total production. Most of the EU's pigmeat exports go to East Asia, in particular China (Commission, 2015).

According to the US department of agriculture (USDA) in January 2012, the United States exported over 501 million pounds of pork (Baer et al., 2013). The United States accounts for 10 % of global pork production, making it the world's third largest pork producer, behind China (46 %) and the EU (24 %) (Lowe and Gereffi, 2008). As the world's third largest pork producer, and second largest consumer, importer and exporter of pork and pork products, the United States had a 2007 inventory of some 67 million hogs. More than 80 % of this inventory was on large farms containing at least 2,000 animals. Between 1985 and 2007 the live weight of pigs in the US has increased by 14 kg and the herd feed conversion has decreased by 0.57 units, respectively (Campbell, 2008).

In a 2008 report by www.safefood.eu more pork meat per capita was consumed on the island of Ireland than any other meats including poultry, beef and mutton/lamb. However, greater than 50 % of pork meat consumed is in a processed form. Eighty-five percent of people on the island consume pork and pork products with consumers in Northern Ireland consuming slightly more than those in the Republic of Ireland. In 2007

in the Republic of Ireland, pigmeat production was valued at €290 (£198) million at farm gate representing five percent of the grass agricultural output and making it the third most important sector in agriculture output after beef and milk.

1.2 Challenges facing the poultry and swine industries

The many challenges facing the animal production industry include rapid human population expansion, increasing demand for limited resources, and animal and human health concerns. The United Nations (UN) has estimated the current world population at 7.2 billion which is projected to increase by almost one billion people within the next twelve years, reaching 8.1 billion in 2025 and 9.6 billion in 2050 (UN, 2014), where 70 % of the population are expected to be urban with an increased income (FAO, 2009a). A challenge to the animal production industry is to be able to meet this increase in demand. The Food and Agriculture Organisation (FAO) in the United States suggests an increase in global food production of 70 % will be necessary to meet these population and income projections (FAO, 2009b).

With the increase in demand for animal protein and the decrease in the quantity of world cereal crops being used for feed, a further challenge to the industry is to have a highly nutritive diet with little wasted energy and improved feed conversion efficiencies. The use of cereals for non-food purposes grew at treble the rate of their use for food purposes between 2000 and 2007 (McLeod et al., 2009). This shift of feed to the fuel production industry left lower quality feed for the animal feed industry. This in turn has led to nutritional problems and challenges in feed conversion efficiencies that need to be addressed.

Disease and animal welfare are a major concern in all sectors involved with animal production. Loss of animal production and reproduction or even a deterioration of health, i.e. “going back”, due to illness both clinical and subclinical, are of major economic importance to the global animal production industry. A decrease in feed efficiencies reduces the margins for financial return on the animal. In addition, the cost of sick animals through medication, vet bills, housing, feed loss, recovery period downtime, loss of product/revenue, and cost of lost time when an animal is ill, is undesirable. The larger the herd/flock the greater the economic impact as disease can

spread throughout a flock or herd relatively quickly. A 2007 analysis conducted by the University of Georgia, United States, calculated that the gross value of production (GVP) of the United States poultry industry in 2005 was US$28.2 billion, and disease losses were 8.2 % of this. The study also showed that for each US$1,000 loss due to mortalities, another US$2,000 is lost elsewhere owing to depressed productivity resulting from disease (FAO, 2013). Pathogenic bacteria can remain undetected at both subclinical levels and high numbers within the digestive tract. In poultry, this type of subclinical infection can result in reduced weight gains of the birds and leads to significant economic losses to the farmers. Reduced supply can drive up the cost of meat for the consumer and the increased load of pathogenic bacteria can lead to increased incidences of zoonotic infection (Santos, 2006). Recently, supermarkets are willing to pay more for healthier/disease free products. This reflects the marketplace's attitude to healthier food and food products and a general concern for food safety with an understanding of the risks of contaminated foods (Baer et al., 2013). Food related disease outbreaks have generated much media attention in recent years increasing consumer awareness.

Zoonotic disease transmission is a continuing challenge to the industry. Microbiological hazards carried primarily by healthy animals have been identified as causing the majority of foodborne risks to human health. These microbes include: *Salmonella enteritidis*, *Campylobacter jejuni*, *Escherichia coli*, *Shigella*, *Cryptosporidium*, *Clostridium perfringens*, *Yersinia enterocolitica*, and *Listeria monocytogenes*, with salmonellosis and campylobacteriosis being the most frequently transmitted zoonotic agents from poultry meat and eggs to humans (Turantas et al., 2015). Avian influenza is a further zoonotic virus that poses a threat to the industry. In regions where the virus has been reported, large decreases in poultry meat consumption were seen in affected countries (ERSA, 2007). Porcine circovirus 2 (PCV2) is recognized as an endemic infection of swine, worldwide. In less than 2 decades, this virus has become one of the most economically important infectious agents in modern swine production. PCV2 has been effectively controlled with conventional vaccines to date (Ellis, 2014). The impact that disease has on the industry is one of its major limiting factors for optimal performance and has resulted in an increase in biosecurity awareness, implementation, and management within the sector. Consumers have also become increasingly

concerned about foodborne illness and infections and the control and possible elimination of this threat is a goal of the poultry industry (Santos, 2006).

Antibiotics have traditionally been used in both poultry and pork feed at sub therapeutic levels to combat sub clinical disease and improve growth (McWilliams, 2013, Zhu et al., 2013, Smith et al., 2002). An increase in food safety concerns resulting from extensive antibiotic use has seen the poultry industry challenged further as safer meat free from antibiotics and disease is a necessity within the EU. Consumer demand for antibiotic free meat has also increased within the United States and other developed countries due to concerns with antibiotic resistance, making it necessary for poultry producers to find suitable alternatives to antibiotics as growth promoters (Sofos, 2008). In the United States, legislation has been introduced that seeks to restrict the use of certain antimicrobial drugs for subtherapeutic or nontherapeutic purposes in food producing animals, such as the cephalosporin class of antimicrobial drugs. In the 112th Congress, the leading bills were the Preservation of Antibiotics for Medical Treatment Act of 2011 (PAMTA, H.R. 965, S. 1211). Most U.S. livestock and poultry producers are opposed to such restrictions because of concerns about animal welfare and food safety, as well as concerns about possible increases in production costs, among other reasons (Johnson, 2011).

1.2.1 Zoonoses associated with animal production

Zoonoses are diseases or infections, which are transmissible from animals, both wild and domestic, to humans. Zoonotic disease can be caused by bacteria, viruses, fungi and parasites and can be transmitted to humans in different ways. These diseases can be acquired directly from animals but are most often acquired through ingestion of contaminated foods (Newell et al., 2010, Norrung and Buncic, 2008). Zoonotic diseases cause losses in goods produced (live animals, milk, meat, eggs, wool) and disability or loss of human life (Narrod et al., 2012). Foodborne pathogens are a major contributor to human illnesses, hospitalizations, and deaths each year. Consumption of food contaminated with zoonotic agents or their toxins is a serious threat for public health. In developed countries, it is estimated that up to one third of the population is affected by microbial foodborne diseases each year (Van Immerseel et al., 2009). The Centre for Disease Control and Prevention (CDC) estimates that approximately 47.8 million

illnesses and 3,000 deaths are caused by foodborne pathogens each year (Baer et al., 2013). Using data from outbreak-associated illnesses for 1998–2008, annual US foodborne illnesses, hospitalizations, and deaths were attributable to each of 17 food commodities: 3 for aquatic animals (fish, crustaceans, and mollusks), 6 for land animals (dairy, eggs, beef, game, pork, and poultry), and 8 for plants (grains/beans; oils-sugars [refined plant foods]; fruits-nuts; fungi; and leafy, root, sprout, and vine-stalk vegetables). Forty six percent of illnesses were linked to food produce and more deaths were attributed to poultry than to any other food commodity (Painter et al., 2013).

Data on foodborne diseases in low income countries are scarce. There is no precise and consistent global information about the full extent of the occurrence of food poisoning and the costs related to unsafe food. Symptoms are often mild and cases are not reported, but their importance is thought to be substantial. Children and people in stress situations, such as those facing malnutrition, war or natural disasters, are especially at risk of foodborne bacterial diseases. The main symptom is diarrhoea, and infection can be fatal (when compared with 0.01 % mortality in infected people in high-income countries). As the causal agent is a bacterium, these diseases can be treated by antibiotics, but access to treatment is difficult in many low-income countries. Another problem is the development of resistance to antibiotics among zoonotic bacteria. The pattern of foodborne disease outbreaks has changed during the last two decades. In the past, most outbreaks were acute and localized, and resulted from a high level of contamination. Now, more outbreaks affect several countries at once, resulting from low level contamination of widely distributed commercial food products. Risks of the contamination of poultry products by residues and bacteria exist everywhere, owing to the globalization of poultry production and trade (FAO, 2013). The economic burden of zoonosis is significant. According to the Institute of Medicine, zoonotic pathogens caused more than 65 % of emerging infectious disease events in the past six decades. The direct cost of zoonotic diseases over the decade up to 2012 has been estimated to be more than $20 billion with over $200 billion in direct losses to affected economies as a whole (Narrod et al., 2012).

Viruses such as Newcastle disease virus and Influenza A virus are the most commonly spread viruses from poultry to humans and are typically resultant from direct contact with birds but may also be spread by the ingestion of contaminated foodstuffs (Pattison et al., 2008). Fungal zoonoses associated with poultry include histoplasmosis and they

are mainly spread through contact with infected faeces (Pattison et al., 2008, Saif and Fadly, 2008). Cryptosporidiosis is a parasite that infects poultry and is also transmissible to humans. The protozoa are shed in faeces and can contaminate water and other sources, where it can be transmitted to humans causing watery non bloody diarrhoea in immuno-competent individuals; while it can cause chronic persistent gastroenteritis in the immuno-compromised. In 2007, 609 cases of cryptosporidiosis were notified in Ireland, a crude incidence rate of 14.4 per 100,000 population, this was the largest annual number of cases notified since human cryptosporidiosis became notifiable in Ireland in 2004 (Pattison et al., 2008, Saif and Fadly, 2008, Garvey and McKeown, 2007).

Bacterial zoonotic agents of poultry origin are the ones that are of most concern to the poultry industry, humans and economists. It is well recognized that pathogens, such as *Campylobacter* and *Salmonella* spp. can be transmitted along the food chain and can be the source of human illness (Gaggia et al., 2010). While *E. coli* is another important zoonotic pathogen it is not as prevalent. *Campylobacte*r and non typhoidal *Salmonella* are the key causes of foodborne illness of bacterial origin, both in the US and in the EU (Van Immerseel et al., 2009). *Salmonella* are major zoonotic pathogens causing over 1 billion cases of infection worldwide each year. Contaminated poultry meat and eggs are important vehicles of infection and there are approximately 100, 000 cases of *Salmonella* infection in the UK every year (Humphrey, 2006). Flock health, the structure of the poultry food chain (short or chilled), the quality of control procedures during production and supply processes and on the final product – all contribute to the marketing of safe poultry meat and eggs (FAO, 2013).

Although pork is less associated with foodborne illness than other meat sources, it remains significant due to its large consumption in a variety of products. As pork is so widely consumed and is an important U.S. export, ensuring a safe pork supply is crucial. Furthermore, heightened consumer awareness of food safety makes the reduction of foodborne pathogens from pork important for producers and processors (Baer et al., 2013). While pigs can be infected with many different serovars, clinical disease is caused mainly by *Salmonella choleraesuis*, *Salmonella typhisuis*, and *Salmonella typhimurium* (Poljak, 2009). Because *Salmonella* resides in the intestinal tract of swine, shedding of the bacteria by asymptomatic carriers on the farm is

inevitable and is the major route for *Salmonella* infection in other animals (Baer et al., 2013).

A recent report compiled by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) presented the results of the zoonoses monitoring activities carried out in 2013 in 32 European countries found that campylobacteriosis was the most commonly reported zoonosis (Table 1.1). A decreasing trend in confirmed salmonellosis cases in humans was observed. The number of confirmed verocytotoxigenic *Escherichia coli* (VTEC) infections in humans increased. The sources of VTEC infections was reported to come from food and animal sources (ECDC, 2015). Although various foods can serve as sources of foodborne illness, meat and meat products are important sources of human infections with *Salmonella* spp., *Campylobacter jejuni*/*coli*, *Yersinia enterocolitica*, VTEC and, to some extent, *L. monocytogenes* (Norrung and Buncic, 2008). Most foodborne outbreaks were caused by *Salmonella*, followed by viruses, bacterial toxins and *Campylobacter*, whereas in 28.9 % of all outbreaks the causative agent was unknown. Important food vehicles in foodborne outbreaks were eggs and egg products, followed by mixed food, and fish and fish products (ECDC, 2015).

Table 1.1 Reported hospitalisation and case-fatality rates due to zoonoses in confirmed human cases in the EU, 2013.

Disease	Number of confirmed[a] human cases	Hospitalisation				Deaths			
		Confirmed cases covered[a],[b] (%)	Number of reporting MS[c]	Reported hospitalised cases	Hospitalisation rate (%)	Confirmed cases covered[a],[b] (%)	Number of reporting MS[c]	Reported deaths	Case-fatality rate (%)
Campylobacteriosis	214,779	12.7	13	11,922	43.6	52.9	14	56	0.05
Salmonellosis	82,694	26.4	12	7,841	36.0	49.6	14	59	0.14
Yersiniosis	6,471	15.3	12	481	48.4	62.4	14	2	0.05
VTEC infections	6,043	41.1	16	922	37.1	59.3	18	13	0.36
Listeriosis	1,763	42.1	15	735	99.1	69.7	19	191	15.6
Echinococcosis	794	22.7	12	127	70.6	28.5	13	2	0.88
Q fever	648	NA	NA	NA	NA	51.2	11	2	0.61
Brucellosis	357	55.2	9	139	70.6	28.3	11	1	0.99
Tularaemia	279	26.9	8	39	52.0	46.2	9	0	0
West Nile fever[a]	250	20.8	3	52	91.7	90.8	6	16	3.4
Trichinellosis	217	74.7	7	106	65.4	82.5	8	1	0.56
Rabies	1	100	1	1	100	100	1	1	100

NA: not applicable as the information is not collected for this disease.
(a): For West Nile fever the total number of cases were included.
(b): The proportion (%) of confirmed cases for which the information on hospitalisation or death was available.
(c): Not all countries observed cases for all diseases.

Source: (ECDC, 2015)

1.2.2 *Salmonella*

Salmonella are Gram-negative, facultatively anaerobic, non spore forming, type 1 fimbriated, rod shaped organisms of the family Enterobacteriaceae and are readily found in the gastrointestinal (GI) tract of humans and animals. The *Salmonella* spp. is divided into more than 2,500 serovars. Salmonlellosis is an infection caused by *Salmonella* and is one of the most common causes of food borne diarrheal disease worldwide (Wegener et al., 2003). The main reservoir of zoonotic *Salmonella* is food animals, and the main sources of infections in industrialized countries are animal derived products (Wegener et al., 2003). Egg and egg products are the largest source of salmonellosis in humans followed by poultry and pork meat at 11 %, 2.7 % and 1.5 % respectively, of all cases reported (Van Immerseel et al., 2009, Norrung and Buncic, 2008).

Human salmonellosis is usually characterised by prolonged fever, diarrhoea, abdominal pain, nausea, stomach pain, spleen enlargement, rash, and severe headache (Niskanen et al., 2015). The majority of human cases are caused by only a few non typhoidal serovars. Approximately 60 % of human cases reported to the CDC in 1995 were caused by four serovars, including *Salmonella enteritidis* (24.7%), *Salmonella typhimurium* (23.5%), *Salmonella newport* (6.2%) and *Salmonella heidelberg* (Rabsch et al., 2001). The incidence of food borne human infections caused by *Salmonella enteritidis* and by multi-drug-resistant strains of *Salmonella typhimurium* increased substantially during the second half of the 20th century in both the US and Europe (Rabsch et al., 2001). In 2013, a total of 82,694 confirmed salmonellosis cases were reported by 27 EU member states, resulting in an EU notification rate of 20.4 cases per 100,000 of the population. The two most commonly reported *Salmonella* serotypes in 2013 were *Salmonella enteritidis* and *Salmonella typhimurium*, representing 39.5 % and 20.2 %, respectively. *Salmonella* was most frequently detected in poultry meat, and less often in pig or bovine meat. *Salmonella* findings were also reported in other animal species, including ducks, geese, sheep and goats (ECDC, 2015). From fowl, *Salmonella infantis* was the most commonly reported isolated serovar in 2013; in broiler meat the most common serotypes were *Salmonella infantis* and *Salmonella enteritidis*, while from feed for fowl, *Salmonella senftenberg* was most commonly reported, followed by *Salmonella typhimurium*. In turkey meat the three most common reported serotypes were *Salmonella derby*, *Salmonella typhimurium* and *Salmonella stanley*. *Salmonella typhimurium* was the most frequently reported serotypes in pigs and pigmeat. In cattle,

Salmonella typhimurium was the most commonly reported, followed by *Salmonella dublin*. Also in bovine meat, *Salmonella typhimurium* were the most frequently reported serotypes, followed by *Salmonella enteritidis* (ECDC, 2015).

A number of these serovars can replicate readily within the host organism however they are non host specific in poultry, normally causing asymptomatic intestinal infections which are therefore difficult to diagnose and treat. Acute outbreaks exhibiting clinical disease along with high levels of mortality may occur in chicks younger than two weeks old. Infection, usually by the faecal oral route, of newly hatched chicks results in rapid and massive multiplication within the alimentary tract due to the absence of competing microflora. Thusresulting in extensive faecal shedding for a number of weeks and cross contamination within the house or hatchery (Pattison et al., 2008). An important means of preventing human salmonellosis is by preventing infection of poultry (Porter, 1998). This strategy can also be applied to the pork industry.

Clinical infection causes diarrhoea in birds along with depression, reduced feed intake, weight loss and possible visual impairment. Subclinical infection may cause an inflammatory response in the intestine with tissue damage and possibly result in systemic infection. Humans, rodents, wild birds, insects, water and feed can all introduce infection into a poultry unit and *Salmonella* spp. can spread from unit to unit through vehicles and equipment such as egg trays and trolleys. The infection cycle can also occur via eggs at the time of hatch by the faecal contamination of egg surfaces when passing through the cloaca. Avian systemic salmonellosis causes considerable worldwide economic loss both through chicken mortality and through loss of productivity (Parsons et al., 2015). The most common disease causing *Salmonella* serotypes in poultry are *Salmonella gallinarum* and *Salmonella pullorum*. Under the new naming system the *Salmonella enterica* serotypes *gallinarum* is divided into biovar *gallinarum* and *pullorum*; these are non motile, host specific salmonellae which are identified to cause fowl typhoid and pullorum disease in poultry respectively, but are not important causes of infection in humans (Cheraghchi et al., 2014, Pattison et al., 2008). These diseases are characterized by anorexia, diarrhoea, dehydration, anaemia and up to 50 % mortality of flocks. Symptoms also include decreased laying and hatchability and high chick mortality (Van Immerseel et al., 2013). Among diseases of poultry, salmonellosis is of great concern and has been responsible for serious economic

losses of poultry through mortality and loss of production (Parsons et al., 2015, Akiba et al., 2011, Kabir, 2010).

1.2.3 *Escherichia coli*

Escherichia coli are Gram-negative bacteria that are a normal constituent of the intestinal tract microbiota of humans and warm blooded animals (Andersson et al., 2012). The niche of commensal *E. coli* is the mucous layer of the mammalian colon (Kaper et al., 2004). They are facultatively anaerobic, flagellated, type 1 fimbriated, and rod shaped bacteria (Pattison et al., 2008). Certain strains are also important disease causing pathogens of both humans and poultry. Pathogenic *E. coli* strains possess specific adherence factors or lectins that allow them to colonize sites that *E. coli* does not normally inhabit, such as the small intestine and the urethra (Kaper et al., 2004). An individual bacterium may co-express more than one lectin e.g. certain strains *of E. coli* are both mannose and galabiose specific (Sharon, 2006). Some strains of *E. coli, e.g.*, O157:H7, are acid tolerant, which permits them to pass through the stomach without being killed (Saif and Fadly, 2008). Many different serotypes of *E. coli* exist and three types of antigens, the O (lippopolysaccharide, LPS), K (capsular), and H (flagellar) antigens are used to identify them. A serogroup is identified by the O antigen only, a serotype by both the O and H (Kaper et al., 2004).

Pathogenic *E. coli* strains use a multi step scheme of pathogenesis that is similar to that used by other mucosal pathogens, which consists of colonization of a mucosal site, evasion of host defences, multiplication and host damage (Kaper et al., 2004). In Europe, antimicrobial resistance in Gram-negative bacteria is on the rise, particularly in *E. coli*, which constitutes a majority of invasive Gram negative isolates in European countries. The emergence and diffusion of multi drug resistant strains of *E. coli* is complicating the treatment of several serious infections. Enterobacteriaceae, particularly *E. coli*, are the most frequent cause of hospital and community acquired infections (Allocati et al., 2013). Pathogenic *E. coli* are characterised based on virulence properties, mechanisms of pathogenicity, clinical symptoms and serology (Mora et al., 2009). Three general clinical syndromes can result from infection with one of the pathogenic strains of *E. coli* pathotypes: enteric/diarrhoeal disease, urinary tract infections (UTIs) and sepsis/meningitis. Among the enteric pathogens there are six well

described categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) or Shigga toxin producing *E. coli* (STEC) or verocytotoxiginic producing *E. coli* (VTEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). UTIs are the most common extraintestinal *E. coli* infections and are caused by uropathogenic *E. coli* (UPEC). An increasingly common cause of extraintestinal infections is the pathotype responsible for meningitis and sepsis, meningitis associated *E. coli* (MNEC) (Kaper et al., 2004). An additional animal pathotype, known as avian pathogenic *E. coli* (APEC), causes extraintestinal infections, primarily respiratory infections, pericarditis, and septicaemia of poultry (Mora et al., 2009, Kaper et al., 2004).

Some pathogenic strains of ETEC cause diarrheal disease with severe symptoms and dehydration, leading to annual mortality of hundreds of thousands of children below the age of five in developing countries (Andersson et al., 2012). *E. coli* O157:H7 is probably the most common source of VTEC infection (Niskanen et al., 2015), is widely known regarding foodborne illness in humans and is transmitted by infected meat and unwashed fruit and vegetables. A report published by the European centre for disease control estimated that the incidences of VTEC in the EU/EAA increased significantly during 2008-2012. The highest number of cases was reported in 2011 due to a large outbreak of VTEC O104:H4 occurring in Germany. Eighty six percent of the confirmed VTEC cases reported in 2010-11 were of domestic origin. The five most common VTEC ‘O’ serogroups (55 % of known data) in 2010-12 were: O157:H7 (26 %), O104 (24 %), O26 (10 %), O103 (3.7 %) and O91 (3.0 %) (Niskanen et al., 2015). During 2010-12, a total of 18,955 confirmed cases of VTEC infections were reported by 26 EU member states and 2 European Economic Area (EAA) countries. The number of confirmed cases of VTEC has increased significantly in the EU since 2008 (Niskanen et al., 2015). In 2013, 6,043 confirmed cases of VTEC infections were reported in the EU. The EU notification rate was 1.59 cases per 100,000 of the population, which was 5.9 % higher than in 2012. The most commonly reported VTEC serogroup in 2013 was, as in previous years, O157 (48.9 % of cases with known serogroup). VTEC serogroup O157 was primarily detected in ruminants (cattle, sheep and goats) and meat thereof (ECDC, 2015).

Among extraintestinal pathogenic *E. coli* (ExPEC) strains, the O1 serogroup is one of the most commonly detected in APEC, UPEC, MNEC and septicemic *E. coli* strains.

APEC strains are responsible for avian colibacillosis in domesticated and wild birds, an illness which starts as a respiratory tract infection and evolves into a systemic infection of internal organs (Mora et al., 2009). Avian colibacillosis is considered one of the principal causes of morbidity and mortality, associated with heavy economic losses to the poultry industry by its association with various disease conditions, either as primary pathogen or as a secondary pathogen (Kabir, 2010). Most of the pathogenic *E. coli* belongs to a small range of serogroups that includes O1:K1, O2:K1 and O78:K80. The O1 carbohydrate capsule has been identified as an important virulence determinant that inhibits phagocytosis (Pattison et al., 2008).

1.2.4 Campylobacterosis

Campylobacter spp. are rod shaped, Gram negative, capsulated, flagellated, microaerophilic organisms and are readily found in the environment and the avian gastrointestinal tract (Evans and Powell, 2008). Symptoms of campylobacterosis in humans include bloody diarrhoea, abdominal pain, fever, headache and nausea. Undercooked meat (poultry, cattle, pigs, and sheep), untreated drinking water and unpasteurised milk have all been recognised as major sources of human infection (Ingresa-Capaccioni et al., 2015, WHO, 2011). *Campylobacter jejuni* and *Campylobacter coli* are the most common species causing foodborne illness. *Campylobacter* spp. infection in broilers usually takes place after three weeks post hatch and infection spreads rapidly throughout the flock. This high rate of colonisation leads to a high degree of carcass contamination at processing plants through spillage of intestinal contents, thus increasing the incidence of campylobacterosis (Sahin et al., 2002). The main site of campylobacter infection in chickens is in the lower intestine particularly the caecum and the cloaca (Beery et al., 1988). Infected birds show few or no signs of clinical illness making the infection hard to treat and therefore difficult to control (Evans and Powell, 2008).

1.3 Pathogen control strategies for reducing zoonoses

In an effort to control zoonotic disease the main strategy is not only to reduce the risk of infection and the bacterial load at the farm level but also to control the spread of disease

causing bacteria from this point throughout the production chain. The use of management processes such as the 'hazard analysis critical control point' (HACCP) approach identifies critical points in the control and prevention of the spread of disease (Unnevehr and Jensen, 1996). Increased biosecurity standards at the farm level can help eliminate the intake of bacterial pathogens. This is achieved by controlling the amount of pathogen carriers coming into contact with the flock/herd, i.e. wild animals, infected members of the flock/herd, as well as maintaining clean sterile bedding, fresh water, and the identification and removal of contaminated feeds. This is difficult as subclinical amounts of bacteria are challenging to detect. Treatment of feed by thermal processing or pelleting can help reduce infection at this level.

The addition of feed additives such as antibiotics, vaccines, organic acids, in-feed enzymes, and/or pre- and probiotics, to the feed is a highly effective way of reducing the bacterial load and decreasing the spread of disease (Sofos and Geornaras, 2010, Norrung and Buncic, 2008). The correct application of these controlling procedures can reduce the burden of zoonoses to the farmer, the consumer and the economy. The most economical and efficient method of preventing contamination is at the farm level through feed supplementation; antibiotic use in feed historically being the most prevalent and successful.

1.3.1 Biosecurity

This is defined as the prevention or reduction of the spread of microbial disease prior to detection. A collection of rules and procedures exist that minimize exposure (security) of a susceptible population to an infectious (biological) agent (Cox, 2005). Exclusion of enteropathogens at the early stages of stock production among elite flocks prevents widespread dissemination. There are numerous codes of practices, standards and guidelines published in many poultry producing nations that underline the importance of biosecurity. At all levels of flock management, ingress of any carrier, including wild birds, mice and rats, insects, as well as humans, should be minimized, as all are potential sources of enteric pathogens (Arsenault et al., 2007). Housing must be designed to prevent entry of any carrier, and pest-control measures such as traps and baits should be used. While human access is necessary, sanitation and hygiene measures such as footbaths should be employed. At the elite flock levels, measures such as

change-in–change-out (farm or shed based apparel) or even shower-in–shower-out (disinfection shower prior to and post-entry to farm) may be used. These precautions are also used for grandparent and parent stock, and some countries, such as Sweden, extend such practices to broiler production (Sternberg Lewerin et al., 2005). Movement of animals also includes the stock; in meat production, stock is usually populated and depopulated on an all-in–all-out basis (Forshell and Wierup, 2006). This approach minimizes the likelihood of cross-contamination between flocks as multi-age stocking increases the risk of colonization in one flock being passed to others (Cox and Pavic, 2010).

1.3.2 Antibiotics

Throughout much of the twentieth century, antibiotics have been a primary defence against bacterial disease. The utilisation of antimicrobial drugs has played an important role in animal husbandry; they have been used in prophylaxis, treatment and growth promotion (Jassim and Limoges, 2014, Page and Gautier, 2012, Dias de Oliveira et al., 2005). Antibiotics, acting as growth promoters, have been included in animal feed at sub-therapeutic levels for approximately 60 years to compensate for the unsanitary conditions in which they are raised (Page and Gautier, 2012, Gaggia et al., 2010, Dibner and Richards, 2005). This results in the exposure of a large number of animals, irrespective of their health, to frequently sub-therapeutic concentrations of antimicrobials (Dias de Oliveira et al., 2005). Antibiotics traditionally used in the animal feed industry include aminoglycoside, penicillin, cephalosporin, inophores, lincosamide, macrolide, streptogramin, sulphonamide, tetracycline, bambermycins, novobiocin, spectinomycin, avoparcin, spiramycin, tylosin, virginiamycin, bacitracin, quinoxaline, flavophosphlipol, avilamycin, salinomycin, and monensin. Multi resistant strains are on the rise worldwide principally due to the spread of genes located on mobile genetic elements, including plasmids, integrons and transposons. Furthermore, the combination of these genes with chromosomally encoded resistance genes frequently results in bacteria that are resistant to all main classes of available antimicrobials (Allocati et al., 2013). The removal of these compounds from animal diets was prophesized to lead to a substantial increase in the use of therapeutic antibiotics (Gaggia et al., 2010). Evidence of the use of therapeutic antibiotics in the

Netherlands was reflected by the number of livestock animals produced remaining stable after the European ban on AGPs. Therefore it can be concluded that the antimicrobial growth promoters were fully replaced by increased therapeutic antimicrobials. However, the Dutch government have introduced and executed plans to reduce the use of therapeutic antibiotics in animal production (Mevius and Heederik, 2014).

Worldwide concern about the development of antimicrobial resistance and the transference of antibiotic resistance genes from animal to human microbiota led to banning the use of antibiotics as growth promoters in the EU since January 2006 (Gaggia et al., 2010). Antibiotics given to animals and closely related compounds used in human therapy have been exerting selective pressure on their target bacteria for decades and have generated a reservoir of antimicrobial resistant bacteria (Dias de Oliveira et al., 2005). Agricultural antibiotic use increases the frequency of antibiotic resistant zoonotic pathogens such as *Salmonella* (FAO, 2013, Dias de Oliveira et al., 2005, Smith et al., 2002). Resistant bacteria from animals can infect humans by direct contact as well as via food products of animal origin. Multidrug resistance is defined as resistance to three or more antimicrobial classes to which bacteria do not show intrinsic resistance (Allocati et al., 2013, Johnson and Nolan, 2009). The development of antimicrobial resistance among pathogenic bacteria has emerged as a major public health concern, which has led to an intensification of discussion about the prudent use of antimicrobial agents, especially in veterinary medicine, nutrition and agriculture (Dias de Oliveira et al., 2005).

The ban on the use of in feed antibiotics in animal feeds in some countries is an effort to arrest the development of antimicrobial resistant strains of pathogens. Resistant bacteria are still detected in meat of livestock animals; however the numbers are decreasing (Marshall and Levy, 2011). In other countries, the number of in feed antibiotics available for use in poultry diets has been restricted. In the US, retail giant Walmart is urging its meat, dairy, seafood and egg suppliers to cut back on the use of antibiotics in their livestock, making it the first multiple grocer to make such a stance. In March of 2015, the fast food chain McDonald's said that in 2 years its US restaurants would stop buying chicken reared on antibiotics (Arnold, 2015). The withdrawal of this preventive measure has serious implications for the productivity of birds, encouraging considerable research effort into finding potential alternatives for antibiotics (FAO, 2013). A need

exists to search for viable alternatives that could enhance the natural defence mechanisms of animals and reduce the massive use of antibiotics (Gaggia et al., 2010).

1.4 Alternatives to antibiotic growth promoters (AGP's)

There are a number of in feed alternatives to the use of AGPs. Improvements in our knowledge of the digestive physiology of the gut as well as the microbiology and microbial ecology of the digestive system has led to the development of alternative strategies and products which are based on a number of well-established principles. These include: diet acidification to maintain gastric pH, increasing gastric proteolysis and nutrient digestibility, promotion of beneficial bacteria at the expense of pathogenic organisms and decreasing intestinal pathogenic bacterial growth. The addition of specific enzymes, such as proteases, amylases and lipases can augment and complement endogenous enzymes, improving nutrient digestion and absorption and hence growth rate for a range of diets. Direct fed microbials, including pro and prebiotics, are added to the diet to influence the microfloral colonisation of the gastro intestinal (GI) tract resulting in competitive exclusion of pathogenic bacteria through competition for nutrients, provision of nutrients for the commensal microflora, changes in pH, and the production of short chain fatty acids (SCFA).

These non-nutritive substances are usually added in amounts of less than 0.05 % to maintain health status, uniformity and production efficiency in intensive animal production systems. These additives have now become vital components of practical diets (FAO, 2013). A combination of probiotics and prebiotics called synbiotics could also be a possible alternative to AGPs. The main effects of these additives are the improved resistance to pathogenic bacteria colonization and enhanced host mucosal immunity; thus resulting in a reduced pathogen load, an improved health status of the animal and a reduced risk of foodborne zoonotic pathogens in foods (Gaggia et al., 2010).

Ideally, alternatives to AGPs should have the same beneficial effect as AGPs. Whatever the mechanism of action of AGPs, the main characteristic of a good alternative from a practical point of view is that it must improve performance at least as well as AGPs (Huyghebaert et al., 2011).

1.4.1 In feed enzymes

The animal feed industry has been confronted with increased costs of raw materials over the last decade, mainly because of increased demands for cereals and oil seeds by emerging markets and the rise in use of these products for energy production (de Vries et al., 2012, FAO, 2011). Utilization of by products from the bio fuel and food industries as feed ingredients is important to reduce feed costs and enhance the sustainable use of feed resources for animal production. Many of these products contain high levels of plant cell wall material, mainly composed of non-starch polysaccharides (NSP), which cannot be digested by mammalian enzymes, but can be partly degraded by the microbial community residing in the intestinal tract of animals (de Vries et al., 2012).

NSP can interfere with digestive processes and associate with other nutrients, thereby reducing digestibility and absorption of these nutrients (de Vries et al., 2012). The presence of high levels of NSP in cereal-based diets results in a poor feed conversion rate, slow weight gain, and sticky droppings by young animals, especially chicks (Bhat, 2000). In poultry there is a greater risk of an outbreak of necrotic enteritis with the use of barley or rye rather than maize. Immerseel et al. (2004) suggested that this was related to the viscous water soluble NSP in barley and rye slowing digestion and thus leaving undigested nutrients for microbial propagation. Hydrolases are the main class of enzymes used in monogastric feed. Hydrolases are used to eliminate anti nutritional factors present in grains or vegetables, degrade certain cereal components in order to improve the nutritional value of feed, or to supplement the animals' own digestive enzymes (e.g. proteases, amylases and glucanases), whenever these enzymes are inadequate, e.g. during post-weaning period, as is often the case with broilers and piglets (FAO, 2013, Bhat, 2000).

In pigs, NSP degradation by microbial enzymes mainly occurs in the large intestine, although some degradation has been reported to occur in the stomach and may reach considerable levels at the terminal ileum. In poultry, NSP degradation mainly occurs in the crop or gizzard (de Vries et al., 2012). Enzymes widely used in the poultry industry are the carbohydrases e.g. cellulases, glucanases, pectinases and xylanases, that cleave the viscous fibre components in cereals and phytases that target the phytic acid complexes in plant ingredients (FAO, 2013). The mechanisms by which feed enzymes

influence the intestinal microbiota have been known for some time, e.g. through enhancing nutrient delivery to the host and by provision of fermentable oligosaccharides (Bedford and Cowieson, 2012). The improvement in the nutritional value of broiler diets by enzyme supplementation facilitates the growth of beneficial gut microflora which in turn produce metabolites such as SCFA's that can be used as an energy source by the host. These SCFA's are also thought to control *Salmonella* and *Campylobacter* populations by reducing the pH of the environment and rendering the growth conditions less favourable (Bedford and Cowieson, 2012).

Proteases have been used in both pig and poultry nutrition to improve protein digestibility of feed ingredients. Most current commercial proteases for animal feed are alkaline proteases of bacterial origin. Feed cost is reduced with the inclusion of proteases through a reduction of crude protein and first limiting amino acids supplied by dietary ingredients. Peek et al. (2009) found that a protease from *Bacillus licheniformis* increased the body weight gain of broilers challenged with three *Eimeria* species, and suggested that the mechanism was a reduction in the attachment of parasites to the mucus layer. This was supported by an increase in the thickness of the adherent mucus layer of the intestine due to protease. Marti et al. (2010) proposed that proteases may dissolve the biofilm matrix and inhibit biofilm formation. Proteases are being used in combination with other feed enzymes such as the carbohydrases.

More recently, technically successful enzyme preparations for use in maize-soybean diets have become available. Future advances in feed enzyme technology will involve the development of enzymes that can be used to target the anti-nutritive factors in non-traditional feedstuffs and improve their feeding value (FAO, 2013).

1.4.2 Vaccines

Vaccination is a widely used preventive measure against many infectious diseases in mammals and poultry (Dahiya et al., 2006). Traditional vaccines are generally classified into live attenuated and inactivated/killed (bacterins) bacteria (Cheng et al., 2014). The immune response of poultry reduces the duration and severity of *Salmonella* infections and helps to prevent reinfection. This response provides the basis for efforts to protect birds against infection by vaccination and for the serological detection of infected flocks

(Gast, 2007). Currently vaccines are used as the primary method for coccidiosis prevention in breeding flocks and to some extent in laying hens and broiler chickens. Vaccines have proved to be a valid addition to coccidiosis control in commercial broilers and it is estimated that approximately 12 % of commercial broilers produced in Europe rely on vaccines alone for coccidiosis control (Taylor-Pickard and Spring, 2008).

Immunisation with live or inactivated *Salmonella* vaccine is regarded as an important prophylactic measure to protect chickens against *Salmonella* infections, thereby protecting human consumers from foodborne disease (Van Immerseel et al., 2009, Gast, 2007, Dahiya et al., 2006). However, the delay in the development of a protective response after vaccination in young broilers with immature immune systems coupled with the early age of slaughter means there is insufficient time to clear the system of *Salmonella* after vaccination. Although vaccination of broilers is not commercially feasible, vaccination of broiler breeders has been successful in transferring maternal immunity. However, these antibodies did not show a reduction in colonisation of *Salmonella* in day-old chicks that were challenged with a low dose of *Salmonella* (Van Immerseel et al., 2009, Gast, 2007). In the case of layers, vaccination has been shown to be very effective in reducing the contamination in the intestine and in eggs (Van Immerseel et al., 2009) leading to an increase in egg production and a reduction in foodborne illnesses.

Neither type of vaccine, bacterin or live, have been able to provide consistent inhibition of *Salmonella* infection, especially against high challenge doses of *Salmonella*. Multivalent bacterins, containing a mixture of serotypes, can provide a wider range of protection. Bacterins are more commonly employed as alternatives to live vaccines as they raise fewer consumer concerns (Cox and Pavic, 2010). Bacterins cannot revert back to a virulent form nor can they carry antibiotic resistance genes. Bacterins, however, do have numerous disadvantages such as the lack of relevant protective antigens due to *in vitro* growth conditions and killing processes, antigenic competition between non protective and protective components, a lack of safety due to potentially harmful components such as liposaccharides, and a lack of broad spectrum protection (Cheng et al., 2014, Gast, 2007).

As a broad generalization, the vaccination of pullets or hens with either killed or live preparations has often reduced but not entirely prevented faecal shedding, organ invasion, and egg contamination following experimental *Salmonella* challenge. Vaccination does not create an impenetrable barrier against *Salmonella* infection. It is important to note that environmental stresses on poultry can have a negative effect on the vaccine performance (Gast, 2007). A combination vaccine, using attenuated then killed *Salmonella* serovars, stimulated both cell-mediated and humoral immune systems, resulting in higher titres than individual vaccination (Cox and Pavic, 2010).

1.4.3 Probiotics

The first proposed definition of a probiotic was suggested by Fuller in 1989 as 'viable microorganisms used as feed additives which lead to beneficial effects for the host by improving its microbial balance' (Fuller, 1989). The current definition as used by the FAO/WHO defines probiotics as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'(Smith, 2014, FAO/WHO, 2001). More precisely, probiotics are live microorganisms of non pathogenic origin and are non toxic in nature, which when administered through the digestive route, are favourable to the host's health . It is believed by most investigators that there is an unsteady balance of beneficial and non-beneficial bacteria in the tract of normal, healthy, non stressed poultry. When a balance exists, the bird performs to its maximum efficiency, but if stress is imposed, the beneficial flora has a tendency to decrease in numbers and an overgrowth of the non-beneficial bacteria occurs. This occurrence may lead to disease, both acute i.e. diarrhoea, or subclinical and reduce production parameters of growth, feed efficiency (Kabir, 2009).

Two basic mechanisms by which probiotics act to maintain a beneficial microbial population include "competitive exclusion" and immune modulation (Yang et al., 2009). Growth promotion of beneficial bacteria is competitive and antagonistic to pathogenic bacteria thus preventing the establishment of bacterial infection and reducing the bacterial load of potentially pathogenic bacteria and, by extension the incidence of zoonosis (Van Immerseel et al., 2009). The competitive exclusion concept developed by Nurmi and Rantala (1973) was a critical stage in the development of probiotics where they found that newly hatched chicks could be protected against

Salmonella enteritidis colonisation by dosing a suspension of gut contents derived from healthy adult chickens. A variety of microbial species have now been established and used as probiotics including *Bacillus* spp., *Bifidobacterium* ssp., *Enterococcus* spp., *E. coli* spp., *Lactobacillus* spp., *Lactococcus* spp., and *Streptococcus* spp. Common human probiotics include *Lactobacillus* spp. and *Bifidobacterium* spp. whereas *Bacillus* spp, *Enterococcus* spp. and *Saccharomyces* spp. are common probiotics in livestock (Chaucheyras-Durand and Durand, 2010). In order for a probiotic to be selected it must meet a number of criteria including: the bacteria must demonstrate non pathogenic behaviour, exhibit resistance to technological processes, withstand the hosts' gastric acids and bile, persist and adhere within the intestinal tract thereby producing inhibitory compounds, modulate immune response, and alter microbial activities. Probiotics must not carry transferable resistance genes; this reduces the problem associated with transferable antibiotic resistance genes between probiotics and microflora (Mathipa and Thantsha, 2015, Smith, 2014, Kabir, 2009, Simmering and Blaut, 2001).

Probiotics containing antibiotic resistance are not generally a safety concern. Specific antibiotic resistance determinants carried on mobile genetic elements, such as tetracycline resistance genes, are often detected in the typical probiotic genera, and constitute a reservoir of resistance for potential food or gut pathogens, thus representing a serious safety issue (Gueimonde et al., 2013). Multi drug resistant bacteria (MDR), resistant to at least one antibiotic drug of three or more antibiotic classes, including extended-spectrum-β-lactamase (ESBL) producing *Enterobacteriaceae*, methicillin resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE), may be transferred from probiotic bacteria to pathogenic bacteria (Petternel et al., 2014). Antibiotic resistance found among the genus *Lactobacillus* include the vancomycin resistant phenotype, chloramphenicol resistance genes, erythromycin resistance genes, aminoglycoside resistance genes, and β-lactam resistance genes. Many of these genetic markers have been found in potentially mobile elements, such as transposons and plasmids, which could spread the antibiotic resistance genes through conjugation. Some of these genes have been found to be transferred *in vitro* between strains of *Lactobacillus* and also from lactobacilli to different Gram-positive bacteria, including food pathogens, such as *Streptococcus* (Gueimonde et al., 2013, Tannock et al., 1994). Bifidobacteria are intrinsically resistant to mupirocin and are not susceptible to aminoglycosides. A few streptomycin resistant strains have also been characterised

(Sato and Iino, 2010). Tetracycline resistance in *Bifdobacterium* are commonly found in this genus and under adequate conditions the gene (*tet)* could be transferred. Tetracycline resistance has also been characterised in mobile elements in *Bacillus* (Gueimonde et al., 2013).

1.4.4 Prebiotics

A prebiotic is a non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid, 1995). This definition was later updated in 2004 and prebiotics are defined thereafter as 'selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health (Li et al., 2015, Swanson et al., 2002). Additionally, prebiotics can induce microbial competition and reduce the populations of harmful intestinal microbiota (Li et al., 2015). It is estimated that the lower intestinal tract of monogastric animals and man harbours as many as 500 to 1,000 different bacterial species (Guarner and Malagelada, 2003). Over 90 % of the known phylogenetic categories in the caecum and colon belong either to the phylum *Firmicutes* (including *Clostridium*, *Enterococcus*, *Lactobacillus* and *Ruminococcus* genera) or to the phylum *Bacteroidetes* (including *Bacteroide*s and *Prevotella* genera) (Ducatelle et al., 2015). Ingested carbohydrates such as lactose and sucrose can hardly reach the colon because they are rapidly digested and adsorbed in the small intestine, and thus they cannot act as prebiotics. However, glycosidic linkages of galactooligosaccharides (GOS) and fructooligosaccharides (FOS) derived from lactose or sucrose make them more resistant to digestion, which might facilitate them arriving at the colon in their intact forms (Li et al., 2015). Availability of carbohydrates that escape metabolism and adsorption in the small intestine have a major influence on the microflora that become established in the colon (Kaplan and Hutkins, 2000). Dietary carbohydrates can be classified by their degree of polymerization (DP) into monosaccharides, oligosaccharides (DP ~ 2, 10), and polysaccharides (DP ≥ 10) (Sako et al., 1999).

Among food ingredients, non digestible carbohydrates (oligo and polysaccharides), some peptides and proteins, and certain lipids (both ethers and esters) are candidate

prebiotics. Because of their chemical structure, these compounds are not absorbed in the upper part of the gastrointestinal tract or hydrolyzed by digestive enzymes. Such ingredients could be called “colonic foods”, i.e., foods entering the colon and serving as substrates for the endogenous colonic bacteria, thus indirectly providing the host with energy, metabolic substrates and essential micronutrients (Gibson and Roberfroid, 1995). Although food ingredients that escape digestion in the upper gastrointestinal tract have the potential to act as a prebiotic, only certain carbohydrates have been shown to provide convincing evidence in favour of this. A range of dietary oligosaccharides, such as lactosucrose, GOS, FOS, transgalactosaccharide, and isomaltooligosaccharides, have been shown to exert prebiotic properties (Li et al., 2015, Shashidhara and Devegowda, 2003). Prebiotics alter the microbial populations of the gut, and consequently, improve the health of the host (Swanson et al., 2002). Mannooligosaccharides (MOS) may soon be added to this list as they were shown by Cuskin et al, (2015) to be fermentable by certain endogenous *Bacteroidetes* spp. in humans. Corrigan et al. (2015) , found an increase in *Bacteroidetes* species in broilers fed a MOS supplemented diet.

1.4.4.1 Inulin

Inulin and oligofructose are natural food ingredients commonly found in varying percentages in dietary foods, they are present in a wide variety of plants as natural storage carbohydrate (≥ 36,000 plant species) (Samanta et al., 2013, Niness, 1999). Inulin and oligofructose are either synthesized from sucrose or extracted from chicory roots; the root of the *Cichorium intybus* plant contains ~ 15–20 % inulin and 5–10 % oligofructose (Niness, 1999). Inulin (Figure 1.2) is a polydisperse β-(2-1) fructan, the fructose units in this mixture of linear fructose polymers and oligomers are each linked by β-(2-1) bonds. The first monomer of the chain is either a β-D-glucopyranosyl or β-D-fructopyranosyl residue. A glucose molecule typically resides at the end of each fructose chain and is linked by an α-(1-2) bond, as in sucrose (Samanta et al., 2013, Roberfroid, 1999, Niness, 1999).

Oligofructose is a subgroup of inulin, consisting of polymers with a degree of polymerization of less than 10. The polyfructan inulin is considered to act as a prebiotic, since it can modulate the composition and metabolic activity of the intestinal microbiota, which might potentially enhance the health of the host organism (Passlack

et al., 2015). The best known nutritional effects of inulin and oligofructose are their actions to stimulate *Bifidobacteria* growth in the intestine (Watson et al., 2013). Nourishing beneficial bacteria, such as *Bifidobacteria*, with inulin or oligofructose allows them to "outcompete" potential detrimental organisms and thereby potentially contribute to the health of the host. Health benefits ascribed to *Bifidobacteria* include inhibiting the growth of harmful bacteria, stimulating of components of the immune system, aiding the absorption of certain ions and the synthesis of B vitamins (Niness, 1999).

Several studies have demonstrated that inulin can modulate the intestinal microbiota in pigs. In particular, *Bifidobacteria* and *Lactobacilli* were increased in different segments of the intestinal tract, when inulin was added in at a concentration of 1.6 % and 4 % to the diets. Moreover, a decrease of *Clostridium perfringens* in the porcine digesta of the colon and rectum or *Clostridium* spp. and members of *Enterobacteriaceae* in the porcine digesta and mucus in different segments of the intestine was reported after the dietary inclusion of inulin (Passlack et al., 2015). Passlack et al. (2015) found that the addition of inulin to a gestation and lactation diet can not only modulate the intestinal microbiota of sows, but also of their offspring. Researchers also demonstrated efficacy of inulin type fructans in combination with competitive exclusion flora in chickens where a reduction in colonisation by the pathogenic bacteria (*Salmonella* and *Campylobacter*) in chickens was observed (Verdonk et al., 2007).

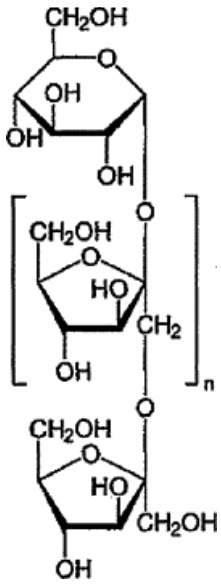


Figure 1.2. Structure of inulin.
The number of repeating units, n, is in the range 0–60 in chicory. Source: (Muzzarelli et al., 2012)

1.4.4.2 Fructooligosaccharides (FOS)

FOS is a common name for fructose oligomers, and these are usually understood as inulin type oligosaccharides or inulin derivatives (Apolinario et al., 2014, Dominguez et al., 2013). The production of FOS is generally through the degradation of inulin or by sucrose transfructosylation to fructooligosaccharide (Paulino and Zuniga-Hansen, 2010). This results in the production of homologous oligosaccharides represented by the formula GFn, which are mainly composed of 1-kestose (GF2), nystose (GF3), and 1F - β-fructofuranosyl nystose (GF4), in which two, three, and four fructosyl units are bound at the β-2,1 position of glucose, respectively, as shown in Figure 1.3. As FOS cannot be digested by the enzymes of the small intestine, due to the β-linkages, they are fermented in the large intestine to selectively stimulate the growth of probiotic like bacteria that are part of the commensal gut microbiota i.e. FOS are thought to act as a growth substrate for *Bifidobacterium* spp. and *Lactobacillus* spp (Ricke, 2015, Oakley et al., 2014, Dominguez et al., 2013, Biggs et al., 2007, Kaplan and Hutkins, 2000). Lactic acid and SCFAs such as formic, acetic, propionic and butyric acids are desirable products of such fermentation reactions, as they can contribute to the improvement of colonic and systemic health (Li et al., 2015, Dominguez et al., 2013).

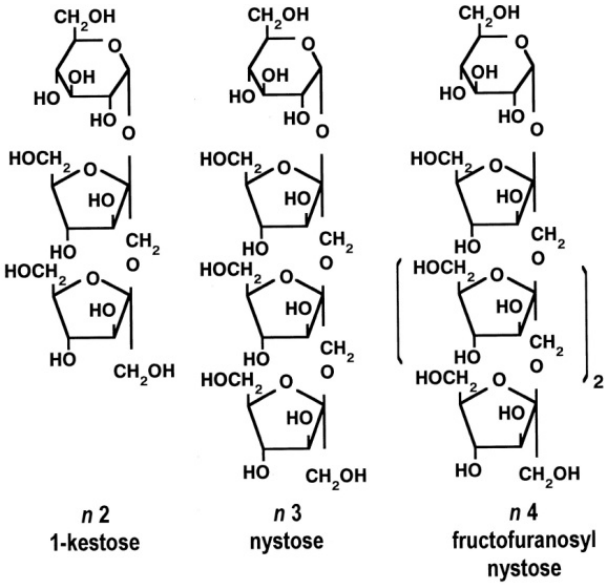


Figure 1.3. Structures of FOS.
Including 1-kestose (n2, left), nystose (n3, centre), and fructofuranosyl nystose (n4, right). Source: (Ohta et al., 1998)

FOS also acts like a growth promoter to commensal bacteria which inhibit the adherence and invasion of pathogens in the colonic epithelia by competing for the same glycoconjugates present on the surface of epithelial cells (Dominguez et al., 2013). The most important property of FOS is their ability to stimulate bifidobacterial growth specifically while suppressing the growth of some other species in the colon, such as *Clostridium perfringens* (Bornet et al., 2002). The ability to control or reduce the growth of *Clostridium perfringens* by FOS, is important to the poultry industry because this bacterium is a primary cause of necrotic enteritis that has been estimated to cost the worldwide poultry industry $2 billion each year (Biggs et al., 2007). FOS has been shown to inhibit *Escherichia coli* and *Salmonella* in the large intestine of broilers (Ricke, 2015, Donalson et al., 2008, Xu et al., 2003, Fukata et al., 1999). It has also been reported to increase growth and improve feed conversion ratio as a consequence of inclusion in broiler diets (Xu et al., 2003). Another interesting feature of FOS is its positive effect on mineral absorption (Dominguez et al., 2013).

Found in trace amounts as natural components in fruits, vegetables, and honey, FOS forms a series of fructose oligomers and polymers derived from sucrose. They occur in many higher plants as reserve carbohydrates. Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato, and rye are well known sources of FOS (Dominguez et al., 2013). Microbial sources of enzymes with transfructosylation activity for the production of FOS from sucrose include *Aspergillus* spp. (Park and Almeida, 1991), *Aureobasidium* spp., and *Fusarium* spp. (Dominguez et al., 2013, Sangeetha et al., 2005, Yun, 1996). Marketed commercially as Raftilose and Nutraflora, FOS has been self-affirmed by the manufacturers as GRAS (generally recognized as safe) and have been added to infant formulas, yogurt, and other food products and food supplements (Kaplan and Hutkins, 2000).

1.4.4.3 Galactooligosaccharides (GOS)

GOS are non digestible carbohydrates, which are resistant to gastrointestinal digestive enzymes, but are fermented by specific colonic bacteria (Sako et al., 1999). This oligosaccharide is a synthetic compound produced from lactose by enzymatic transgalactosylation and consists of lactose and several galactose molecules. It passes through to the hindgut undigested due to its β-(1, 6) and β-(1, 4) linkages that avoid

digestion by β-galactosidase (Figure 1.4) (Biggs et al., 2007, Sako et al., 1999). It is stable at high temperature and low pH, and has a long shelf life (Sako et al., 1999).

In the livestock industry, GOS is a relatively unstudied compound. However, it has been examined for its bifidogenic effect in humans (Ito et al., 1993). Diets enriched with GOS increase populations of *Bifidobacterium* spp. and *Lactobacillus* spp. and their fermentation products in the colon. The fermentation products are mainly SCFAs, which improve the energy supply to the colonic epithelium, and facilitate calcium and magnesium absorption. Moreover, glycoconjugates containing GOS have been implicated in interactions between epithelial and bacterial cells, implying that these compounds also have the capacity to inhibit the binding of pathogens to cell surfaces by acting as competitive receptors (Tzortzis et al., 2005). GOS formulations have the potential to reduce the adherence of enteropathogens such as EPEC and *Salmonella typhimurium in vitro* and *in vivo* (Searle et al., 2009). GOS addition has shown strong inhibition of EPEC and *Salmonella* attachment to HT29 cells *in vitro.* Shoaf et al. (2006) demonstrated that purified GOS exhibited adherence inhibition of EPEC on both Caco-2 and Hep-2 cells by 65 % and 70 %, respectively. The effect of GOS on the microbial population of the major bacterial genera in the proximal and distal colonic contents of pigs included significant increases in *Bifidobacter* spp. and *Lactobacilli* spp., while no change was observed for *Clostridium histoyticum* or *Bacteroidetes* spp. (Tzortzis et al., 2005).

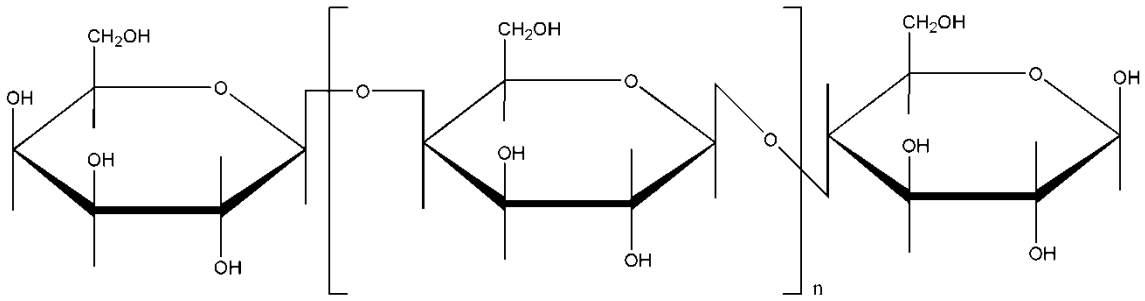


Figure 1.4. Structure of galactooligosaccharide.
Source: (Souza et al., 2015)

1.4.4.4 Mannan oligosaccharides (MOS)

Mannan oligosaccharides (MOS) are sugars composed of polymers of mannose (Figure 1.5) that have been shown to affect gut health by pathogen adsorption, immune

modulation and improving intestinal morphology. A widely known property of MOS is their ability for the agglutination of Gram-negative pathogenic bacteria containing type 1 fimbriae. These pathogens can be absorbed to the MOS instead of attaching to intestinal epithelial cells and move through the intestine without colonisation (Luquetti et al., 2012, Hooge and Connolly, 2011, Oliveira et al., 2009, Yang et al., 2009, Ferket et al., 2002, Spring et al., 2000).

Many enteric pathogens must attach to the mucosal surface of the gut wall to establish themselves in the GI tract (Spring et al., 2000). As attachment is often mediated through binding of bacterial type 1 fimbriae to receptors containing D-mannose (Eshdat et al., 1978), it may be possible to block the lectins with mannose or similar sugars and inhibit bacterial attachment (Spring et al., 2000). Some bacteria recognize binding sites on prebiotics instead of on the intestinal mucosa, and the colonisation by pathogenic bacteria in the intestine is thus reduced (Ganguly, 2013). Type 1 fimbriae are common on numerous species of *E. coli* and *Salmonella* (Swanson et al., 2002). Specifically MOS prevent the pathogen from adhering to enterocytes, making it difficult to establish an infection, thus reducing colonization of the intestinal epithelium (Lourenco et al., 2015, Ganguly, 2013).

As relatively high concentrations of mannose are required to control colonization of pathogenic bacteria, the cost of using pure mannose in commercial production is prohibitive. However, mannose-based carbohydrates occur naturally in many products, such as yeast cell walls or different gums, which are available at reasonable prices. One MOS product contains yeast cell wall fragments derived from *Saccharomyces cerevisiae*. The cell wall fragments are obtained by centrifugation from a lysed yeast culture. The pellet containing the yeast cell wall fragments is then washed and spray dried (Spring et al., 2000). MOS derived from yeast cell wall are more complex than the name suggests; they are components of the outer layer of yeast cell walls and their components include: proteins, glucans and phosphate radicals as well as mannose (Yang et al., 2009).

Three major modes of action by which broiler performance is improved by MOS are proposed: control of pathogenic or potential pathogenic bacteria which possess type 1 fimbriae (mannose sensitive lectin), immune modulation based on the antigenicity characteristics of its mannan and glucan components, and modulation of intestinal

morphology and expression of mucin and brush border enzymes (Yang et al., 2009). New evidence by Cuskin et al. (2015) suggests that MOS may be fermentable by *Bacteroidetes* spp. in humans. Yang et al. (2009) showed that MOS inhibited the development of *Lactobacilli* spp. and coliforms; in particular the colonization of mucosa associated coliforms in broilers was inhibited by MOS as early as 7 days of age (Yang et al., 2009, Cuskin et al., 2015). Research by Corrigan et al. (2015) , found that MOS supplementation consistently and reproductively altered the cecal microbiota and increased the levels of *Bacteroidetes* in the broiler cecum.

The MOS derived from the outer cell wall of yeast, and its evaluation in diets for breeders is of particular interest because it not only shifts gastrointestinal microflora balance toward beneficial organisms but also has immunomodulatory properties. The yeast cell wall has powerful antigenic stimulating properties, and it is well established that this property is a characteristic of the mannan chain (Shashidhara and Devegowda, 2003). Studies by Tohid et al. (2010) and Shashidhara and Devegowda (2003) investigating the immunomodulatory properties of MOS found improved antibody response in broilers and layers, stimulation of the humoral response, and increased maternal immunity in the progeny of MOS treated poultry. MOS supplemented pigs were also found to have an improvement in total Ig levels (IgG, IgM and IgA) in the colostrums. In a series of experiments by Spring et al. (2000) dietary MOS has been shown to reduce intestinal *Salmonella* and coliform concentrations in broiler chicks. The reduction in pathogenic bacteria with the inclusion of dietary MOS has been demonstrated consistently (Baurhoo et al., 2009). It has been reported that the inclusion of MOS at 0.05 % in turkey diets from 0 to 105 days of age significantly reduce litter moisture and numerically lowered coliform counts versus the negative control (Navidshad et al., 2015, de Barros et al., 2015, Ganguly, 2013, Wasilewska et al., 2010, Sims et al., 2004). MOS has been studied to control necrotic enteritis in chickens by promoting commensal bacteria while controlling pathogen invasion (Yitbarek et al., 2012).

In the search for alternatives to AGPs for poultry production, MOS has demonstrated its potential to reduce enteric disease in poultry and subsequent contamination of poultry products. A relatively cheap source of MOS exists as the cell wall of *Saccharomyces cerevisiae*.

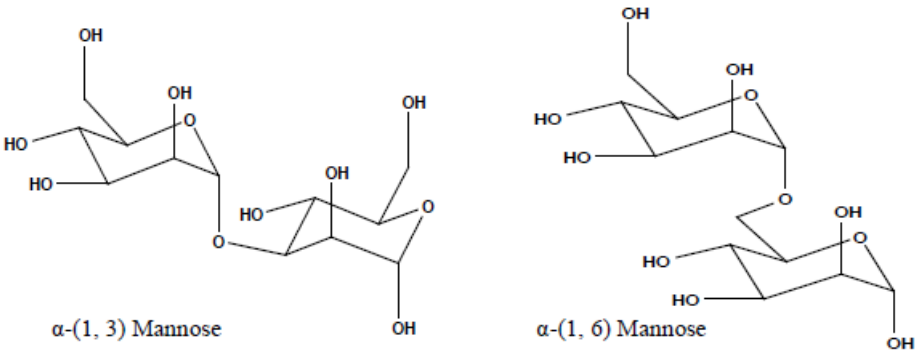


Figure 1.5. Chemical structure of α-(1, 3) Mannose and α-(1, 6) Mannose linkages.
Source: (Davidson et al., 2000)

1.4.5 Synbiotics

A synbiotic is, in its simplest definition, a combination of probiotics and prebiotics (de Vrese and Schrezenmeir, 2008). This combination could improve the survival of the probiotic organism, because its specific substrate is available for fermentation. This could result in advantages to the host through the availability of the live microorganism and the prebiotics. Examples of synbiotics are fructooligosaccharides (FOS) and *Bifidobacteria*, and lactitol and *lactobacilli* (Yang et al., 2009). A combination of *Bifidobacterium breve* strain Yakult and *Lactobacillus casei* strain Shirota and galactooligosaccharides in patients with systemic inflammatory response syndrome had significantly greater levels of beneficial *Bifidobacterium* and *Lactobacillus* and SCFAs and lower incidence of infectious complications such as enteritis, pneumonia and bacteremia than those who received no synbiotics (Shimizu et al., 2013). The combination of FOS and competitive exclusion flora was more effective at reducing *Salmonella* in broilers than either of the material on their own (Yang et al., 2009). Li et al, (2008) reported that application of a combination of FOS and bacillus to a corn soybean basal diet improved the average daily gain (ADG) and feed conversion ratio (FCR) by 6 % and 2 %, respectively. Furthermore, diarrhoea and mortality rate were reduced by 58 % and 67 %, respectively, which were comparable to aureomycin (chlortetracycline) treatment (the relative changes were 4 % for ADG, 2 % for FCR, 69 % for diarrhoea rate and 33 % for mortality rate) (Li et al., 2008).

There are some reports on the effect of synbiotics on the physiological and biochemical indexes of piglets including the enhancement of immune function, the improvement of ADG and digestibility, the reduction of diarrhoea, morbidity and mortality, the ease of weaning stress response, and the significant promotion of piglet performance. However, the reports of the beneficial effects of synbiotics on swine production are still limited. So far, the synergy mechanism of probiotics and prebiotics has not been thoroughly understood. As such the extensive application of synbiotics has a long way to go (Cheng et al., 2014).

1.5 Yeast cell wall

Yeast cell structure extends beyond the plasma membrane into an extracellular network of proteins and polysaccharides. Fungi have extracellular matrices containing polysaccharide protein complexes termed cell walls allowing them to build structure. Much of the diversity between fungi comes at the level of the extracellular matrices themselves, while the underlying mechanisms are conserved. Fungal cell walls are highly diverse so yeast study will only capture a fraction of the fungal cell wall repertoire (Lesage and Bussey, 2006). The cell wall of *Saccharomyces cerevisiae* is a sturdy structure providing physical protection, osmotic support (Klis et al., 2002) and is required for cell viability (Orlean, 1997). It defines cell shape during budding, growth, mating, sporulation, pseudohypha formation, presents adhesive glycoproteins to other yeast cells, and participates in cell to cell recognition (Orlean, 2012, Klis et al., 2006, Orlean, 1997). The cell wall, far from being a static structure armouring the cell, represents a highly dynamic carbohydrate moiety which grows and constantly undergoes modifications in shape, chemical composition and physical properties (Teparic and Mrsa, 2013). In addition, it provides a matrix for a variety of enzymes involved in the hydrolytic processes, nutritional uptake, end metabolite secretion and cellular maintenance (Aguilar-Uscanga and Francois, 2003).

The cell wall comprises 15-30 % of the dry weight of the cell, is as wide as 110-200 nm, with the major components being β-(1, 3) glucan, β-(1, 6) glucan, chitin and proteins that bear N- and O-linked glycans (mannoproteins) and a glycolipid anchor (GPI). These components are linked to form macromolecular complexes, which are assembled to form the intact cell wall as illustrated in Figure 1.6 (Klis et al., 2002,

Kollar et al., 1997). β-(1, 6) glucan has a central role in cross linking wall components. Wall composition and degree of cross-linking vary during growth and development and change in response to cell wall stress (Orlean, 2012). The yeast cell wall is typically composed of approximately 85 % polysaccharides and approximately 15 % proteins but the relative amount of wall components can vary depending on growth conditions, yeast species, growth phase, and stress conditions (Teparic and Mrsa, 2013).

The cell wall has two layers, a mannan outer layer and glucan inner layer with different structures, different physicochemical properties, and different physiological roles (Teparic and Mrsa, 2013). The outer layer is electron dense, has a brush like surface, and can be removed by proteolysis; it therefore consists mostly of mannoproteins. These heavily glycosylated mannoproteins emanating from the cell surface are involved in cell-cell recognition events. This layer also limits the accessibility of the inner part of the wall and the plasma membrane to foreign enzymes such as cell wall degrading enzymes in plant tissue. The outer protein layer accounts for about one third of the wall dry weight and may at anytime consist of at least 20 different glycoproteins, the composition of this protein layer may vary depending on growth conditions (Klis et al., 2006, Klis et al., 2002). The inner layer, more electron transparent, is microfibrillar and is β-glucanase digestible, indicating that its major components are glucans (Orlean, 2012). The mechanical strength of the wall is mainly due to the inner layer, which consists of β-(1, 3) glucan and chitin, and represents about 50-60 % of the wall dry weight (Klis et al., 2002).

Glucan provides osmotic stability and serves as an anchoring moiety for wall proteins (Teparic and Mrsa, 2013). The protein population that is anchored to the stress bearing polysaccharides of the cell wall, and forms the interface with the outside world, is highly diverse. This diversity is believed to play an important role in adaptation of the cell to environmental conditions, in growth mode and in survival (Klis et al., 2006). Cell wall proteins allow the cells to flocculate, recognize mating partners, form a biofilm and grow pseudohyphally. They also help the cells to retain iron and facilitate sterol uptake and are required for growth under anaerobic conditions (Klis et al., 2006).

The majority of cell wall proteins are glycophosphatidylinositol (GPI)-modified and are thus indirectly linked to the β-(1, 3) glucan network (CWP-GPI) (Klis et al., 2006). In addition to the GPI-CWPs, a smaller group of proteins are directly linked to the β-(1, 3)

glucan network through an unidentified linkage that is sensitive to mild alkali, as β-(1, 3) glucan is alkali insoluble. These proteins are called ASL (alkali-sensitive linkage)-CWPs and includes the family of Pir-CWPs (Pir, proteins with internal repeats). The Pir-CWPs seem to be uniformly distributed throughout the inner skeletal layer, which is consistent with their being directly connected to β-(1, 3) glucan macromolecules. It seems conceivable that Pir-CWPs may interconnect two or even more β-(1, 3) glucan molecules. This would considerably strengthen the wall. It may be advantageous to the cell to use Pir-CWPs during a period of isotropic growth (Klis et al., 2006). The cell wall also contains proteins that are either retained in a noncovalent fashion, such as Bg12p, or via disulphide bridges to other proteins (Klis et al., 2006).

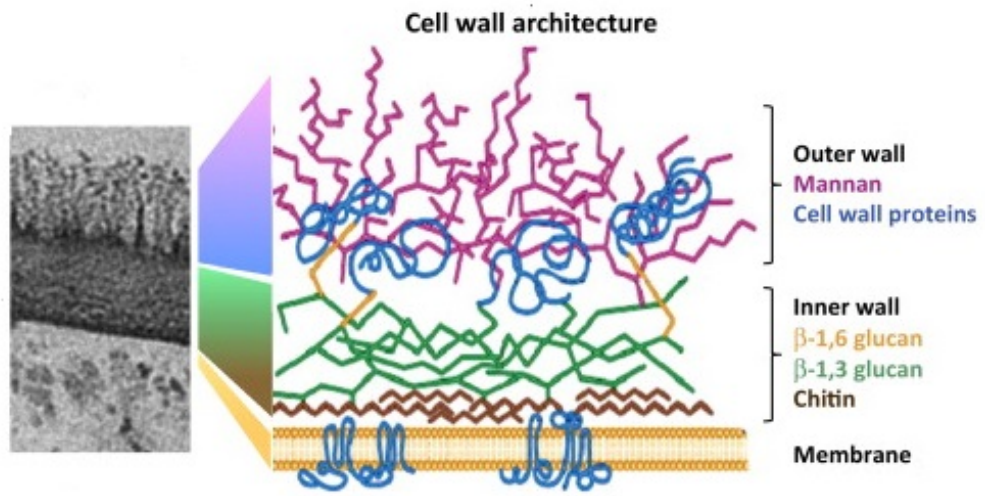


Figure 1.6. Yeast cell wall architecture.
Transmission electron micrograph of yeast cell wall on the left and the cartoon on the right illustrates the structure. Source: (Brown et al., 2014)

1.5.1 β-glucan

β-glucans generally described as polymers of glucose, form the major structural polymer in the yeast cell wall and encompass the entire yeast cell with a microfibrillar net (Lipke and Ovalle, 1998, Kollar et al., 1997). β-glucans compose 30-60 % of the dry weight of the wall and can be separated into three fractions. The first fraction which makes up approximately 35 % of the dry weight of the wall, is an acid- and alkali-insoluble β-(1, 3) glucan with a degree of polymerization of approximately 1500 and β-(1, 3) linked glucan side chains initiated at branching β-(1, 6) linked glucoses that

represent approximately 3 % of the whole polymer. The non reducing ends of β-(1, 3) glucan chains in this fraction can be linked to the reducing end of chitin through β-(1, 4) linkages, rendering the β-glucan insoluble; up to 50 % of chitin chains could be linked in this way. One chitin-β-glucan linkage per 8000 hexoses would have a major impact on the solubility of β-glucan (Orlean, 2012). A second β-glucan fraction, representing approximately 20 % of the dry weight of the wall, is similar in size and composition to the alkali-insoluble β-(1, 3) glucan, but soluble in alkali because it is not cross linked to chitin (Orlean, 2012). A third fraction, making up approximately 5 % of the dry weight of the wall, can be released from alkali-insoluble glucan by extraction with acid or digestion with endo-β-(1, 3) glucanase. This fraction is a β-(1, 6) glucan with a degree of polymerization of 140, in which 14 % of the β-(1, 6) linked residues bear a side branching β-(1, 3) glucan (Orlean, 2012). Three types of β-glucan linkages are shown in Figure 1.7

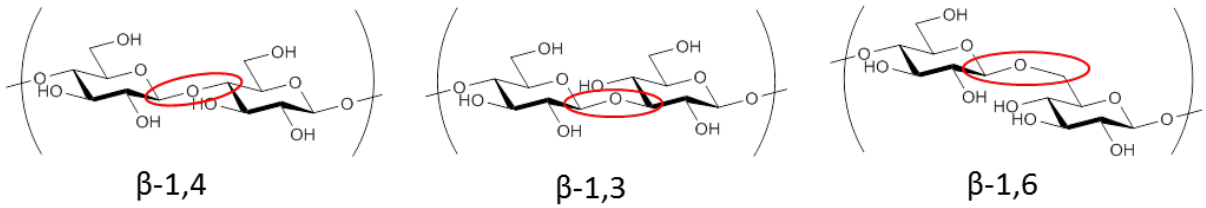


Figure 1.7. β glucan linkages.
Source: (Megazyme, 2015)

1.5.2 Chitin

Chitin, as pictured in Figure 1.8 is a polymer of β-(1, 4) linked N-acetylglucosamine (GlcNAc) and contributes only 1-2 % of the dry weight of the wall of unstressed wild type cells. The structure of α-chitin is similar to α-cellulose, with hydrogen bonded anti parallel chains of GlcNAc units (Lipke and Ovalle, 1998). Chitin is normally deposited in a ring in the neck between a mother cell and its emerging bud, in the primary division septum, and in the lateral walls of newly separated daughter cells. In addition to free chitin, some is bound to β-(1, 3) glucan and present mainly in the neck between mother and daughter cell, whereas a lesser amount found in lateral walls, is bound to β-(1, 6) glucan. Chitin is glycosidically linked to non reducing branches of the β-(1, 3) glucan

and β-(1, 6) glucan (Kollar et al., 1997). Chitin levels increase in response to mating pheromones and delocalized chitin in lateral walls can increase to as much as 20 % of the wall in *Saccharomyces cerevisiae* mutants mounting the cell wall stress response (Orlean, 2012).

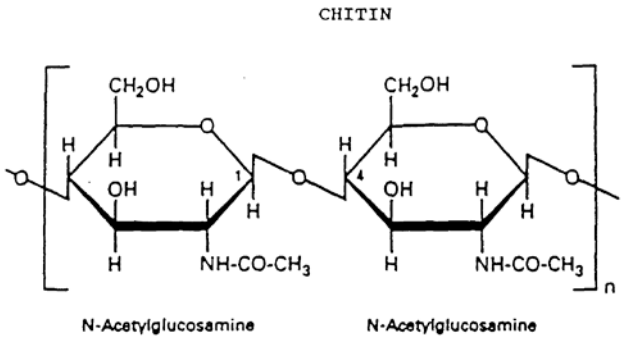


Figure 1.8. Chemical structure of chitin.
Source: (Rhee and Berg, 1995)

1.5.3 Mannoproteins

Mannoproteins are cell wall polysaccharides that form yeast mannan oligosaccharides (MOS), the electron dense, fibrillary outer layer of the cell wall. The polysaccharide structure on *Saccharomyces cerevisiae* mannoproteins can vary considerably among yeast species (Lesage and Bussey, 2006). Mannoproteins represent 25-50 % of the yeast cell wall and determine the cell surface properties (Lipke and Ovalle, 1998). Yeast cell wall proteins can bear the asparagine (N-) linked glycans, serine/threonine (O-) linked mannan oligosaccharides and often a glycosylphosphatidylinositol anchor as well. Cell wall and secreted mannoproteins are extensively mannosylated in the Golgi. The N-linked glycans can be extended with an outer chain of 50 or more α-(1, 6) linked mannose residues, extending from the N-glycan core, that is extensively decorated with short α-(1, 2) mannan side branches terminated in α-(1, 3) mannan forming a highly branched structure containing as many as 200 mannose residues. Phosphodiester linked mannose can also be attached to α-(1, 2) linked residues as depicted in Figure 1.9. Mannosylphosphate outer chains result in a negative net charge (Orlean, 2012, Lesage and Bussey, 2006). Mannan attached to proteins, in a process of protein glycosylation, creates a chemically inert external shield which defines the porosity of the cell wall (Teparic and Mrsa, 2013). Many glycoproteins also bear mannosyl glycans, which are

often present in Ser/Thr-rich stretches, these O-mannose saccharides are thought only to exist in yeast such as *Saccharomyces cerevisiae*, where they occur as linear chains of one to six mannose units on a wider variety of proteins (Haltiwanger and Lowe, 2004). Polymannose structures found on secreted or cell wall mannoproteins may contribute to 95 % of the molecular mass of these glycoproteins. Intracellular glycoproteins are short Man_9-$Man_{13}GlcNAc_2$ (Dean, 1999).

Cell wall proteins are thought to form three groups of proteins: the first contains those with potential to participate in wall construction as hydrolyases or glycosidases. The second contains non enzymatic agglutinins, flocculins, or β-(1, 3) glucan cross connectors. Most of these proteins are glycosylated. Proteins covalently attached to the cell wall are referred to as CWP and fall into GPI proteins, mild alkali releasable proteins, and disulphide linked proteins. The third group consist of single pass plasma membrane proteins with short C-terminal cytoplasmic domains and long Ser/Thr rich extracellular regions, which also have N-terminal cysteine rich domains. These are the mechanosensors that detect cell wall stress and activate the cell wall integrity signalling pathway (CWI) (Orlean, 2012). The CWI pathway is responsible for glucan synthesis at the site of wall remodelling, gene expression related to cell wall biogenesis, organization of the actin cytoskeleton, and secretory vesicle targeting to the growth site (Levin, 2005). Some mannoproteins have, or are predicted to have, enzymatic activity as hydrolases or cross linkers; others may have structural roles or mediate “social activity” by serving as mating agglutinins or flocculins (Teparic and Mrsa, 2013).

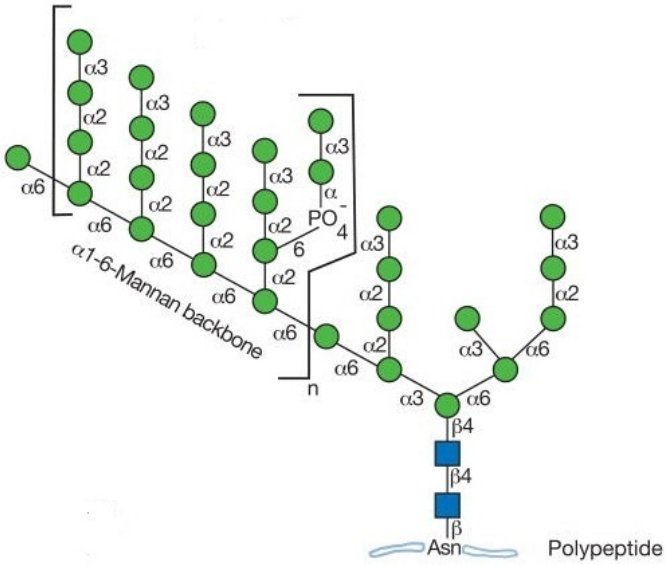


Figure 1.9. N-linked mannan oligosaccharide
Source: (Nani, 2007)

1.6 MOS and the control of pathogenic bacteria

Adhesion of pathogenic organisms to host tissues is the prerequisite for the initiation of the majority of infectious diseases (Sharon, 2006). Adhesion is required so that the organisms are not swept away by the natural cleansing mechanisms of the host, like airflow in the respiratory tract or urine flow in the urinary tract, leading eventually to the penetration of bacteria into the tissues. The most common means of adhesion is mediated by lectins present on the surface of the infectious organism that bind to complementary carbohydrates on the surface of the host tissues (Sharon, 2006). These filamentous structures were recognized and characterized for several Gram-negative species by two research groups, one led by James Duguid, who referred to these structures as fimbriae (derived from the Latin word for fringe); and the other led by Charles Brinton, who preferred to call these structures pili (derived from the Latin word for hair or fur) (Telford et al., 2006). These bacterial lectins are typically in the form of elongated submicroscopic multi-subunit protein appendages, expression of which is dependent on growth conditions (Sharon, 2006).

Compelling evidence for the role of fimbriae in bacterial infection derives from experiments in which blocking the fimbriae by suitable sugars provided protection against such infection. Fimbriae bind mono- and oligosaccharides reversibly and with

high specificity, but are devoid of catalytic activity, and in contrast to antibodies, are not products of an immune response. On the basis of their specificity, fimbriae are classified into five groups, according to the monosaccharide for which they exhibit the highest affinity: mannose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose, and N-acetylneuraminic acid. These sugars are typical constituents of the surfaces' of eukaryotic cells. Studies with fimbriated *E. coli* (as well as purified fimbriae) and uroepithelial cells suggested that adherence induces a two way flow of biological cross talk via the fimbrial bridge, affecting both partners. The target cell is activated, with resultant production of cytokines that engender acute inflammation and symptoms of disease. In the bacterium the interaction leads to up regulation of two signal transduction systems that allow responses to the changing environment (Lis and Sharon, 1998).

Since anti adhesive agents do not act by killing or arresting the growth of the pathogens, it is very likely that strains resistant to such agents will emerge at a markedly lower rate than of strains that are resistant to antibiotics. Saccharides are ideal for this purpose as they are unlikely to be toxic or immunogenic, since many of those that inhibit bacterial adhesion are normal constituents of cell surfaces or body fluids (Sharon, 2006).

Yeast agglutination tests have been used to show the binding of pathogenic bacteria to yeast and yeast cell wall (Tiago et al., 2012, Perez-Sotelo et al., 2005, Spring et al., 2000). Spring et al. (2000) demonstrated that the MOS fraction of yeast cell wall was responsible for the agglutination of type 1 fimbriae containing *Salmonella* and *E. coli* spp. *in vitro* and agglutination was inhibited in the presence of mannose. In addition, their study reported a reduction of *Salmonella typhimurium* colonisation *in vivo* when MOS was added to the basal diet of newly hatched chicks. However, as agglutination tests are qualitative they do not provide a means to quantify the amount of bacteria bound to MOS.

1.7 Actigen®

Alltech, Inc. (Nicholasville, Kentucky, USA) has pioneered industrial extraction procedures that are currently used to produce functional mannanoligosaccharides extract from the cell wall of *Saccharomyces cerevisiae* var. *boulardii* and this is marketed as

Actigen®. It is a prebiotic; a non digestible food ingredient which may be fermented in the hind gut (Cuskin et al., 2015, Nurmi and Rantala, 1973). It added to animal diets and results have shown that it improves feed efficiencies, weight gain, reduces mortalities, decreases pathogen load and improves overall gut health (Hooge, 2004a, Hooge, 2004b). Actigen® can bind enteropathogenic bacteria such as *Salmonella* and *E. coli* spp. and reduce colonisation (Luquetti et al., 2012, Biggs et al., 2007). *Saccharomyces cerevisiae*, a budding yeast, is commercially produced under controlled conditions in an aerobic environment. The yeast cells are autolysed and the cell wall material is separated from the intracellular contents. The outer cell wall consists of mannan and glucan fractions; the mannan portion is extracted for Actigen® production. The fractionated liquid product is pumped to low temperature spray driers where the liquid is evaporated at the lowest temperature possible in the shortest time. The final dried product is collected in a cooling chamber and held for the quality analysis and packaging.

1.8 Type 1 Fimbriae

Type 1 fimbriae are the most abundant surface structures both in pathogenic and non-pathogenic Gram-negative bacteria (Bernardi et al., 2013). Adhesion fimbriae are specialized to sustain attachment of bacterial cells under the environmental conditions surrounding their preferred host target tissue (Andersson et al., 2012). A single bacterial isolate often can express more than one type of fimbriae (Althouse et al., 2003). The best characterised fimbriae are the type 1 fimbriae of *E. coli*. Several genera of bacteria possess type 1 fimbriae, which are specific for mannose and allow the attachment to mannose receptors on various host tissues. In short, bacteria utilize the sugar decoration of cells, to colonize the cell surface, wherever cells are in contact with the outside environment, as in the case of epithelial cells (Hartmann and Lindhorst, 2011, Krogfelt et al., 1990). Type 1 fimbriae are expressed by a large number of *E. coli* strains, and are found in more than 95 % of *E. coli* isolates from the intestinal and extraintestinal infections such as urinary ones. They are also produced by other enterobacterial species including: *K. pneumonia*, *Salmonella typhimurium* and *Salmonella enteritidis* (Sharon, 2006). *In vitro* studies have shown that 80 % of *Salmonella typhimurium*, 67 % of *Salmonella enteritidis*, and over 65 % of *E. coli* possess fimbriae which attach to

mannose receptors (Duguid et al., 1966). The affinity of different phenotypes of *E. coli* type 1 fimbriae to mannose may differ within a factor of 15 and they can be functionally subdivided into either low mannose binding (M_1L) or high mannose binding (M_1H) phenotypes. These two basic phenotypes have been found to predominate in different niches (Sharon, 2006, Sokurenko et al., 1998). The galabiose specific P fimbriae and the N-acetylglucosamine specific F17 fimbriae of *E. coli* are also well characterised (Sharon, 2006). The K88 (F4) type 4 fimbrial adhesions are common in enterotoxigenic *E. coli* and are reported to bind to galactose residues (Payne et al., 1993).

Type 1 fimbriae, shown in Figure 1.10 A, are threadlike appendages consisting of about 1000 subunits of the FimA protein as well as a few percent of minor components. Structurally, the fimbriae are 1-2 μm long and 7 nm thick fibres, that are rod like, made up largely of repeating immunoglobulin like FimA subunits (mw 17 kDa) that are helically arranged in a structure referred to as a "shaft" (Madison et al., 1994). The shaft, anchored to an outer plasma membrane pore assembly platform termed usher (FimD), is joined to a short 3 nm thick distal tip fibrillum that consists of two adaptor proteins, FimF and FimG, and a third of a different kind, FimH (m.w. 29-31 kDa) (Figure 1.10 B). The latter is the only subunit that possesses a carbohydrate binding site and is thus responsible for the sugar binding activity of the fimbriae. FimH is also present in small numbers at intervals along the fimbrial filament, but only the subunit at the tip appears to be able to mediate mannose specific adhesive interactions, whereas the subunits at the other positions are inaccessible to the carbohydrate ligand (Sharon, 2006). Recombinant bacteria expressing fimbriae consisting of FimA only or FimA, FimF, and FimG but not FimH do not exhibit any binding ability (Krogfelt et al., 1990). Nearly all mutations in the combining site of FimH abolished or decreased its binding not only to mannose, but also to urinary epithelial cells, indicating that the site may be highly conserved (Sokurenko et al., 1998). Since the FimH binding site can accommodate only one α-mannoside, multivalency effects which have been observed in the inhibition of type 1 fimbriae mediated bacterial adhesion cannot be explained on the basis of the structure of mannose specific lectin FimH. Nevertheless, avidity effects have been frequently observed with a variety of multivalent mannose containing glycomimetics. Such avidity can originate from statistical effects arising from a higher concentration of mannose in the proximity of the carbohydrate binding site, the existence of additional carbohydrate binding sites on the lectin FimH, or the occurrence

of the natural multivalent process, since fimbriae occur on the bacterial surface in several hundreds of copies (Bernardi et al., 2013).

In vitro work has shown that the presence of D-mannose can inhibit type 1 fimbriae mediated association of *E. coli* to human mucosal cells (Ofek et al., 1977) and *Salmonella* to small intestinal enterocytes of the rat (Lindquist et al., 1987). Typically, type 1 fimbriae of *E. coli* have a considerably higher affinity for oligosaccharides such as the constituents of cell surface glycoproteins compared to mannose (Sharon, 2006). Type 1 fimbriae have been shown to cause an immune response through the activation of the target cells and production of high levels of certain cytokines, in particular TNF-α, can be induced by the purified type 1 fimbriae, as well as by the isolated carbohydrate binding subunit of the fimbriae (FimH) (Lis and Sharon, 1998).

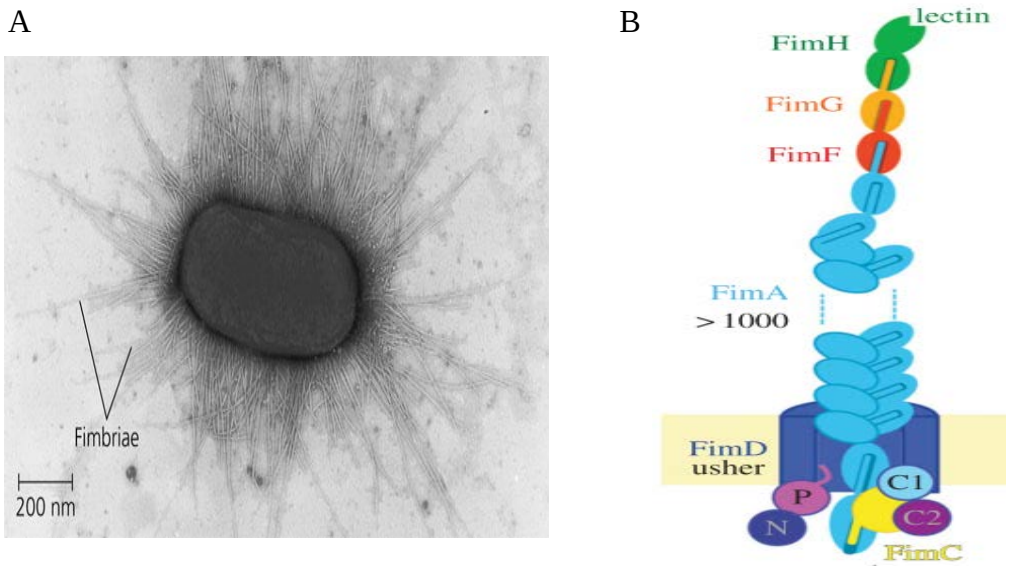


Figure 1.10. A: Type 1 fimbriated *E. coli*, and B: structure of Type 1 fimbriae.
Source: A:Sharon (2006) and B: Busch et al. (2015)

1.9 Assay development

During assay development, or assay optimisation, an analytical process or idea is defined and optimised into a robust and reproducible device that delivers results as intended. An assay's development requires continuous evaluation that should be clearly distinct from the validation phase. Optimising an assay involves choosing the optimal

format. With the intended use in mind, a new assay's appropriate performance characteristics are then defined. The optimisation phase is a continuous cycle that begins with defining these initial performance characteristics and continues until the performance metrics are established and there is confidence in the results that are obtained for the assay. Once a final optimised and feasible prototype design is completed, it proceeds to validation (Derzko, 2005).

1.9.1 Specificity/selectivity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present (ICH, 1995, ICH, 1996). Typically these may include impurities, degradants and matrix among others. The term "specific" generally refers to a method that produces a response for a single analyte only, while the term "selective" refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be specific. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.

1.9.2 Precision

Precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions (ICH, 1995). Precision may be considered at two levels:

Repeatability:

An assay's repeatability expresses the precision under the same operating conditions over a short interval of time and can also be termed intra-assay precision. The level of repeatability of an assay is assessed when the analysis is carried out in one laboratory by one operator using one piece of equipment over a relatively short time span (ICH, 1995).

Reproducibility:

Reproducibility is expressed as within laboratory variation. The extent to which reproducibility should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variables to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually (ICH, 1996).

1.10 Objectives of this study

A rise in food safety and production standards (e.g. the ban on antibiotics) has led to a search for alternative natural products to achieve a high yielding, more efficient method of meat production. Mannan oligosaccharides are one such class of product that can potentially provide this. Mannan oligosaccharides are abundant in the cell wall of *Saccharomyces cerevisiae* as well as other yeast species. Yeast cell wall mannan oligosaccharides have the capacity to inhibit type 1 fimbriae containing pathogens from binding to host tissue and prevent biofilm development. The mannan oligosaccharide acts as an alternative, more attractive, binding site for the pathogens and the mannan oligosaccharide – pathogen complex are removed from the host system.

A review of the literature on these yeast cell wall products has indicated that other than *in vivo* animal studies very few studies exist which demonstrate the efficacy of these types of products. Of the assays that do exist the majority of these tests are qualitative e.g. agglutination (Spring et al., 2000).

The initial aim of this project was the production and characterisation of cell wall samples from a variety of yeast species in search of a new or next generation MOS product. Alterations in the growing conditions of several yeast strains were examined to determine if by altering simple fermentation constituents it was possible to alter yeast cell wall composition. The saccharide content of the yeast cell wall material samples was determined. Another objective of this study was to develop a quantitative assay to assess the efficacy of yeast cell wall material to bind pathogens *in vitro*. This assay could be used to screen other yeast cell wall material samples in the search for a second generation yeast cell wall product. Correlation of the saccharide content with the

pathogen binding capacity was undertaken in an effort to understand some of the factors involved in the binding of type 1 fimbriae containing pathogens by yeast cell wall material. The specific goals of this thesis were:

- Development of a second generation yeast cell wall product by growing a number of GRAS listed yeasts with augmented growth medium. The altered growth conditions included carbon source supplementation. Saccharide composition of these yeast strains was determined by HPLC.
- Development and optimisation of an assay to quantify the capacity of YCWM to bind pathogens *in vitro*.
- Screening of samples using the developed pathogen binding assay.
- Analysis of the complexity of the glycan profiles of selected samples was performed.

Section 2

Materials and Methods

2. Materials and methods

2.1 Materials

2.1.1 Chemicals, solvents, and other reagents

2, 2'-azio-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABSA), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), acetonitrile, ammonium peroxisulphate, bovine serum albumin (BSA), calcium carbonate, D-(+)-fructose, D-(+)-galactose, D-(+)-glucose, D-(+)-maltose, D-(+)-mannose, D-(+)-trehalose, D-(+)-xylose, dithiothreitol (DTT), formic acid, glycerol, Gram stain kit, iodoacetamide (IAA), Jack Bean mannosidaes, oxalic acid, peptide N-glycosidase F (PNGase F), peptone (from soya), phosphate buffered saline, potassium chloride, potassium phosphate (dibasic), sodium chloride, sodium phosphate (monobasic), streptavidin peroxidase polymer, sucrose, sulfuric acid, tetramethylethylenediamine (TEMED), and TWEEN 20 were all supplied from Sigma-Aldrich, Arklow, Ireland.

Galactosidase was provided by Megazyme (Bray, Ireland)

Becton Dickinson (BD) Difco agar, nutrient broth (NB), peptone, yeast extract, and yeast extract-peptone-dextrose (YPD) broth were all supplied by Unitech, Dublin, Ireland.

LudgerTag™ 2-AB (2-aminobenzamide) Gycan labelling kit was purchased from LudgerTag, Oxford, U.K.

Phytips were ordered from PhyNexus, CA, USA.

RapiGest SF surfactant was supplied by Waters, Dublin, Ireland.

Amicon YM-10 regenerated cellulose 10 KD MWCO filters were purchased from Fisher Scientific, Dublin, Ireland.

Rabbit polyclonal antibody to *Salmonella* group antigen (HRP labelled) (ab53299) and rabbit polyclonal antibody to *Escherichia coli* (Biotin labelled) (ab20640) were obtained from abcam® (Cambridge, UK).

Escherichia coli 10778, *Escherichia coli* 17076, *Escherichia coli* 30083, *Schizosaccharomyces pombe* 70572, *Rhodotorula mucilaginosa* 70825, *Rhodotorula*

mucilaginosa 18184, *Rhodotorula mucilaginosa* 70404, and *Candida utilis* 70167 were supplied by DSMZ GmbH, Braunschweig, Germany.

Salmonella enterica var. *dublin* 15480 was supplied by ATCC, American Type Culture Collection, VA, U.S.A.

Salmonella enterica var. *enteritidis* 12694 was supplied by NCTC, National Collection of Type Cultures, Salisbury, U.K.

Salmonella enterica var. *gallinarum* 2931, and *Salmonella enterica* var. *typhimurium* 2514 were supplied by KCTC, Korean Collection for Type Cultures, Yuseong-gu, Daejeon 305-806, Korea.

Saccharomyces cerevisiae 72, *Saccharomyces cerevisiae* 358, *Saccharomyces cerevisiae* 88, *Saccharomyces pastorianus* 203, *Saccharomyces cerevisiae* 695, *Pichia membranifacieus* 326, and *Kluyveromyces lactis* 752 were supplied by NCYC, National Collection of Yeast Cultures, Institute of Food Research, Norwich, U.K.

Kluyveromyces marxianus 2415, *Kluyveromyces marxianus* 701, *Saccharomyces cerevisiae* 67, Alltech SC1, Alltech SC2, and Alltech SC 3, Alltech SC 4 were obtained from Alltech Inc., Nicholasville, Kentucky, U.S.A.

Kluyveromyces lactis 141 and *Saccharomyces cerevisiae* 4070 were supplies by CBS, CBS-KNAW, Fungal Biodiversity Centre, Utrecht, The Netherlands.

Yeast cell wall material was made available by Alltech Inc. (Nicholasville, KY 40356, USA).

2.2 Methods

2.2.1 Growth and maintenance of bacteria

A bacterial suspension was prepared from a stored glycerol stock by aseptically transferring 100 μL to a 20 mL fresh sterile nutrient broth (NB) and incubated at 37 °C for 24 hours at 150 RPM a Grant-bio orbital shaker incubator ES 20. Aliquots of the suspension were used to subculture fresh nutrient agar plates and incubated for 24 hours at 37 °C. Single colony isolates (SCI) were used to inoculate fresh agar plates and were incubated at the appropriate conditions. These plates were kept for short term storage at 4 °C from which liquid starter cultures of pathogen were inoculated. For long term storage, frozen stocks of bacteria (liquid culture) were prepared and stored at -70 °C in 10 % (v/v) growing culture in 70 % (v/v) glycerol.

A liquid culture of pathogen was inoculated from a SCI and incubated under the appropriate conditions. Cells were harvested by centrifugation at 4 °C and washed three times with ice cold sterile 10 mM phosphate buffered saline (PBS), pH 7.4, to remove any residual growth medium. The cell pellet was resuspended in 10 mM PBS and the optical density at 650 nm (OD_{650nm}) for each pathogen was determined using a Shimadzu 1601 PC UV-visible Spectrophotometer. The samples were adjusted to their required OD_{650nm} using 10 mM sterile PBS for further analysis.

2.2.2 Microscopic imaging of pathogen and cell wall material binding

The ability of yeast cell wall material to agglutinate pathogens was evaluated according to the protocol used by Perez-Sotelo et al. (2005) with the following modifications: an overnight bacterial culture was washed to remove any growth medium and suspended in 10 mM PBS, pH 7.4, to an OD_{650nm} of approximately 1.0. Ten μL of this was mixed with 10 μL of (1 % w/v) yeast cell wall material suspension in 10 mM PBS, pH 7.4, on a microscope slide. A control sample contained equal volumes (10 μL) of testing bacteria and 10 mM PBS, pH 7.4, and was analysed in parallel with the other slides. All the slides were stained with Safranin O using a standard method. Slides were observed at 10, 40, and 100 X magnifications by light microscopy (Olympus BX53). Adhesion

was subjectively evaluated by observing the amount of bacteria bound to the yeast cell wall material (Perez-Sotelo et al., 2005).

2.2.3 Sample and standard preparation

2.2.3.1 Preparation of yeast cell wall material standard

Exact quantities of yeast cell wall material were weighed into 500 mL of sterile 10 mM PBS, pH 7.4, making a concentration of 10 μg 100 μL^{-1}. The mixture was stirred for 1 hour at room temperature to ensure homogenous mixing. A 40 mL aliquot of the yeast cell wall material suspension was collected. The suspension was sonicated twice, using a Branson sonifier 150, at a power setting of 15 Watts for 30 seconds, keeping the sample on ice between rounds of sonication. The sample was stored at 4°C prior to use or at -70 °C for long term storage. These samples were utilized in the yeast cell wall material activity assay.

2.2.3.2 Preparation of yeast samples

Yeast samples were prepared and harvested as described in Section 2.2.8. Samples were weighed into cold, sterile 10 mM PBS, pH 7.4, 5 % (w/v). The yeast cells were lysed by sonication to break open the yeast cells. The suspension was sonicated for ten rounds of 30 seconds at a power setting of 15 Watts, keeping the suspension on ice between rounds. The sonicated samples were centrifuged to separate the cell wall material from the intercellular matrix. The pellet containing the cell wall material was freeze dried and stored at room temperature until needed.

2.2.4 Quantification of bacteria

2.2.4.1 Determination of colony forming units of a bacterial strain by plate count method

To quantify the colony forming units (CFUs) of a bacterial strain suspension, nutrient agar plates were prepared according to the manufacturer's instructions. A standard curve of dilutions was prepared for the pathogen suspension in sterile 10 mM PBS, pH

7.4. The OD_{650nm} of the dilutions were recorded on a Shimadzu 1601 PC UV-visible spectrophotometer. Serial dilutions were made of the bacterial strain suspension before inoculation onto agar plates in order for the CFUs to be within the countable range of 30-300 CFUs. The agar plates were inoculated, in triplicate, with 100 µL of the bacterial strain dilutions and spread using a sterile T-spreader. The agar plates were incubated overnight at 37 °C. The CFUs were counted, recorded and the CFUs were adjusted to CFU mL^{-1}. A standard curve was plotted using the optical density values versus the CFU mL^{-1} values.

2.2.4.2 Quantification of bacteria using an enzyme linked immunosorbent assay

A liquid culture of pathogen was prepared under the appropriate conditions and cells were harvested as outlined in Section 2.2.1. A standard curve for each bacterial strain was created by dilution with sterile 10 mM PBS, as in Section 2.2.4.1. The OD_{650nm} of the pathogen dilutions were recorded on a Shimadzu 1601 PC UV-visible spectrophotometer. Each dilution of the bacterial solution at OD_{650nm} (100 µL $well^{-1}$), were mixed together in a well of a NUNC Maxisorb 96-well micro-titre plate with an equal volume of sterile 10 mM PBS, bringing the final volume to 200 µL $well^{-1}$. The plate was incubated overnight at 37 °C with constant shaking (150 RPM in a Grant-bio orbital shaker incubator ES 20). After the overnight incubation, the micro-titre plate was washed twice with 300 µL wash buffer (10 mM PBS, pH 7.4).

For the *Salmonella* strains, 200 µL of blocking solution (3 % (w/v) bovine serum albumin (BSA) in 10 mM PBS) was added to each well and incubated at room temperature for 1 h, to prevent non-specific binding. After blocking, the plate was washed twice with 300 µL washing buffer. Each well was treated with 100 µL of antibody solution (0.4 µg L^{-1} HRP labelled rabbit polyclonal antibody to *Salmonella* group antigen in 10mM PBS containing 1 % (w/v) BSA and 0.05 % (v/v) Tween). The plate was incubated at room temperature for 1 hour.

After four additional post-incubation washes, 100 µL of substrate (2, 2'-Azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABSA)) was added to each well. Following incubation at room temperature for 20 min, the reaction was stopped by the addition of stopping reagent (100 µL of 0.5 M oxalic acid) to each well. The optical density was

read at 410 nm, using a BioTek Synergy HT micro-titre plate reader (BioTek Instruments Inc., Winooski, Vermont, USA). The absorbance values at 410 nm for each point on the standard curve were recorded and plotted against the CFU mL^{-1} value for the corresponding standard point determined by the plate count method (Section 2.2.4.1).

For the *E. coli* strains, strains were plated as outlined for *Salmonella*. Blocking solution (200 µL), containing 4 % (w/v) BSA in 10 mM PBS for O157:H7 and O2:K1:H-, and containing 3 % (w/v) BSA in 10 mM PBS for O1:K1:H7, was added to each well and incubated at room temperature for 1 h, to prevent non-specific binding. After blocking, the plate was washed twice with 300 µL washing buffer. Each well was treated with 100 µL of antibody solution (0.4 µg mL^{-1} of biotin labelled rabbit polyclonal antibody to *E. coli* for O157:H7, 4 µg mL^{-1} of the biotin labelled for O2:K1:H-, and 1.6 µg mL^{-1} of the biotin labelled antibody for O1:K1:H7, in 10mM PBS containing 1 % BSA (w/v) and 0.05 % (v/v) Tween). The micro-titre plate was then incubated at room temperature for 1 hour.

The plate was washed four times with 300 µL 10 mM PBS, pH 7.4. Each well was treated with 100 µL of streptavidin peroxidise solution (1 µg mL^{-1} streptavidin peroxidise polymer in 10mM PBS containing 1 % (w/v) BSA and 0.05 % (v/v) Tween) and was incubated at room temperature for one hour. After four additional post-incubation washes, 100 µL of substrate (ABSA) was added to each well. Following incubation at room temperature for 20 min, the reaction was stopped by the addition of stopping reagent (100 µL of 0.5 M oxalic acid) to each well. The optical density was read at 410 nm, using a BioTek Synergy HT micro-titre plate reader. The absorbance values at 410 nm for each point on the standard curve were recorded and plotted against the CFU mL^{-1} value for the corresponding standard point determined by the plate count method (Section 2.2.4.1).

2.2.5 Yeast cell wall material binding activity assay for *Salmonella* and *E. coli* serotypes

A yeast cell wall material standard (10 µg 100 μL^{-1}) and a bacterial solution (100 µL $well^{-1}$, of 0.03 OD_{650nm} for each strain of *Salmonella*, and an OD_{650nm} of 0.08 for *E. coli*

O157:H7, 0.12 for *E. coli* O2:K1:H-, and 0.06 for *E. coli* O1:K1:H7), were prepared as described in Sections 2.2.3.1 and 2.2.1, respectively. The yeast cell wall material and the pathogen were mixed together in a well of a NUNC Maxisorb 96-well micro-titre plate and incubated overnight at 37 °C at 150 RPM in a Grant-bio orbital shaker incubator ES 20. A yeast cell wall material control was prepared by mixing 100 μL of yeast cell wall material standard and 100 μL of 10 mM PBS, pH 7.4, in a well of the plate; while a pathogen control was prepared by mixing 100 μL of the bacterial solution and 100 μL of 10 mM PBS, pH 7.4, in a well of the plate. The plate was incubated overnight at 37 °C at 150 RPM in a Grant-bio orbital shaker incubator ES 20. The plate was washed twice with 300 μL washing buffer (10 mM PBS, pH 7.4). The quantity of bacteria remaining on the plate was determined as outlined in Section 2.2.4.2.

As a known quantity of bacteria was added to the plate initially, the quantity of bacteria bound to yeast cell wall material is calculated by subtracting what is left on the plate from what was added to the plate initially. The activity of the yeast cell wall material was calculated as follows:

$$\%\ Activity = \frac{CFU\ bound\ to\ YCWM}{CFU\ added\ initially} \times 100$$

2.2.6 Determination of the optimum bacterial cell number for use in the yeast cell wall material activity assay

These determinations were conducted by preparing a serial dilution of the pathogen suspension in sterile 10 mM PBS, each pathogen was cultured as described in Section 2.2.1. The optical density for each dilution was recorded on a Shimadzu 1601 PC UV-Vis spectrophotometer. A yeast cell wall material standard (10 μg 100 μL^{-1}), prepared as in Section 2.2.3.1, was mixed with 100 μL of each dilution of the pathogen solution in a well of a micro-titre plate. A control was prepared by mixing 100 μL of each dilution of the pathogen solution with 100 μL of 10 mM PBS, pH 7.4. The mixture was incubated overnight at 37 °C at 150 RPM in a Grant-bio orbital shaker incubator ES 20. The plate was analysed according to the ELISA protocols in Section 2.2.5. The percent of pathogen bound by the yeast cell wall material standard was determined for each of the dilutions of the pathogen solution as described in Section 2.2.5.

2.2.7 Growth and maintenance of yeast strains

2.2.7.1 Yeast maintenance and storage

Yeast strains, as shown in Table 2.1, were cultured in 1 % (w/v) yeast extract, 2 % (w/v) peptone, and 2 % (w/v) dextrose medium (YPD), and sterilised by autoclaving at 105 °C for 30 minutes. SCIs of yeast isolated on YPD agar plates were used to aseptically inoculate 20 mL seed cultures which were cultivated overnight at 28 °C in a Gallenkamp (cat. 10X400XX2.C) shaking incubator chamber at 200 RPM.

Sterile inoculation loops were used to subculture fresh agar plates from the seed culture, which were incubated accordingly. These agar plates of actively growing cultures were used to inoculate liquid starter cultures or stored at 4 °C for up to a month. For long term storage, frozen stocks of yeast (liquid culture) were prepared and stored at -70 °C, 10 % (v/v) growing culture in 70 % (v/v) glycerol.

Table 2.1. Yeast strains and their reference identification code.

Yeast	Reference
Saccharomyces cerevisiae	72
Saccharomyces cerevisiae	88
Saccharomyces cerevisiae	SC 4
Saccharomyces cerevisiae	4070
Saccharomyces cerevisiae	695
Saccharomyces pastorianus	203
Saccharomyces cerevisiae	SC1
Saccharomyces cerevisiae	SC2
Saccharomyces cerevisiae	SC 3
Kluyveromyces marxianus	2415
Kluyveromyces marxianus	701
Kluyveromyces lactis	752
Pichia membranifaciens	326
Rhodotorula mucilaginosa	18184
Rhodotorula mucilaginosa	70825
Candida utilis	70167
Schizosaccharomyces pombe	70572

2.2.7.2 Yeast liquid fermentation conditions

A starter culture was prepared in 20 mL of YPD culture medium by adding a SCI of actively growing yeast isolated on YPD agar prepared according to Section 2.2.7.1. The yeast was allowed to grow in the medium at an appropriate temperature at 200 RPM in a Gallenkamp incubator for 1 day until confluent growth was achieved. From this starter culture of yeast, 1 mL was used to inoculate 100 mL of any subsequent liquid medium.

2.2.8 Carbohydrate supplementation of yeast fermentation

A liquid starter culture of yeast was prepared in fresh sterile medium (Section 2.2.7.2) according to the optimal growth conditions for yeast production. On the second day, the starter culture was distributed into test flasks in triplicate and diluted to 1 % (v/v). Fresh sterile modified YPD medium (100 mL) was set up in 250 mL Erlenmeyer flasks. The dextrose was omitted from the medium and the medium was supplemented with individual carbon sources (1.0 and 2.0 %, w/v). The carbon sources used were fructose, galactose, glucose, maltose, mannose and sucrose. Liquid cultures were grown at the appropriate conditions for 1 day, following which the yeast biomass was separated from the culture medium by centrifugation and washing with deionised water. This was repeated twice to ensure complete removal of growth medium. The yeast biomass was freeze dried and stored in sealed containers at room temperature until needed.

2.2.9 Compositional analysis of the yeast cell wall material

2.2.9.1 Acid hydrolysis and sample preparation

Dry biomass (0.3 ± 0.01 g) was accurately weighed in triplicate and placed in a test tube. Following careful mixing, the biomass was hydrolysed with 3 mL of 72 % (v/v) H_2SO_4 for 2 hours at 30 °C and mixed intermittently throughout the hydrolysis. Each hydrolysate was transferred to its own 100 mL serum bottle and diluted to a 4 % (v/v) sulphuric acid concentration by adding 84 mL deionised water. Each bottle was sealed using a crimper (Wheaton corporation, Millville, New Jersey) and autoclaved for 1 hour at 121 °C. After completion of the autoclave cycle, the samples were allowed to cool at room temperature before removing the seals and stoppers. Aliquots of the hydrolysate

(20 mL) were neutralised with calcium carbonate to a pH of between 5 and 6. The neutralised hydrolysate was filtered through a 0.2 μM filter (Chromafil ultra RC). A dual-component series of sugar calibration standards were prepared in deionised water. This contained D-(+) glucose and D-(+) mannose. Recovery standards of glucose, mannose, and an in house reference material (cell wall material) were hydrolysed in parallel with the yeast biomass samples.

2.2.9.2 Carbohydrate compositional analysis by high performance liquid chromatography

Separation by HPLC was performed using an Agilent Infinity 1290 liquid chromatography system with a refractive index detector. The mobile phase consisted of filtered, deionised water at a constant flow rate of 0.6 mL min^{-1} for 30 minutes. The column used for separation was a Bio-rad Aminex® HPX-87p column (300 X 7.8 mm), thermostatted at 80 °C, whilst accompanying column guards were maintained at room temperature. Samples were analysed for glucose and mannose and were quantified against reference standards of known concentration.

2.2.10 N-glycan analysis of yeast samples

2.2.10.1 Protein extraction from yeast cell wall material

Yeast proteins were extracted using CHAPS extraction buffer (150 mM NaCl, 20 mM sodium phosphate monobasic, pH 7.4, and 1 % CHAPS) and sonicated at 15 Watts for three rounds of 30 seconds, keeping the samples on ice between rounds of sonication. The sonicated material was agitated at 4 °C for 1 hour to maximise protein extraction. The samples were centrifuged at 16600 x *g* for 40 minutes at 4 °C to pellet the cellular debris. The supernatant containing the protein fraction was dried in a vacuum centrifuge.

2.2.10.2 In-gel block release of N-glycans with PNGase F.

The vacuum dried protein samples (Section 2.2.10.1) were reduced by adding 4 μL of 5 X sample buffer (0.625 mL of 0.5 M Tris, pH 6.6., 1 mL of 10 % SDS, and 3.375 mL of water), 15 μL of water, and 1 μL of 1.0 M DL-dithiothreitol (DTT) and incubated at 65 °C for 15 minutes. The samples were then alkylated by adding 1 μL of 100 mM indoleacetic acid (IAA) and were incubated for 30 minutes in the dark at room temperature. The sample was set into a gel block by adding 45 μL 30 % (w/w) acrylamide/ 0.8 % (w/v) bis-acrylamide stock solution, Tris (pH 8.8), 10 % SDS, 10 % ammonium peroxodisulfate (APS), and finally N, N, N, N'-tetramethyl-ethylenediamine (TEMED), mixed, and left to polymerize for 15 minutes. The gel samples were washed with 1 mL 100 % acetonitrile (ACN) for 10 minutes. The ACN was removed and the samples were washed with 1 mL of 20 mM $NaHCO_3$ for 10 minutes. This wash procedure was repeated once more, with a final wash with 1 mL ACN for 10 minutes. The gel pieces were centrifuged briefly to remove any traces of ACN. N-glycans were released by adding 25 μL PNGase F (2 U mL^{-1}), mixed gently with pipette action, and left at room temperature for 5 minutes. An additional 25 μL of PNGase was then added followed by 50 μL of 20 mM $NaHCO_3$ and was incubated over night at 37 °C. The released glycans were collected by washing the gel pieces with three by 200 μL of deionised water for 10 minutes, 200 μL of ACN for 10 minutes, 200 μL of water for 10 minutes and finally 200 μL of ACN for 10 minutes. The liquid fraction was collected after each wash step. The N-glycans were dried using a vacuum centrifuge overnight. The glycans were converted back to reducing aldoses with 20 μL 1 % (v/v) formic acid and the mixture was incubated at room temperature for 40 minutes. The samples were then dried for further processing.

2.2.10.3 2-AB glycan labelling and clean-up

The in-gel block released dried glycan samples were labelled by adding 5 μL 2-AB labelling solution (LudgerTag™ 2-AB labelling kit) and thoroughly mixed for 2 hours at 65 °C. Excess 2-AB was removed using Phytips. The glycans were dried overnight in a vacuum centrifuge. The samples were suspended in 75 % (v/v) ACN for HILIC UPLC analysis.

2.2.10.4 Hydrophilic interaction chromatography (HILIC) UPLC analysis of N-glycans

The N-glycans were analysed using an Agilent 1290 Infinity Ultra Pure Liquid Chromatography (UPLC) system, coupled to a fluorescence detector (UPLC-FLD). Separation was achieved on an Acquity UPLC 1.7 μm BEH Glycan column (2.1 X 150 mm), thermostatted at 40 °C. The samples were kept at 5 °C and the volume injection was 20 μL. A solvent system consisting of 50 mM ammonium formate, pH 4.4, (solvent A) and acetonitrile (solvent B) was used with the following gradients: 30 minute linear gradient with a flow rate of 0.561 mL min^{-1} (except for wash step): 30 % solvent A for 1.47 minutes, increasing to 47 % solvent A over 23.34 minutes, increasing to 70 % solvent A over 0.69 minutes; 70 % solvent A for 0.75 minutes and then for a further 0.3 minutes at a reduced flow rate of 0.4 mL min^{-1}, returning to 30 % solvent A over 0.3 minutes at a flow rate of 0.4 mL min^{-1}, then equilibrating with 30 % solvent A for 1.95 minutes with the flow rate returned to 0.561 mL min^{-1}. The sample loop was washed and purged with 80 % acetonitrile. Glycans were identified by comparison of retention times of a dextran standard used to generate glucose unit (GU) values, standard units for carbohydrate analysis, and relative percentage peak areas were calculated. Detection was performed with a fluorescence detector, set at the following wavelengths: excitation λ_{330nm} and emission λ_{420nm}. The UPLC system was calibrated by running an external standard of 2-AB dextran ladder (2-AB labelled glucose homopolymer) alongside the sample runs.

2.2.10.5 Confirmation of high mannose N-glycan identification

The 2-AB labelled glycan samples were digested with Jack Bean Mannosidase (JBM) to confirm the presence of high mannose N-glycans. Exoglycosidase digestion was carried out, as described by Royal et al. (Royle et al., 2008), on aliquots of the 2-AB labelled N-glycan pool, where pool refers to the combined sample from experiments performed in triplicate during the initial release of N-glyans (Section 2.2.10.2). On completion of the enzymatic digestion the enzyme was removed using 10 kD MWCO filters and centrifuged at 13, 105 x g for 2 minutes and then a further 5 minutes after a wash with deionised water. The supernatant was dried in a vacuum centrifuge and suspended in 66 % (v/v) ACN for HILIC UPLC analysis.

2.2.10.6 Confirmation of galactose N-glycan identification

The 2-AB labelled glycan samples were digested with galactosidase to confirm the presence of galactose N-glycan. Exoglyconsidase digestion was carried out as described by Royle et al. (2008) on aliquots of the 2-AB labelled N-glycan pool. On completion of the enzymatic digestion the enzyme was removed using 10 kDa MWCO filters as described in Section 2.2.10.5. The supernatant was dried in a vacuum centrifuge and suspended in 66 % (v/v) ACN for HILIC UPLC analysis.

2.2.11 Statistical analysis

Statistical analyses of results were performed using Minitab statistical software package version 17 (Coventry, U.K.). One way analysis of variance (ANOVA) and Tukey's multiple comparisons were carried out to test any significant differences among means, where the confidence level was set at 95 %. One way analysis of variance (ANOVA) and Dunnett's comparisons were carried out to test any significant differences between each test mean and a control mean, where the confidence level was set at 95 % (Tucker, 2003, Minitab, 2010). Significant levels were defined using $p \leq 0.05$. Spearman Rho correlation coefficient (r) between the content of total glucose and mannose, to the activity (%) of each yeast cell wall material sample was also determined. (The data was tested for normality) (Minitab, 2010).

Section 3

Results and Discussion

3 Results and Discussion

3.1 The effect of yeast growth medium augmentation on yeast cell wall saccharide composition.

Yeasts have been a component of the human diet for the last 7000 years (Cuskin et al., 2015). Food grade yeasts are used as sources of high nutritional value proteins, enzymes and vitamins, with applications in the health food industry as nutritional supplements. They are also used as food additives, conditioners and flavouring agents, for the production of microbiology media, as well as livestock feeds. Yeasts are included in starter cultures, for the production of specific types of fermented foods like cheese, bread, sourdoughs, fermented meat and vegetable products, vinegar, etc. (Bekatorou et al., 2006).

It is well documented that the cell wall of various yeast species vary in their oligosaccharide composition (Schiavone et al., 2015, Bzducha-Wrobel et al., 2013, Ene et al., 2012b, Xie and Lipke, 2010, Backhaus et al., 2010, Liu et al., 2009b, Nguyen et al., 1998, Alsteens et al., 2008). Glucan and mannan are the main oligosaccharide components of yeast cell wall, the quantities of which can be affected by environmental stress and growth conditions (Ene et al., 2012a, Backhaus et al., 2011, Liu et al., 2009b, Rodrigues et al., 2006, Aguilar-Uscanga and Francois, 2003). In addition to using *Saccharomyces cerevisiae* var. *boulardii*, the parent strain from which Actigen® is derived; it was decided to subject a number of different species of yeast to a varied regime of culture conditions as outlined in Section 2.2.8. Many of the strains chosen for this are used industrially. Fifteen ascomycetes, including species from the genera: *Saccharomyces*, *Candida*, *Kluyveromyces*, *Pichia*, and *Schizosaccharomyces*; and two species of basidiomycetes (*Rhodotorula*), were assessed and their cell wall saccharide content analysed. The cell wall composition of *Saccharomyces cerevisiae* and *Candida albicans* are well studied, no other yeasts have had the depth of biochemical, genetic, or genomic analysis as these two strains (Xie and Lipke, 2010). Even with the depth of current work on both strains very little published research exists on the effect of supplementation of various carbon sources into the growth medium on their cell wall composition (Aguilar-Uscanga and Francois, 2003). For this reason the effect different

carbon sources in the growth medium had on the cell wall composition of a number of strains of yeast was investigated.

3.1.1 Effect of carbon sources on *Saccharomyces* spp. cell wall saccharide content

The addition of 1 and 2 % (w/v) of simple carbon sources to the basal cultivation medium was studied to determine how it affected the saccharide composition of the cell wall. The basal medium consisted of 1 % (w/v) yeast extract and 2 % (w/v) peptone (YP); to this 1 or 2 % (w/v) carbon sources were added. The basal medium containing 2 % (w/v) glucose as the sole carbon source was chosen as the control as this is a common lab medium for the cultivation of yeast. Carbohydrate content of the cell wall samples was determined following acid hydrolysis and analysis of the hydrolysate by HPLC as described in Section 2.2.9. Acid hydrolysis of the yeast cell wall breaks the cell wall oligosaccharides into their component monosaccharides i.e. glucose and mannose (Bzducha-Wrobel et al., 2013, Klis et al., 2006). This method has been used extensively in the literature to determine the carbohydrate content of biomass (Ruiz and Ehrman, 1996). Tables 3.1-3.6 list the total cell wall glucose and mannose content of the 17 species of yeast used in this study following cultivation with various carbon sources at 1 % and 2 % (w/v) inclusion. The cell wall glucose and mannose content of *Saccharomyces* strains grown in 1 and 2 % (w/v) carbon source are shown in Tables 3.1 and 3.2.

Table 3.1. Saccharide composition of *Saccharomyces* cell wall following cultivation in growth medium containing a sole carbon source (1 % w/v)

Strain	Carbon source	% Glucose (w/w)	% Mannose (w/w)
Saccharomyces cerevisiae (72)	***Control***	11.4 ± 0.6	14.8 ±1.8
	Fructose	7.8 ± 4.3	14.2 ± 3.2
	Galactose	11.0 ± 0.3	14.5 ± 2.0
	Glucose	10.7 ± 0.8	14.6 ± 1.5
	Maltose	15.3 ± 3.0	14.6 ± 1.0
	Mannose	9.6 ± 1.1	16.7 ± 1.1
	Sucrose	12.2 ± 1.6	16.8 ± 3.0
Saccharomyces pastorianus (203)	***Control***	20.7 ± 4.5	15.7 ± 5.8
	Fructose	23.6 ± 0.5	19.6 ± 2.3
	Galactose	24.7 ± 0.7	17.0 ± 4.7
	Glucose	23.1 ± 3.6	20.6 ± 2.8
	Maltose	27.3 ± 3.1	20.4 ± 2.5
	Mannose	21.6 ± 0.3	17.7 ± 1.7
	Sucrose	22.2 ± 1.2	17.8 ± 4.8
Saccharomyces cerevisiae (4070)	***Control***	10.1 ± 3.5	14.0 ± 4.7
	Fructose	**21.1 ± 2.1***	14.0 ± 1.2
	Galactose	9.3 ± 2.0	16.6 ± 4.9
	Glucose	15.4 ± 6.1	16.4 ± 4.3
	Maltose	12.2 ± 5.3	14.0 ± 4.0
	Mannose	16.4 ± 6.1	16.7 ± 4.8
	Sucrose	13.2 ± 3.5	15.4 ± 2.5
Saccharomyces cerevisiae (SC 4)	***Control***	13.7 ± 0.1	12.4 ± 2.3
	Fructose	15.8 ± 3.3	13.9 ± 3.8
	Galactose	16.6 ± 4.9	13.7 ± 4.6
	Glucose	16.5 ± 3.7	12.5 ± 2.1
	Maltose	15.0 ± 4.6	14.3 ± 5.2
	Mannose	17.1 ± 2.0	14.7 ± 2.9
	Sucrose	14.4 ± 1.8	12.9 ± 3.4
Saccharomyces cerevisiae (695)	***Control***	10.6 ± 1.5	10.9 ± 1.9
	Fructose	14.1 ± 6.3	10.8 ± 1.9
	Galactose	10.9 ± 5.4	12.5 ± 2.1
	Glucose	12.7 ± 6.0	11.9 ± 2.7
	Maltose	14.2 ± 4.4	13.0 ± 2.2
	Mannose	9.1 ± 1.2	12.0 ± 1.4
	Sucrose	14.0 ± 4.0	11.2 ± 3.2

Results displayed are a representative of the mean of triplicate cultivations ± standard deviation. Statistical analysis was performed using Dunnett's test to evaluate the significance of samples when compared to the control and values with an asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the control. An independent sample t-test was performed to evaluate the significance of 1 and 2 % (w/v) carbon source in the growth medium. Values in **bold** denotes a significant change ($p \leq 0.05$) in the saccharide composition between the two concentrations.

Table 3.1 cont. Saccharide composition of *Saccharomyces* cell wall following cultivation in growth medium containing a sole carbon source (1 % w/v)

Strain	Carbon source	% Glucose (w/w)	% Mannose (w/w)
Saccharomyces cerevisiae (88)	**Control**	13.9 ± 0.8	14.8 ± 2.7
	Fructose	15.1 ± 1.2	14.3 ± 1.3
	Galactose	**11.0 ± 1.0**	12.0 ± 2.1
	Glucose	16.2 ± 0.3	14.7 ± 1.3
	Maltose	16.9 ± 0.3	16.3 ± 0.4
	Mannose	12.1 ± 1.3	16.9 ± 2.3
	Sucrose	9.9 ± 4.4	10.2± 6.3
Saccharomyces cerevisiae (SC 3)	**Control**	17.0 ± 1.4	13.9 ± 1.2
	Fructose	14.4 ± 2.5	13.1 ± 0.6
	Galactose	14.6 ± 2.3	11.7 ± 1.0
	Glucose	14.8 ± 1.4	13.6 ± 0.8
	Maltose	14.0 ± 1.2	13.2 ± 1.0
	Mannose	15.0 ± 1.3	14.6 ± 1.4
	Sucrose	15.7 ± 1.3	13.5 ± 0.9
Saccharomyces cerevisiae (SC 1)	**Control**	16.1 ± 0.5	18.5 ± 2.4
	Fructose	19.4 ± 2.7	13.8 ± 2.7
	Galactose	16.5 ± 0.7	19.2 ± 1.5
	Glucose	16.5 ± 1.6	17.4 ± 1.6
	Maltose	17.7 ± 0.6	20.2 ± 3.3
	Mannose	**23.5 ± 4.2***	**16.1 ± 2.1**
	Sucrose	16.4 ± 0.9	18.2 ± 1.7
Saccharomyces cerevisiae (SC 2)	**Control**	9.9 ± 1.7	12.2 ± 0.3
	Fructose	10.5 ± 1.1	13.0 ± 1.0
	Galactose	15.5 ± 4.0*	13.6 ± 2.3
	Glucose	10.2 ± 1.4	12.3 ± 0.4
	Maltose	11.5 ± 3.1	11.4 ± 1.4
	Mannose	10.1 ± 1.6	12.4 ± 0.7
	Sucrose	10.7 ± 2.4	12.0 ± 0.8

Results displayed are a representative of the mean of triplicate cultivations ± standard deviation. Statistical analysis was performed using Dunnett's test to evaluate the significance of samples when compared to the control and values with an asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the control. An independent sample t-test was performed to evaluate the significance of 1 and 2 % (w/v) carbon source in the growth medium. Values in **bold** denotes a significant change ($p \leq 0.05$) in the saccharide composition between the two concentrations.

A few changes were apparent when strains were cultured with 1 % (w/v) carbon source in the growth medium (Table 3.1). The cell wall glucose content of *S. cerevisiae* 4070 was significantly higher (21.3 %, w/w) than the control (10.1 %, w/w) when grown in 1 % (w/v) fructose as the sole carbon source. Mannose (1 %, w/v) as the sole carbon source significantly increased the glucose content of *S. cerevisiae* SC 1 (23.5 %, w/w) compared to the control (16.1 %, w/w). The glucose content of *S. cerevisiae* SC 2 was

significantly increased (15.5 %, w/w) compared to the control (9.9 %, w/w) when grown in medium containing 1 % (w/v) galactose. Although there were few significant differences observed between the cell wall glucose and mannose content of the samples grown in 1 % (w/v) carbon sources compared to the control, there were a number of interesting observations. The glucose cell wall content for all the strains of *Saccharomyces* ranged from 7.8 % to 27.3 % (w/w) and the mannose cell wall content ranged from 10.2 % to 20.2 % (w/w) depending on the carbon source used. Growth medium containing 1 % (w/v) fructose as the sole carbon source for *S. cerevisiae* 72 yielded the lowest amount of cell wall glucose content (7.8 %, w/w) for all the strains of *Saccharomyces*, however, it was not significantly different to the control. Strains *S. pastorianus* 203 and *S. cerevisiae* SC 1 had higher overall cell wall glucose and mannose content while *S. cerevisiae* 72 and SC 4 had lower overall cell wall glucose content, and *S. cerevisiae* 695 had the lowest overall cell wall mannose content compared to the other strains of *Saccharomyces*. The cell wall monosaccharide content of *S. pastorianus* 203 grown in 1 % (w/v) carbon sources was between 21.6 and 27.3 % (w/w) glucose content and between 17.0 and 20.6 % (w/w) mannose content. While all the values of cell wall glucose and mannose content were higher than the control values for *S. pastorianus* none were significantly different. *S. cerevisiae* SC 1 cell wall content was found to approach similarly high levels of glucose and mannose (16.4 – 23.5 %, w/w and 13.8 – 20.2 %, w/v) as *S. pastorianus* 203.

Table 3.2 shows the cell wall glucose and mannose content for all strains of *Saccharomyces* grown in 2 % (w/v) of the various carbon sources. Some variation was noted between the cell wall saccharide content of the *Saccharomyces* strains. The cell wall glucose content for all strains ranged from 8.9 % to 31.8 % (w/w), and the overall cell wall mannose content ranged from 10.9 % to 22.5 % (w/w). *S. pastorianus* 203 was again found to have the highest levels of cell wall glucose content of all the *Saccharomyces* strains, while *S. cerevisiae* SC 1 had the highest mannose content of all the *Saccharomyces* strains when cultured in growth medium containing 2 % (w/v) carbon source.

Differences exist between *S. cerevisiae* and *S. pastorianus*, for example, *S. pastorianus* is an allotetraploid hybrid yeast derived from *S. cerevisiae* and *S. bayanus* (Rainieri et al., 2006). It is a bottom fermenting yeast used in the production of lager, while *S. cerevisiae* (brewer's yeast) is a top fermenting yeast (Yamagishi and Ogata, 1999); each

strain is known to have different surface biochemical composition, surface hydrophobicity and aggregation properties. *S. cerevisiae* strains are richer in proteins and more hydrophobic (Dengis et al., 1995). The two strains have similar surface ultrastructure but have different cell wall elasticity and polysaccharide properties. Cell wall mannans are much more extended on *S. cerevisiae* compared to *S. pastorianus* (Alsteens et al., 2008). *S. pastorianus* also contains slightly different glucan linkages, β-(1, 4) linkages, in the cell wall than those of *S. cerevisiae* (Bastos et al., 2015, Pinto et al., 2015). All of these differences between the two species may contribute to the variations in their monosaccharide content.

Table 3.2 Saccharide composition of *Saccharomyces* cell wall following cultivation in growth medium containing a sole carbon source (2 % w/v)

Strain	Carbon source	% Glucose (w/w)	% Mannose (w/w)
Saccharomyces cerevisiae (72)	**Control**	11.4 ± 0.6	14.8 ±1.8
	Fructose	11.7 ± 2.4	15.9 ± 0.7
	Galactose	14.2 ± 2.4	15.9 ± 0.7
	Maltose	13.3 ± 2.3	16.8 ± 0.4
	Mannose	11.9 ± 2.2	18.4 ± 0.6
	Sucrose	11.7 ± 2.7	15.7 ± 0.6
Saccharomyces pastorianus (203)	**Control**	20.7 ± 4.5	15.7 ± 5.8
	Fructose	20.7 ± 3.8	14.7 ± 4.9
	Galactose	20.0 ± 4.4	15.1 ± 5.9
	Maltose	31.8 ± 5.1	22.5 ± 0.3
	Mannose	23.4 ± 1.0	13.5 ± 1.0
	Sucrose	20.6 ± 2.9	16.4 ± 4.6
Saccharomyces cerevisiae (4070)	**Control**	10.1 ± 3.5	14.0 ± 4.7
	Fructose	**15.0 ± 1.0**	14.4 ± 1.3
	Galactose	8.9 ± 0.7	16.4 ± 2.1
	Maltose	9.9 ± 1.5	17.0 ± 2.3
	Mannose	12.3 ± 1.2	17.4 ± 2.2
	Sucrose	10.1 ± 2.1	14.8 ± 2.9
Saccharomyces cerevisiae (SC 4)	**Control**	13.7 ± 0.1	12.4 ± 2.3
	Fructose	13.0 ± 0.8	12.8 ± 2.7
	Galactose	11.0 ± 0.9	12.9 ± 2.7
	Maltose	11.7 ± 1.7	11.2 ± 2.3
	Mannose	14.1 ± 1.5	12.2 ± 2.6
	Sucrose	13.9 ± 0.7	11.8 ± 2.3
Saccharomyces cerevisiae (695)	**Control**	10.6 ± 1.5	10.9 ± 1.9
	Fructose	11.3 ± 1.0	14.2 ± 3.7
	Galactose	10.4 ± 1.1	13.5 ± 3.1
	Maltose	13.0 ± 1.6	12.5 ± 3.0
	Mannose	9.3 ± 1.9	13.8 ± 3.1
	Sucrose	12.5 ± 3.1	11.8 ± 2.6

Results displayed are a representative of the mean of triplicate cultivations ± standard deviation. Statistical analysis was performed using Dunnett's test to evaluate the significance of samples when compared to the control and values with an asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the control. An independent sample t-test was performed to evaluate the significance of 1 and 2 % (w/v) carbon source in the growth medium. Values in **bold** denotes a significant change ($p \leq 0.05$) in the saccharide composition between the two concentrations.

Table 3.2 cont. Saccharide composition of Saccharomyces cell wall following cultivation in growth medium containing a sole carbon source (2 % w/v)

Strain	Carbon source	% Glucose (w/w)	% Mannose (w/w)
Saccharomyces cerevisiae (88)	**Control**	13.9 ± 0.8	14.8 ± 2.7
	Fructose	14.3 ± 0.1	16.6 ± 0.4
	Galactose	**14.4 ± 0.4**	13.8 ± 1.4
	Maltose	17.2 ± 0.3	16.2 ± 0.8
	Mannose	13.3 ± 1.9	16.8 ± 2.2
	Sucrose	12.0 ± 2.7	13.5 ± 3.4
Saccharomyces cerevisiae (SC 3)	**Control**	17.0 ± 1.4	13.9 ± 1.2
	Fructose	16.0 ± 1.7	14.0 ± 1.0
	Galactose	14.3 ± 2.2	13.4 ± 0.9
	Maltose	15.9 ± 1.6	13.2 ± 1.2
	Mannose	15.2 ± 2.4	14.9 ± 1.2
	Sucrose	17.9 ± 1.8	14.3 ± 1.3
Saccharomyces cerevisiae (SC 1)	**Control**	16.1 ± 0.5	18.5 ± 2.4
	Fructose	14.5 ± 0.9	20.5 ± 3.3
	Galactose	15.1 ± 2.6	20.1 ± 2.7
	Maltose	16.5 ± 0.8	20.1 ± 3.2
	Mannose	**14.8 ± 1.1**	**21.2 ± 0.8**
	Sucrose	14.2 ± 1.4	18.0 ± 0.5
Saccharomyces cerevisiae (SC 2)	**Control**	9.9 ± 1.7	12.2 ± 0.3
	Fructose	9.9 ± 1.6	13.3 ± 1.2
	Galactose	10.1 ± 1.8	11.6 ± 0.6
	Maltose	11.2 ± 1.3	11.8 ± 0.7
	Mannose	10.4 ± 1.9	13.5 ± 1.0
	Sucrose	10.8 ± 1.5	11.5 ± 1.0

Results displayed are a representative of the mean of triplicate cultivations ± standard deviation. Statistical analysis was performed using Dunnett's test to evaluate the significance of samples when compared to the control and values with an asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the control. An independent sample t-test was performed to evaluate the significance of 1 and 2 % (w/v) carbon source in the growth medium. Values in **bold** denotes a significant change ($p \leq 0.05$) in the saccharide composition between the two concentrations.

Conversely to 1 % (w/v) fructose as the sole carbon source, 2 % (w/v) fructose as the sole carbon source did not cause any significant changes in the cell wall glucose content of *S. cerevisiae* 4070 compared to the control. However, the cell wall glucose content of *S. cerevisiae* 4070 was significantly decreased from 21.5 % to 15.0 % (w/w) with an increase in fructose concentration in the growth medium from 1 to 2 % (w/v), indicating that the increased concentration of fructose significantly affects the glucose content in the cell wall. No other significant differences were observed for cell wall saccharides of

the other strains of *Saccharomyces* when the growth medium contained fructose as the sole carbon source.

The metabolic pathway of fructose fermentation is similar to that of glucose. After phosphorylation, fructose-6-phosphate readily enters glycolysis by conversion into fructose-1, 6-bisphosphate, while glucose-6-phosphate still has to be converted first into fructose-6-phosphate by phosphogluco-isomerase (PGI). The differences in glucose and fructose metabolism appears to be located in the transport and/or phosphorylation steps (Berthels et al., 2004). The transporters are shared although their affinity for glucose is higher than for fructose. Yeast cells possess glucose sensor proteins in the plasma membrane of which at least one, Gpr1, is known to have a different affinity for glucose and fructose (Rolland et al., 2001). It is not known whether yeast cells posses a specific fructose sensor. *S. cerevisiae* cells grown on medium lacking glucose but containing fructose were found to exhibit enhanced agar invasion (Palecek et al., 2002). Fructose also induces the expression of Snf1 in the absence of glucose (Palecek et al., 2002); Snf1 kinase complex, a key regulator of yeast carbohydrate metabolism, also plays a role in the maintenance of cell wall integrity. An increase in Snf1 kinase was shown to cause a decrease in yeast cell wall thickness with an increase in carbon source concentration (Backhaus et al., 2013). In this study, an increase in fructose concentration in the growth medium caused a decrease in cell wall glucose content of one strain of *Saccharomyces* i.e. *S. cerevisiae* 4070. A decrease in cell wall glucose may be associated with a decrease in cell wall thickness as glucan is a major part of the cell wall structure (Orlean, 2012).

Galactose as the sole carbon source at 2 % (w/v) in the growth medium had one significant effect on the cell wall saccharide content of these strains of *Saccharomyces*. The glucose content of *S. cerevisiae* 88 was found to increase significantly from 11.0 to 14.4 % (w/w) with an increase in galactose concentration from 1 to 2 % (w/v). Galactose is known to be a slow fermenting sugar in *S. cerevisiae*. In contrast to glucose and fructose, which are directly incorporated into glycolysis, galactose needs some additional metabolic steps; it is metabolised by the enzymes of the Leloir pathway (Fonseca et al., 2013). This pathway requires five enzymes to convert galactose to glucose-6-phosphate. In order for galactose to be metabolised it must first be transported into the cells. Specific sugar transporters are expressed under certain conditions. The high affinity galactose transporter Gal2p is expressed in the presence of

galactose and repressed in the presence of glucose (Timson, 2007). An increase in cell wall glucan grown in galactose as the single carbon source for a single strain of *S. cerevisiae* (CEN.PK133-7D) was described by Aguilar-Uscanga and Francois (2003). Regulation of cell wall integrity proteins such as β-(1, 3) and β-(1, 6) glucan synthases Fks1, Fks2, and Kre5 by galactose in some strains of yeast, including *S. cerevisiae*, have been reported (Ovalle et al., 1998). These syntheses are involved in cell wall construction (Sekiya-Kawasaki et al., 2002). *S. cerevisiae* cells grown on medium lacking glucose but containing galactose exhibited enhanced agar invasion (Palecek et al., 2002), suggesting morphological changes occur in the presence of galactose which may affect the cell wall saccharide content of the yeast. This was seen with *S. cerevisiae* SC 2 and 88 in this study although the effect was seen with 1 % (w/v) galactose for *S. cerevisiae* 88 and with 2 % (w/v) galactose for *S. cerevisiae* SC 2.

Glucose is the preferred carbon source of *S. cerevisiae*, if present even at low levels compared to alternative carbon sources it will be metabolised exclusively (Bisson et al., 1993). The central pathways of carbohydrate metabolism have evolved to process the hexose monosaccharide glucose (Timson, 2007). Glucose in the growth medium did not cause any significant changes in the cell wall saccharide content of these *Saccharomyces* spp. nor was it found to have any significant effects on *Saccharomyces* cell wall saccharide content with an increase in the glucose concentration from 1 to 2 % (w/v). Perhaps, lower or even higher concentrations of glucose would cause changes in the cell wall saccharide content not observed at the concentrations used in this study.

No significant differences were observed for the cell wall glucose (w/w) content for the *Saccharomyces* strains when grown in growth medium containing 1 or 2 % (w/w) maltose. Maltose as the sole carbon source was found to cause changes in the glucose and mannose content in the cell wall of *S. cerevisiae* (Aguilar-Uscanga and Francois, 2003), but no such changes were seen in this study with maltose as the sole carbon source in the growth medium.

Mannose as the sole carbon source in the growth medium of 2 % (w/v) did not cause any significant changes in the cell wall content of the *Saccharomyces* strains analysed in this study compared to the control. Even so, the differences in the cell wall saccharide content of *S. cerevisiae* SC 1 between samples grown in 1 and 2 % (w/v) mannose were significant. The cell wall glucose content increased and the cell wall mannose content

decreased with the increase of mannose concentration in the growth medium. Many of the enzymes of the glycolytic pathway are so specific for glucose that other sugars, even other hexoses, are not processed at any appreciable rate (Timson, 2007). Mannose is metabolised through different pathways compared to glucose and fructose. In all eukaryotes, the activation of mannose initiates from formation of mannose 6-phosphate (Man-6-P), which occurs by one of two routes: direct phosphorylation of mannose or interconversion from fructose 6-phosphate via phosphomannose isomerase. Excess mannose concentrations can lead to an accumulation of Man-6-P which inhibits the activity of phosphoglucose isomerase and thus represses glycolysis, protein biosynthesis, and cell wall biogenesis (Jin, 2012, Pitkanen et al., 2004). This may explain the significant decrease in cell wall glucose content of SC 1 when the concentration of mannose was increased from 1 to 2 % (w/v) in the growth medium. Palecek et al. (2002) found that changing the carbon source from glucose to mannose caused the cell wall composition of a single strain of *S. cerevisiae* to change leading to the conclusion that mannose induced a number of genes related to cell wall structure and maintenance proteins. Aguilar-Uscanga and Francois (2003) found that mannose supplementation caused changes in the glucan and mannan content of the cell wall compared to a glucose supplemented control for a strain of *S. cerevisiae*. Nonetheless, only one strain of *S. cerevisiae* (SC 1) was significantly affected in this study. The difference in the metabolism of mannose by *S. cerevisiae* compared to other hexoses was not reflected in the cell wall saccharide content of the majority of the strains used in this study apart from *S. cerevisiae* SC 1.

No statistically significant findings were observed between the monosaccharide content of the control and that of the samples grown in sucrose as the sole carbon source or indeed due to an increase in sucrose concentrations. Sucrose is rapidly hydrolysed to fructose and glucose by extracellular invertase followed by uptake and incorporation into the glycolytic pathway by *S. cerevisiae* (Liu et al., 2009b, Lorenz et al., 2000). Interestingly, neither of the disaccharides used in this study (sucrose or maltose) caused any significant changes in the cell wall glucose or mannose content of any of the *Saccharomyces* strains used compared to the control. It appears from this work that sucrose and maltose act similarly to glucose in terms of their effect on the cell wall glucose and mannose content in these strains of *Saccharomyces*.

As strains of *S. cerevisiae* differ slightly from each other, changes in the cell wall architecture and content can vary (Xie and Lipke, 2010). Schiavone et al. (2015) found that the glucan, mannan and chitin composition between two strains of *S. cerevisiae* were different when grown in a lab medium containing yeast extract, peptone and glucose under the same conditions. The glucan content was higher and mannan content lower in one strain compared to the other. Differences in the cell wall glucose and mannose content of *Saccharomyces* strains and their controls were also observed in this research. A number of studies on the effects of various carbon sources, including monosaccharides and disaccharides, on the cell wall composition of *S. cerevisiae,* found that the cell wall glucan and mannan content did change depending on the carbon source (Liu et al., 2009b, Palecek et al., 2002, Aguilar-Uscanga and Francois, 2003). Growth medium and especially carbon source can influence the cell wall composition (Jaehrig et al., 2008).

The absence of glucose and the presence of different carbon sources have been shown to cause metabolic changes in *Saccharomyces* strains. About 163 genes from *S. cerevisiae* are up regulated in glucose limited conditions. Many of these genes are still poorly characterized and their function unknown (Weinhandl et al., 2014). The effect of various carbon sources on the metabolic pathways of yeast may be a factor in the variation of the cell wall monosaccharide content of individual strains grown in different carbon sources.

3.1.2 Effect of carbon source on *Kluyveromyces* spp. cell wall saccharide content

Three strains from the genus *Kluyveromyces* were grown in the modified medium and the glucose and mannose content of their cell wall was analysed as described previously for the *Saccharomyces* strains. The outer surface of *Kluyveromyces* spp. were shown to have a three layered ultra structure cell wall consisting of a plasma membrane, a polysaccharide glucan layer and an electron dense mannoprotein outer layer (Backhaus et al., 2010), similar to *S. cerevisiae* (Klis et al., 2010, Uccelletti et al., 2005) with comparable polysaccharide and mannoprotein composition (Lukondeh et al., 2003a, Lukondeh et al., 2003b). The monosaccharide content of the *Kluyveromyces* samples used in this study are shown in Tables 3.3 and 3.4 for 1 and 2 % (w/v) carbon sources.

When considering *Kluyveromyces*, the relatively close phylogenetic relationship with *Saccharomyces* might be expected to facilitate direct comparisons, whereas, with sugar metabolism, *Saccharomyces* is quite unique and has developed a whole series of idiosyncrasies not found in other yeast (Lane and Morrissey, 2010). *Kluyveromyces*, in contrast to *S. cerevisiae*, did not undergo a whole genome duplication event (Fares et al., 2013). Unlike *S. cerevisiae*, *Kluyveromyces* are Crabtree negative yeast (Lane et al., 2011), lacking the ability to produce ethanol under aerobic condition, and preferentially direct metabolism towards the TCA cycle and optimum energy generation. *Kluyveromyces* are one of the few strains that can use lactose as a carbon and energy source (Lane and Morrissey, 2010, Dong and Dickson, 1997). As such, variation in the cell wall monosaccharide content between *Kluyveromyces* and *Saccharomyces* species were expected. *K. lactis* grows faster on lactose as a carbon source than on glucose (Madinger et al., 2009, Dong and Dickson, 1997). *K. marxianus* is a species that is substantially different to the related *K. lactis* (Lane et al., 2011). The dairy yeast *K. marxianus*, a GRAS organism, has the fastest growth rate of any eukaryotic microbe, thermotolerance, and the capacity to assimilate a wide range of sugars (Fonseca et al., 2013, Lane and Morrissey, 2010).

Table 3.3. Saccharide composition of *K. lactis* and *K. marxianus* cell wall following cultivation in growth medium containing a sole carbon source (1 % w/v)

Strain	Carbon source	% Glucose (w/w)	% Mannose (w/w)
Kluyveromyces lactis (752)	**Control**	16.4 ± 1.1	14.2 ± 1.9
	Fructose	12.7 ± 1.4	13.9 ± 0.7
	Galactose	17.6 ± 0.6	14.2 ± 1.6
	Glucose	19.2 ± 1.2	12.7 ± 1.5
	Maltose	19.5 ± 3.9	10.4 ± 0.4*
	Mannose	12.1 ± 1.1	15.2 ± 0.7
	Sucrose	20.0 ± 1.5	14.7 ± 1.5
Kluyveromyces marxianus (701)	**Control**	14.5 ± 4.1	11.6 ± 2.3
	Fructose	12.6 ± 1.2	14.0 ± 0.7*
	Galactose	13.9 ± 1.5	13.3 ± 0.5
	Glucose	13.2 ± 1.8	13.7 ± 1.3*
	Maltose	12.8 ± 1.7	12.7 ± 0.6
	Mannose	13.5 ± 1.7	**14.0 ± 0.2***
	Sucrose	12.7 ± 1.3	12.9 ± 0.4
Kluyveromyces marxianus (2415)	**Control**	**11.2 ± 0.9**	**10.3 ± 0.5**
	Fructose	**34.3 ± 2.9***	**13.6 ± 0.8***
	Galactose	**10.9 ± 0.5**	15.6 ± 0.7*
	Glucose	30.3 ± 1.8*	13.6 ± 0.5*
	Mannose	10.4 ± 0.8	17.4 ± 3.0*
	Sucrose	**10.8 ± 0.7**	**9.9 ± 0.8**

Results displayed are a representative of the mean of triplicate cultivations ± standard deviation. Statistical analysis was performed using Dunnett's test to evaluate the significance of samples when compared to the control and values with an asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the control. An independent sample t-test was performed to evaluate the significance of 1 and 2 % (w/v) carbon source in the growth medium. Values in **bold** denotes a significant change ($p \leq 0.05$) in the saccharide composition between the two concentrations.

Table 3.4. Saccharide composition of *K. lactis* and *K. marxianus* cell wall following cultivation in growth medium containing a sole carbon source (2 % w/v)

Strain	Carbon source	% Glucose (w/w)	% Mannose (w/w)
Kluyveromyces lactis (752)	**Control**	16.4 ± 1.1	14.2 ± 1.9
	Fructose	13.3 ± 1.2	13.9 ± 0.6
	Galactose	24.1 ± 6.0*	13.5 ± 1.1
	Maltose	22.7 ± 3.1	12.2 ± 1.9
	Mannose	13.1 ± 1.1	14.7 ± 0.5
	Sucrose	19.5 ± 0.7	13.8 ± 0.6
Kluyveromyces marxianus (701)	**Control**	14.5 ± 4.1	11.6 ± 2.3
	Fructose	14.9 ± 2.0	13.9 ± 0.6*
	Galactose	13.2 ± 1.8	13.1 ± 0.6
	Maltose	15.6 ± 1.4	13.1 ± 0.2
	Mannose	16.0 ± 2.4	**15.8 ± 0.1***
	Sucrose	16.0 ± 1.5	13.5 ± 0.6
Kluyveromyces marxianus (2415)	**Control**	11.2 ± 0.9	10.3 ± 0.5
	Fructose	**10.6 ± 1.3**	**12.6 ± 0.3**
	Galactose	**12.2 ± 1.3**	15.1 ± 1.3*
	Maltose	21.1 ± 6.8*	10.2 ± 0.1
	Mannose	9.6 ± 2.0	15.8 ± 3.0*
	Sucrose	**12.3 ± 1.3**	**11.6 ± 0.5**

Results displayed are a representative of the mean of triplicate cultivations ± standard deviation. Statistical analysis was performed using Dunnett's test to evaluate the significance of samples when compared to the control and values with an asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the control. An independent sample t-test was performed to evaluate the significance of 1 and 2 % (w/v) carbon source in the growth medium. Values in **bold** denotes a significant change ($p \leq 0.05$) in the saccharide composition between the two concentrations.

Each of the strains of *Kluyveromyces*, grown in medium containing 1 and 2 % (w/v) carbon source, were found to have varying cell wall saccharide content between the strains. The cell wall glucose and mannose content ranged from 10.4 – 34.3 % (w/w) glucose and 9.9 – 17.4 % (w/w) mannose with 1 % (w/v) carbon source in the growth medium. Two percent (w/v) carbon source in the growth medium resulted in cell wall glucose content ranging from 9.6 – 24.1 % (w/w) and mannose content ranging from 10.2 – 15.8 % (w/w). A number of significant differences were observed in cell wall glucose and mannose content compared to the control for each of the strains. The ability to utilize particular sugars, and the pathways used to generate energy from sugars are defining features of different yeasts (Lane and Morrissey, 2010).

A significant difference was observed in the cell wall saccharide content of *K. marxianus* 701 and 2415, but not with *K. lactis* 752, compared to the control when

grown in fructose containing growth medium. The cell wall glucose content of *K. marxianus* 2415 was increased significantly in 1 % (w/v) fructose compared to the control. In fact, there was a 3 fold increase in the cell wall glucose content compared to the control demonstrating a dramatic difference in the effect of increasing glucose concentration as the sole carbon source on the cell wall glucose content; this effect may be due to catabolite repression. The cell wall mannose content of both *K. marxianus* 2415 and 701 was also significantly increased with 1 % (w/v) fructose as the sole carbon source in the growth medium. Fructose (2 %, w/v) as the sole carbon source also produced a significant increase in cell wall mannose content of *K. marxianus* 701, but not in 2415. *K. marxianus* expresses the *Frt1* gene, encoding a hexose transporter with a high affinity for fructose, and its product mediates active transport of fructose in symport with protons (Horak, 2013). Fonseca et al. (2013) found that the growth rate of *K. marxianus* was slower when grown in fructose as the sole carbon source compared to glucose. Interestingly, an increase in fructose concentration from 1 to 2 % (w/v) caused a significant decrease in the cell wall saccharide content of *K. marxianus* 2415, suggesting a catabolite repression effect. No significant differences in the cell wall saccharide content were observed for *K. lactis* 752, illustrating a difference in the metabolism of fructose between the species of *Kluyveromyces* and the effect on cell wall saccharide content.

Galactose (2 %, w/v) as the sole carbon source in the growth medium significantly increased the glucose content of *K. lactis* 752 (24.1 %, w/w), compared to the control values of 16.4 % (w/w), while having no effect on the mannose content. The mannose content was significantly increased in *K. marxianus* 2415 with both 1 and 2 % (w/v) galactose concentrations in the growth medium compared to the control. An increase in galactose concentration from 1 to 2 % (w/v) as a sole carbon source caused a significant increase in the glucose content of *K. marxianus* 2415. As in *S. cerevisiae*, galactose is metabolised to glucose-6-phosphate via the Leloir pathway in *Kluyveromyces* (Dong and Dickson, 1997). However, there are differences between the species; the basal expression of the *Lac/Gal* genes is higher in *Kluyveromyces* compared to *S. cerevisiae*. Mechanistically, there are also differences, for example, *Kluyveromyces* lack a specific galactose transporter present in *S. cerevisiae* (Lane and Morrissey, 2010). *K. marxianus* was found to have a slower growth rate in galactose compared to glucose as the sole carbon source, suggesting that galactose uptake is less efficient than other sugars

(Fonseca et al., 2013). The Snf1 and Mig1 proteins play similar roles in regulating galactose metabolism in *S. cerevisiae* and *K. lactis*. The way in which these proteins are integrated into the regulatory circuits are unique to each as is the degree to which each is controlled by the two proteins (Dong and Dickson, 1997). The Snf1 kinase has been associated with cell wall integrity in *Kluyveromyces* spp., expression of which can cause changes in the cell wall composition (Backhaus et al., 2013). This may explain the significant changes in the cell wall glucose content of *K. lactis* 752 and the cell wall mannose content of *K. marxianus* 2415 seen in this study with the use of galactose as the sole carbon source in the growth medium.

Glucose as the sole carbon source had very different effects on the cell wall glucose content of both strains of *K. marxianus*. Growth medium containing 1 % (w/v) glucose significantly increased the cell wall glucose content of *K. marxianus* 2415 compared to the control from 11.2 – 34.3 % (w/w). This 3 fold increase in cell wall glucose content at 1 % (w/v) glucose as the sole carbon source in the growth medium compared to the control may be due to glucose repression at higher concentrations of glucose. Interestingly, no such effect was observed with the cell wall glucose content of *K. marxianus* 701. The cell wall mannose content of both strains was significantly increased compared to the controls. The content of *Kluyveromyces* cell wall proteins vary in a carbon source dependent manner, with many proteins more readily found in the walls of glucose grown cells (Backhaus et al., 2010). As most yeast cell wall proteins are glycosylated with mannose residues this may affect the cell wall mannose content (De Groot et al., 2005). No significant difference in the cell wall glucose or mannose content was observed for *K. lactis* 752 with glucose as the sole carbon source in the growth medium. The glucose uptake pathway is simpler in *Kluyveromyces* than *Saccharomyces*, with only two glucose transporters and little if any gene redundancy (Cairey-Remonnay et al., 2015, Boles and Hollenberg, 1997). *Kluyveromyces* metabolizes glucose predominantly by respiration. Hexose transporter isoforms and polymorphisms within the species of *K. marxianus* (Belloch et al., 2002) may explain the differences in cell wall glucose and mannose content seen between the strains in the presence of glucose as the sole carbon source. *Kluyveromyces* spp. are reported to express different metabolic sugar transporters (Kht1 and Kht2) in the presence of different concentrations of glucose (Milkowski et al., 2001). The concentration of glucose in the growth medium was shown to have a dramatic effect on *K. marxianus*

2415 cell wall glucose content in this study. In addition, catabolite repression and strain dependent variations have been attributed to the presence of different isoforms of sugar transporters, as well as polymorphism within the *Kluyveromyces* spp. (Rodicio and Heinisch, 2013, Belloch et al., 2002), as was seen in this study with *K. marxianus* 2415. From these results glucose as the sole carbon source in the growth medium effected each strain of *Kluyveromyces* differently in terms of cell wall saccharide content. A wide spectrum of biotechnological studies on *K. marxianus* also found that diversity is present within the species (Lane et al., 2011).

Maltose (1 %, w/v) as the sole carbon source in the growth medium caused a significant decrease in the cell wall mannose content of *K. lactis* 752 compared to the control, 14.2 to 10.4 % (w/w). Maltose at 2 % (w/v) significantly increased the cell wall glucose content of *K. marxianus* 2415 to 21.1 % (w/w) compared to the control (11.2 %, w/w). Maltose is hydrolyzed by maltase located in the inner side of the cytoplasmic membrane; therefore maltose has to be transported into the cell by a transporter. In *K. lactis Mal22* encodes a maltase and *Mal21* encodes a maltose transporter. These are structurally very similar to the *S. cerevisiae Mal* genes. Maltase synthesis in *S. cerevisiae* is inducible, while in *K. lactis* induction is strain dependent (Goffrini et al., 2002). The difference in maltose metabolism and the induction of specific genes may be responsible for the changes in the cell wall monosaccharide of *K. lactis* 752 and *K. marxianus* 2415 compared to the control.

The mannose content was significantly increased in each strain of *K. marxianus* (701 and 2415) when both 1 and 2 % (w/v) mannose was used as the sole carbon source compared to the control. No significant affect was detected for the cell wall glucose content. The increase of mannose concentration from 1 to 2 % (w/v) also caused a significant decrease in the cell wall mannose content of *K. marxianus* 701. In addition to the induction of mannose specific genes in the presence of mannose and the absence of glucose, mannose is also metabolised differently to glucose. After mannose phosphorylation by hexokinase (Hxk2), mannose is converted to mannose-6-phosphate and then isomerized to fructose-6-phosphate by phosphomannose isomerase (Rodrussamee et al., 2011). The increase in cell wall mannose content of *K. marxianus* 2415 and 701 for both concentrations of mannose as the sole carbon source compared to the control is evidence of the effect of mannose metabolism on the cell wall mannose content. Interestingly, *K. lactis* 752 was not subject to any significant changes due to

mannose as the sole carbon source in the growth medium, illustrating that differences exist between the species of *Kluyveromyces*.

Growth medium containing 1 or 2 % (w/v) sucrose as the sole carbon source did not have any significant effect on the cell wall saccharide content compared to the control for any of the strains of *Kluyveromyces*. However, increasing the concentration of sucrose from 1 to 2 % (w/v) caused a significant increase in the cell wall mannose content of *K. marxianus* 2415 from 10.8 to 12.3 % (w/w). In *K. marxianus*, inulinase, which is closely related to invertase in *S. cerevisiae* is responsible for fructose hydrolysation and is activated by sucrose or inulin (Weinhandl et al., 2014). These enzymes exhibit corresponding hydrolytic activities towards sucrose. The majority of the inulinase activity is found in the extracellular medium (Lim, 2010). The inulinase of *Kluyveromyces* is in part associated with the cell wall, but, in contrast to the invertase of *Saccharomyces*, much more of the enzyme is actually secreted into the culture fluid (Rouwenhorst et al., 1990). The significant increase in cell wall glucose and mannose content of *K. marxianus* 2415 corresponding to an increase in sucrose concentration may be influenced by an increase in inulinase activity in certain *Kluyveromyces* strains.

Differences in the cell wall glucan and mannoprotein content of two strains of *K. marxianus*, R157 and 1586, were also observed by Nguyen et al. (1998). Further metabolic differences were documented in a separate study, as the rate of lactose utilization was shown to vary among 13 strains of *K. marxianus*, indicating that differences exist between the metabolic capabilities of different strains (Lane et al., 2011). In agreement with this study, that carbon source type can influence the cell wall glucose and mannose content of *Kluyveromyces* spp. Backhaus et al. (2010) found that different carbon sources could influence the cell wall glucose and mannose content of *K. lactis*. For example, ethanol (3 %, w/v) as the sole carbon source in the growth medium was shown to significantly decrease the cell wall mannan content of *K. lactis* compared to a growth medium containing 2 % (w/v) glucose as the sole carbon source. These studies support the observations in this study that the influence of the carbon source is, for the most part, strain specific.

3.1.3 Effect of carbon source on *Schizosaccharomyces, Candida, Rhodotorula,* and *Pichia* spp. cell wall saccharide content

The remaining strains of yeast assessed in this study included *Schizosaccharomyces pombe, Candida utilis, Rhodotorula mucilaginosa,* and *Pichia membranifaciens*. The cell wall of these yeast strains were shown to have a similar structure to *S. cerevisiae* consisting of glucan polysaccharides and a mannoprotein layer (Klis et al., 2010). The cell wall glucose and mannose content of each strain grown in 1 and 2 % (w/v) carbon source was determined as described in Sections 2.2.8-9. *S. pombe* is a fission yeast with a number of distinctive features compared to budding yeast. The monosaccharide cell wall composition of *S. pombe* includes galactose, as galactomannan (Hamilton and Gerngross, 2007), a typical yeast mannan oligosaccharide with galactose units bound at the terminal positions (Perez and Ribas, 2004). Galactosylation of glycoproteins is a unique feature of fission yeast, and does not occur in the budding yeast *S. cerevisiae* (Matsuzawa et al., 2011). As this study was concerned with the glucose and mannose content of the yeast cell wall, the galactose content was not quantified for this strain. *S. pombe* and *Rhodotorula* species are also reported to contain relatively low amounts of cell wall mannose compared to other yeast strains such as *Saccharomyces, Candida,* and *Pichia* (Biswas et al., 2001). Table 3.5 and 3.6 show the cell wall glucose and mannose content for these yeast strains grown in growth medium containing 1 and 2 % (w/v) of the various carbon sources. A number of yeast strains are unable to assimilate certain carbon sources and were excluded from the study.

Table 3.5. Saccharide composition of *S. pombe*, *C. utilis*, *R. mucilaginosa*, and *P. membranifaciens* cell wall following cultivation in growth medium containing a sole carbon source (1 % w/v)

Strain	Carbon source	% Glucose (w/w)	% Mannose (w/w)
Schizosaccharomyces pombe (70572)	**Control**	27.6 ± 4.0	6.5 ± 2.5
	Fructose	27.5 ± 6.1	5.4 ± 2.7
	Galactose	-	-
	Glucose	31.1 ± 0.9	5.4 ± 0.7
	Maltose	30.0 ± 3.0	4.9 ± 2.5
	Mannose	-	-
	Sucrose	21.9 ± 1.5	4.5 ± 2.4
Candida utilis (70167)	**Control**	14.8 ± 3.0	10.8 ± 1.5
	Fructose	**11.2 ± 0.1***	12.5 ± 0.1
	Galactose	-	-
	Glucose	11.3 ± 0.1	12.1 ± 0.5
	Maltose	**18.7 ± 1.0***	11.9 ± 0.4
	Mannose	11.7 ± 0.5	12.4 ± 0.6
	Sucrose	11.4 ± 0.4*	11.4 ± 0.7
Rhodotorula mucilaginosa (70825)	**Control**	23.2 ± 1.5	8.6 ± 0.7
	Fructose	18.7 ± 1.0*	5.6 ± 1.8
	Galactose	22.6 ± 1.1	7.8 ± 2.7
	Glucose	**26.2 ± 0.3***	9.2 ± 0.4
	Maltose	24.6 ± 3.4	8.2 ± 0.7
	Mannose	24.1 ± 1.0	5.8 ± 2.4
	Sucrose	**20.1 ± 0.2**	5.7 ± 2.3
Rhodotorula mucilaginosa (18184)	**Control**	21.7 ± 2.9	8.9 ± 0.4
	Fructose	14.8 ± 1.8*	8.9 ± 1.2
	Galactose	13.9 ± 2.9*	6.9 ± 0.2
	Glucose	21.9 ± 2.7	8.4 ± 2.0
	Maltose	16.9 ± 3.0	5.6 ± 1.5
	Mannose	**21.9 ± 2.4**	8.7 ± 1.6
	Sucrose	**20.3 ± 0.2**	**10.0 ± 0.0**
Pichia membranifaciens (326)	**Control**	24.1 ± 5.8	18.6 ± 0.8
	Fructose	23.0 ± 7.3	18.6 ± 2.7
	Galactose	-	-
	Glucose	23.9 ± 1.0	18.6 ± 2.7
	Maltose	-	-
	Mannose	15.8 ± 1.9	17.7 ± 1.9
	Sucrose	-	-

Results displayed are a representative of the mean of triplicate cultivations ± standard deviation. Statistical analysis was performed using Dunnett's test to evaluate the significance of samples when compared to the control and values with an asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the control. An independent sample t-test was performed to evaluate the significance of 1 and 2 % (w/v) carbon source in the growth medium. Values in **bold** denotes a significant change ($p \leq 0.05$) in the saccharide composition between the two concentrations.

Table 3.6. Saccharide composition analysis of *S. pombe, C. utilis, R. mucilaginosa,* and *P. membranifaciens* cell wall following cultivation in growth medium containing a sole carbon source (2 % w/v)

Strain	Carbon source	% Glucose (w/w)	% Mannose (w/w)
Schizosaccharomyces pombe (70572)	**Control**	27.6 ± 4.0	6.5 ± 2.5
	Fructose	23.4 ± 6.1	5.2 ± 1.4
	Galactose	-	-
	Maltose	30.9 ± 2.0	4.0 ± 2.0
	Mannose	-	-
	Sucrose	27.5 ± 3.7	3.7 ± 0.4
Candida utilis (70167)	**Control**	14.8 ± 3.0	10.8 ± 1.5
	Fructose	**12.0 ± 0.0**	12.7 ± 0.3
	Galactose	-	-
	Maltose	**15.2 ± 2.1**	9.8 ± 2.2
	Mannose	12.0 ± 1.0	8.3 ± 4.3
	Sucrose	10.8 ± 0.5*	9.9 ± 3.9
Rhodotorula mucilaginosa (70825)	**Control**	**23.2 ± 1.5**	8.6 ± 0.7
	Fructose	17.4 ± 2.8*	5.1 ± 2.7
	Galactose	20.9 ± 0.7	8.9 ± 1.3
	Maltose	20.7 ± 0.7	7.5 ± 0.2
	Mannose	24.4 ± 0.2	6.3 ± 2.1
	Sucrose	**19.3 ± 0.2***	5.2 ± 1.8
Rhodotorula mucilaginosa (18184)	**Control**	21.7 ± 2.9	8.9 ± 0.4
	Fructose	17.7 ± 4.8	7.5 ± 0.8
	Galactose	12.2 ± 3.6*	6.1 ± 0.1*
	Maltose	18.1 ± 2.2	8.4 ± 1.3
	Mannose	**15.7 ± 1.9***	7.0 ± 1.2
	Sucrose	**22.9 ± 0.1**	**9.6 ± 0.0**
Pichia membranifaciens (326)	**Control**	24.1 ± 5.8	18.6 ± 0.8
	Fructose	22.2 ± 5.7	18.1 ± 1.2
	Galactose	-	-
	Maltose	-	-
	Mannose	16.2 ± 2.4	17.8 ± 1.9
	Sucrose	-	-

Results displayed are a representative of the mean of triplicate cultivations ± standard deviation. Statistical analysis was performed using Dunnett's test to evaluate the significance of samples when compared to the control and values with an asterisk [*] denotes a significant change ($p \leq 0.05$) in the composition compared to the control. An independent sample t-test was performed to evaluate the significance of 1 and 2 % (w/v) carbon source in the growth medium. Values in **bold** denotes a significant change ($p \leq 0.05$) in the saccharide composition between the two concentrations.

With the strain *S. Pombe* 70572, no significant differences were found between the cell wall glucose and mannose content of the samples grown in 1 or 2 % (w/v) for each of the carbon sources as the sole carbon source in the growth medium and the control.

Nevertheless, a number of observations were made about the glucose and mannose content of the cell wall material. *S. pombe* had the second highest level of glucose in its cell wall (31.1 %, w/w) of all the strains of yeast examined in this study. It also had the lowest mannose content (3.7 %, w/w) of all the strains. There were no significant differences in the cell wall glucose and mannose content between 1 % and 2 % (w/v) concentration of carbon source in the growth medium for *S. pombe*. *S. pombe* is unable to metabolise galactose (Matsuzawa et al., 2011), therefore, it was not cultured in its presence. In addition, very weak growth was observed for *S. pombe* 70572 in mannose containing medium and further investigation was not pursued with this carbon source.

Unlike *S. cerevisiae*, regulatory mechanisms in glucose sensing and signalling are not fully understood in fission yeast (Palabiyik et al., 2013). In *S. pombe* glucose may be fermented under aerobic conditions (Crabtree effect), and a reduction in glucose concentration strongly affects cell metabolism and gene expression (Flores et al., 2000). The cell wall integrity pathway, a MAPK cascade, that in *S. pombe* regulates processes like cell wall construction and maintenance, becomes activated with glucose withdrawal (Madrid et al., 2013). However, in this study no changes were observed in the cell wall saccharide content between the concentrations of the carbon sources.

Candida utilis is a popular "fodder yeast" used to produce single cell protein for the animal feed industry (García et al., 2014). This strain was reported to be incapable of assimilating galactose as the sole carbon source (CBS, 2015), for this reason the strain was not cultured in its presence. A significant decrease in cell wall glucose content was observed when grown in 1 % (w/v) fructose (11.2 %, w/w) and sucrose (11.4 %, w/w) compared to the control (14.8 %, w/w). An increase in the fructose concentration from 1 to 2 % (w/v) caused a significant increase in cell wall glucose content. The cell wall glucose content was significantly increased with 1 % (w/v) maltose as the sole carbon source (18.7 %, w/w), compared to the control. It was evident that increasing the concentration of maltose in the growth medium from 1 to 2 % (w/v) caused a significant decrease in the cell wall glucose content; suggesting increased maltase activity may influence the cell wall glucose content. As the sole carbon source 2 % (w/v) sucrose also yielded a significant decrease in the cell wall glucose content compared to the control. Various carbon sources are metabolised differently in *Candida* spp. through the induction of required enzymes in the presence of the substrate. Ene et al. (2012b) demonstrated that changes in carbon source have been shown to affect *Candida* cell

wall by influencing the cell wall content and structure. When fermentable carbon sources are present in high concentrations excess carbon may be directed via hexose phosphates to biosyntheses of β-glucan and mannan for cell wall generation (Ene et al., 2012a). In this study, 2 % (w/v) glucose as the sole carbon source produced significantly higher cell wall glucose than was produced by lower concentrations of 1 % (w/v) fructose and sucrose in the growth medium. The cell wall β-glucan and mannoprotein layer in a number of *C. utilis* strains was also dependant on the species and strain (Bzducha-Wróbel et al., 2012). Culture conditions were shown to induce changes in the content of polymers in the glucan and mannoprotein layer, and the thickness of the wall when glycerol was used as a carbon source, was thought to be a response to stimuli received through stress sensors (Bzducha-Wróbel et al., 2014).

Rhodotorula mucilaginosa 70825 had high cell wall glucose content (17.4 – 26.2 %, w/w) and relatively low mannose content (5.6 – 9.2 %, w/w). *Rhodotorula* are reported to have low cell wall mannose content compared to *Saccharomyces, Candida, Kluyveromyces,* and *Pichia* strains (Biswas et al., 2001). There was a significant decrease in the glucose content of the cell wall when grown in 1 % (w/v) fructose (18.7 %, w/w) as the sole carbon source compared to the control (23.2 %, w/w). A significant increase in the cell wall glucose content was also observed with 1 % (w/v) glucose as the sole carbon source in the growth medium (26.2 %, w/w) compared to the control. No other significant differences were observed for growth medium containing 1 % (w/v) carbon source. With 2 % (w/v) fructose and sucrose as the sole carbon source, respectively, the monosaccharide content of the cell wall of *R. mucilaginosa* 70825 was significantly decreased (17.4 %, w/w) and (19.3 %, w/w), compared to the control (23.2 %, w/v). A significant decrease in the cell wall glucose content of *R. mucilaginosa* 70825 was produced by an increase in glucose and sucrose concentration in the growth medium; this effect may have been mediated through catabolite repression. There was no significant change in the mannose content of the cell wall when the concentration of the carbon sources was increased.

R. mucilaginosa 18184 had significantly less cell wall glucose content compared to the control when the growth medium contained 1 % (w/v) fructose and galactose. The cell wall glucose and mannose content was significantly decreased (12.2 and 6.1 %, w/w) when 2 % (w/v) galactose was used as the sole carbon source compared to the control values (21.7 and 8.9 %, w/v). This large decrease in cell wall glucose content was not

observed in *R. mucilaginosa* 70825 with 2 % (w/v) galactose was the sole carbon source in the growth medium. A number of significant differences were observed in the cell wall glucose and mannose content of *R. mucilaginosa* 18184 when the concentration of mannose and sucrose were increased from 1 to 2 % (w/v). An increase in mannose concentration in the growth medium caused a significant decrease in the cell wall glucose content from 21.9 to 15.7 % (w/w). However, an increase in mannose concentration in the growth medium did not have a significant effect on the cell wall glucose content of *R. mucilaginosa* 70825, suggesting differences exist between these two strains' cell wall composition in response to increased mannose concentration as a carbon source. An increase in sucrose concentration in the growth medium also increased the cell wall glucose content of *R. mucilaginosa* 18184 significantly while its mannose content was significantly decreased; in contrast to *R. mucilaginosa* 70825 where an increase in sucrose concentration in the growth medium significantly decreased the cell wall glucose content of 70825. Interestingly, the cell wall glucose content of *K. marxianus* 2415 also increased significantly with a rise in sucrose concentration in the growth medium.

R. mucilaginosa is a strictly aerobic yeast, unlike *S. cerevisiae* or *Kluyveromyces* species. *Rhodotorula* species have been shown to successfully metabolise various carbon sources, both hexose and pentose sugars, including glucose, xylose, sucrose and lactate (Aksu and Eren, 2007, Aksu and Eren, 2005, Xu et al., 2011). Various carbon sources are metabolised differently in *Rhodotorula* spp. Induction and repression of particular enzymes occur for the metabolism of specific carbon sources in the absence of glucose (Xu et al., 2011, Gupta et al., 1986). These changes may influence the cell wall content of the cells as was seen with *R. mucilaginosa* 70825 and 18184 where 1 % (w/v) fructose as the sole carbon source in the growth medium caused a significant decrease in the cell wall glucose content compared to the control. Galactose as the sole carbon source in the growth medium at both concentrations had a significant effect on the cell wall glucose content of *R. mucilaginosa* 18184, decreasing the cell wall glucose and mannose content, but not in *R. mucilaginosa* 70825. Research on *Rhodotorula* species industrially is focused mostly on the production of carotenoids and data on cell wall composition is scarce.

P. membranifaciens can be used as a biological control organism against *Botrytis cinerea,* the causal organism of the grey mould disease of the grapevine (Masih et al.,

2001), and for the control of the spoilage yeast *Brettanomyces bruxellensis*. This strain of *P. membranifaciens* could not assimilate galactose, maltose or sucrose as a carbon source (NCYC, 2015), for this reason the yeast was not cultured in the presence of these carbon sources. Certain strains of *Pichia* lack the functional pathway for galactose metabolism (Weinhandl et al., 2014). There was no significant difference in either the cell wall glucose or mannose content when grown in 1 or 2 % (w/v) carbon source for *P. membranifaciens*. Neither was there any significant differences observed in the cell wall glucose and mannose content of *P. membranifaciens* between 1 and 2 % (w/v) carbon source concentrations.

In the present investigation, the total glucose and mannose content of the cell wall varied, depending on the species and strain, and represented 8.2 – 34.3 % (w/w) glucose and 3.7 – 21.2 % (w/w) mannose of the total carbohydrate composition regardless of the sugar source. In addition, each carbon source and concentration of carbon source affected the composition of the cell wall components differently between species and between strains of the same species. However, a clear relationship between the glucose and mannose content of yeast cell wall material and the carbon source for all strains of yeast used in this study was difficult to ascertain. Perhaps an increased concentration of the carbon sources, greater than 2 % (w/v), would lead to more significant differences in the glucose and mannose content of the yeast cell wall composition of the yeast strains when compared to the control.

It was established by Nguyen et al. (1998), using compositional analysis of the cell walls of several yeast species that the portions of the different fractions within the cell wall varied with the species and strain. In agreement with their findings, the data from this study suggests that the glucose and mannose composition of each of the selected yeast varied depending on each species and strain. Several studies have provided evidence that the cell wall is not a static entity; rather, it is dynamically remodelled in response to its environment (Ene et al., 2015, Teparic and Mrsa, 2013). Yeast cell wall polysaccharides and mannoproteins are continually adapted to environmental conditions in terms of their abundance and organization (Hall, 2015, Ene et al., 2012a, Ene et al., 2012b, Backhaus et al., 2011, Xie and Lipke, 2010, Liu et al., 2009b, Aguilar-Uscanga and Francois, 2003, Klis et al., 2002). Carbon catabolite repression plays a significant role in the regulation of a host of yeast genes (Gancedo, 1998), many of which influence the structure and maintenance of yeast cell wall.

The purpose of this work was to investigate the possibility of developing a second generation Acitgen® product. Actigen® is produced from the mannan oligosaccharide (MOS) fraction of *S. cerevisiae* cell wall. Yeast cell wall MOS have the capacity to inhibit type 1 fimbriae containing pathogens from binding to host tissue by acting as an alternative binding site for the pathogen. Gedek (1999) suggested that pathogens bound to MOS are not capable of binding to the target host and are subsequently removed by the flow of secretions. Yeast agglutination tests have been used to demonstrate the binding of pathogenic bacteria to yeast and yeast cell wall (Tiago et al., 2012, Perez-Sotelo et al., 2005, Spring et al., 2000). In the search for a second generation product, it was necessary to assess whether the samples generated in this study were effective at agglutinating bacteria. A sample or samples with increased bacterial binding properties compared to Actigen® would be suitable candidates for further research and development as a potential new product. Prior to assessing each of the samples discussed in this section, the ability of a yeast cell wall material control (YCWM) sample to bind selected pathogenic strains of *Salmonella* and *E. coli* with relevance to the animal health industry was investigated.

3.2 Microscopic imaging of yeast cell wall material agglutination of bacteria.

Despite functional similarity between the fimbriae of *Salmonella* and *E. coli*, the fimbriae in these two species are not evolutionarily related, with a lack of virtually any significant sequence homology (Kisiela et al., 2013, Kisiela et al., 2012). Fimbriae play a critical role in the colonization of specific host cells and tissues by facilitating the initial attachment to enterocytes and promoting intestinal colonisation (Borowsky et al., 2009, Althouse et al., 2003, Baumler et al., 1997, Muller et al., 1991, Eshdat, 1981). Type 1 fimbriae are associated with most *Salmonella enterica* serovars (80 %) and are prevalent also in many *E. coli* spp. (Wu et al., 2012, Wright et al., 2007, Althouse et al., 2003). *E. coli* and *Salmonella* strains containing type 1 fimbriae have been shown to agglutinate to *Saccharomyces cerevisiae* (Tiago et al., 2012, Stevens et al., 2009, Eshdat, 1981). The mannan oligosaccharides of *S. cerevisiae* cell wall (YCWM) act as high affinity ligands for the mannose specific type 1 fimbriae of *E. coli* spp. and *Salmonella* spp. thus preventing their attachment to the mucosal lining of the intestinal tract and the subsequent development of enteric disease (Newman, 1994).

The objective of this aspect of the research was to identify if YCWM had the ability to bind specific strains of *E. coli* and *Salmonella in vitro*. Four *Salmonella* spp., classified as *Salmonella enterica* subspecies *enterica* serotype *enteritidis*, *typhimurium*, *gallinarum* and *dublin*, and three *E. coli* serotypes, designated O157:H7, O2:K1:H-, and O1:K1:H7, were used in this study. Each strain was chosen due to its pathogenicity either to domestic food animals or as a zoonotic agent to humans through foodborne infection or through direct contact with infected animals. Each of these pathogens can have a negative effect on animal production and/or public health. All the strains were analysed for positive adhesion to YCWM, this was observed by light microscopy. For this study all strains of bacteria were grown under identical culture conditions, as described in Section 2.2.1.

YCWM is based on an extraction of cell wall oligosaccharides from a strain of *S. cerevisiae*. *S. cerevisiae* cells, produced under strictly controlled conditions, are autolyzed and the resulting cell wall material is carefully separated from the intracellular yeast contents. YCWM is the basis of Alltech's Actigen® product. YCWM was stained with Safranin O in the absence of any bacteria (Figure 3.1). This control sample was prepared using YCWM and 10 mM PBS buffer as outlined in Section 2.2.2.

Safranin O is used to test yeast viability (Meledina et al., 2015, Krzepilko, 2009), as it stains dead or lysed yeast cells. Safranin O stained the exposed plasma membrane phospholipids (Byrne, 1962) attached to the YCWM aiding in the microscopic visualisation of the YCWM. It is clear from Figure 3.1 that YCWM when examined using light microscopy aggregates with little dispersion on the slide.

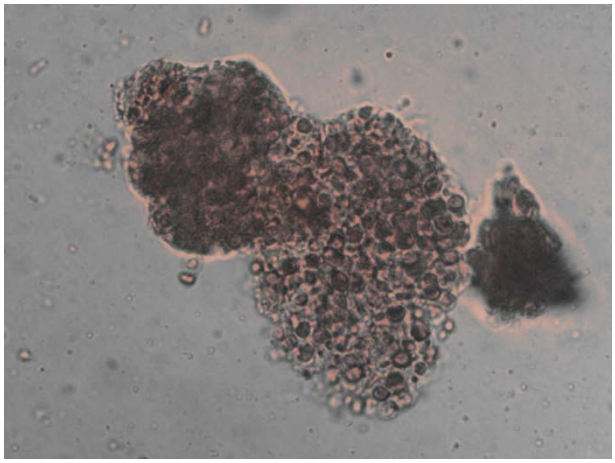
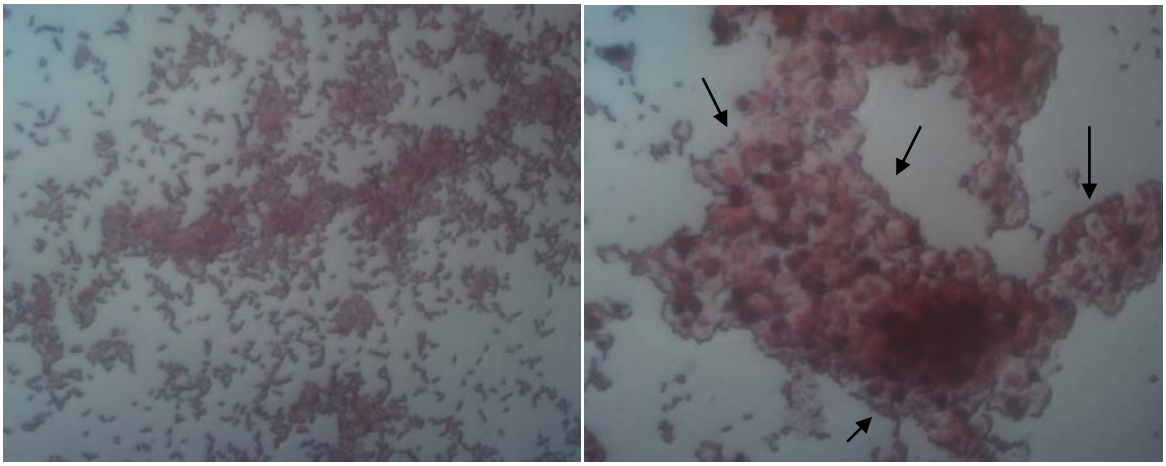


Figure 3.1. Cell wall material (YCWM) (1.0 % w/v) fixed to a microscope slide and stained with Safranin O.

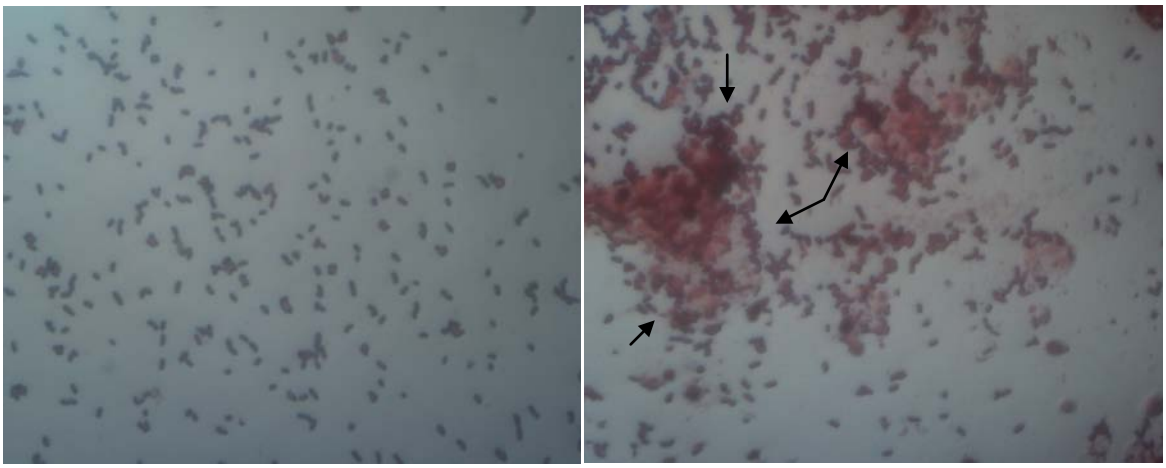
Magnification 1000 X

The subsequent images show strains of *Salmonella* (Figure 3.2) and *E. coli* (Figure 3.3) in the absence of YCWM (A, C, E, and G) and in the presence of YCWM (B, D, F, and H). The strains of *Salmonella* include *Salmonella enteritidis* (Figure 3.2 A-B), *typhimurium* (Figure 3.2 C-D), *gallinarum* (Figure 3.2 E-F), and *dublin* (Figure 3.2 G-H), and the *E. coli* strains include *E. coli* O157:H7 (Figure 3.3 A-B), O2:K1:H- (Figure 3.3 C-D), and O1:K1:H7 (Figure 3.3 E-F). The YCWM and bacteria cells were mixed and allowed to agglutinate on a glass microscope slide before being fixed and stained with Safranin O. Safranin O stains Gram negative bacteria, such as *E. coli* and *Salmonella*, by binding to the thin peptidoglycan layer in their cell wall (Dmitriev et al., 2004). In the case of *Salmonella* and *E. coli* strains the layer is on the surface of the bacteria and accessible to the dye without further treatment.

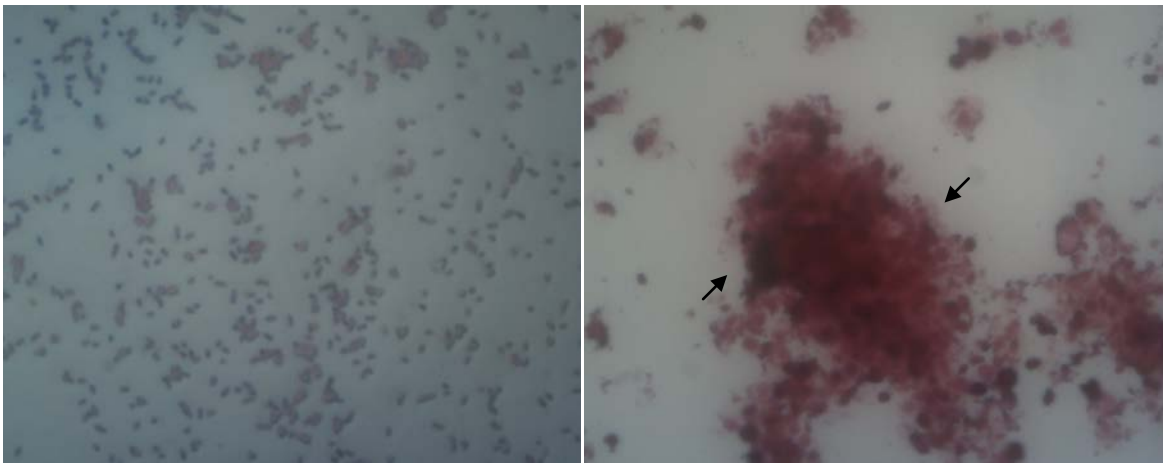
A B



C D



E F



G H

Figure 3.2. Microscopic images of *Salmonella* strains and *Salmonella* strains + YCWM stained with Safranin O.

(A) *Salmonella enteritidis* (B) *Salmonella enteritidis* + YCWM, (C) *Salmonella typhimurium*, (D) *Salmonella typhimurium* + YCWM, (E) *Salmonella gallinarum*, (F) *Salmonella gallinarum* + YCWM, (G) *Salmonella dublin*, (H) *Salmonella dublin* + YCWM. Examples of bacteria bound to YCWM are indicated with black arrows. Magnification 1000X.

Each of the bacteria disperses evenly throughout the glass slides in the absence of YCWM. The bacteria were not as evenly distributed in the slides when combined with YCWM (Figure 3.2 B, D, E, and H) compared to the slides without YCWM (Figure 3.2 A, C, E, and G), where there is obvious clumping of the bacteria to the YCWM. There are observable differences between the agglutination and the distribution of the cells between the *Salmonella* serovars when mixed with YCWM. Fewer free cells of *Salmonella enteritidis* and *gallinarum* were visible when mixed with YCWM (Figures 3.2 B and F) indicating that the cells are adhered to YCWM. However, the agglutination to YCWM by *Salmonella typhimurium* and *dublin* does not look as comprehensive (Figure 3.2 D and H); this is evident by the larger number of free bacterial cells visible on the slide.

The differences in the agglutination to YCWM by the *Salmonella* serovars shown in Figure 3.2 may be explained by the heterogeneity of type 1 fimbriae. Type 1 fimbriae of *Salmonella* are composed primarily of structural protein FimA with a lectin like protein (FimH) attached to the tip (Kisiela et al., 2006). FimH is directly involved in binding to high mannose oligosaccharides carried by surface glycoproteins of eukaryotic cells, including yeast (Jones, 1995, Krogfelt et al., 1990). Significant heterogeneity among

FimH adhesins from different serovars and even strains of the same serovar exists and are responsible for the selective binding to different mannosylated oligosaccharides. These differences are most likely associated with differences in the structure of FimH adhesins, and may be partially responsible for the tropism of various *Salmonella* serovars to different species, tissues and cells (Grzymajlo et al., 2013, Guo et al., 2009). It has also been shown that amino acid replacements resulting from single nucleotide polymorphisms (SNPs) throughout the FimH gene of *E. coli* or *Salmonella enterica* (Kisiela et al., 2006), conferred different binding phenotypes (Boddicker et al., 2002). *Salmonella typhimurium* and *enteritidis*, expressing allelic variants of FimH protein, were shown to differ by four amino acid residues (Kisiela et al., 2006). This variation in amino acids may explain the difference in the agglutination to YCWM seen between these four strains in Figure 3.2. Different strains of *Salmonella typhimurium* containing 2 different allelic variants of FimH were shown to adhere weakly and strongly to human HEp-2 cells (Boddicker et al., 2002). Grzymajlo et al. (2013) also demonstrated differences in binding profiles to cell lysates from sheep, pig and cattle enterocytes between FimH proteins from host restricted serovars (*Salmonella dublin*) and broad host range serovars represented by FimH adhesin from *Salmonella enteritidis*. This agreed with the current study where differences between the agglutination of *Salmonella dublin* and *enteritidis* to YCWM were observed in Figure 3.2. Avian adapted *Salmonella gallinarum* contains a FimH variant with a single point mutation at position 78, wherein threonine is replaced by isoleucine. This was shown to alter its oligosaccharide binding properties (Kisiela et al., 2012, Kisiela et al., 2006). This change was responsible for the increased type 1 fimbriae mediated binding of *Salmonella gallinarum* to chicken leukocytes (Kuzminska-Bajor et al., 2012). This FimH variant may be responsible for the differences seen in agglutination of *Salmonella gallinarum* in Figure 3.2 compared to the other strains of *Salmonella* used in this study.

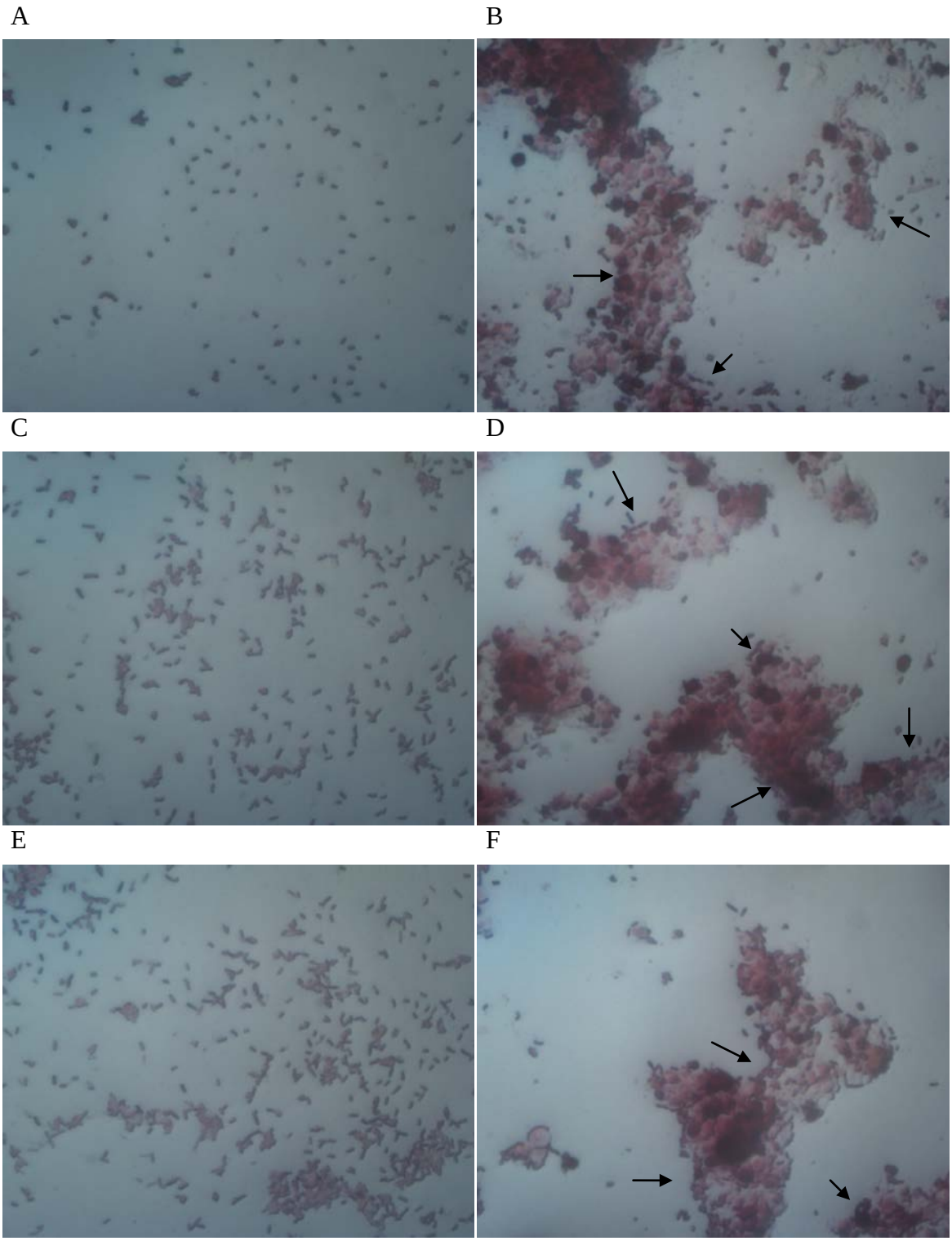


Figure 3.3. Microscopic images of *E. coli* strains and *E. coli* + YCWM stained with Safranin O.

(A) *E. coli* O157:H7 (B) *E. coli* O157:H7+ YCWM, (C) *E. coli* O2:K1:H-, (D) *E. coli* O2:K1:H- + YCWM, (E) *E. coli* O1:K1:H7, (F) *E. coli* O1:K1:H7 + YCWM. Examples of bacteria bound to YCWM are indicated with black arrows. Magnification 1000X.

Microscope analysis of *E. coli* O157:H7, O2:K1:H-, and O1:K1:H7 cells in the absence of YCWM showed an even dispersion throughout Figure 3.3 A, C, and E. While the *E. coli* cells in the presence of YCWM (Figure 3.3 B, D, and F) are not evenly distributed, there was clumping of the bacteria to the YCWM. The agglutination to YCWM by the *E. coli* strains was different for each of the strains used in this study, O157:H7, O2:K1:H-, and O1:K1:H7, as was observed with the *Salmonella* strains. In Figure 3.3, the agglutination to YCWM by *E. coli* O157:H7 (Figure 3.3 B) shows more free bacteria cells compared to *E. coli* strains O2:K1:H- and O1:K1:H7.

In vitro studies by Mirelman et al. (1980) and Firon et al. (1983) observed that *E. coli* agglutinated to *S. cerevisiae*. *E. coli* strains containing type 1 fimbriae agglutinated to yeast, while strains without type 1 fimbriae did not result in agglutination. They concluded that this agglutination was mannose sensitive. Similar work was carried out by Nachin et al. (2005), concluding that fimbriation was essential for agglutination to *S. cerevisiae*. Each of the *E. coli* strains used in this study contained type 1 fimbriae (Reiland et al., 2014, Monroy et al., 2005, Iida et al., 2001, Roe, 2001, Gedek, 1999). The agglutination patterns observed in Figure 3.3 may be explained by the reported differences in FimH or FimH variants of *E. coli* strains as described by Pizarro-Cerda and Cossart (2006). Variants of FimH found on different strains of *E. coli*, bind with varying affinity to different mannose oligosaccharides such as mono or trimannose residues. Allelic variants of the FimH adhesin from *E. coli* strains O2 and O1, differing only in one amino acid residue, have different receptor specificities and in addition to high mannose type oligosaccharides can bind monomannnose residues (Kisiela et al., 2006, Weissman et al., 2006, Sokurenko et al., 1995). Structural and functional heterogeneity occurs within the class of type 1 fimbriae and adhesive diversity will lead to a broader spectrum of receptive surfaces for potential colonization. Differences associated with specific amino acids were proposed to be responsible for observed differences in the binding to mannan as well as variability in binding specificities (Sokurenko et al., 1995). Based upon the amino acid sequences of the *E. coli* FimH adhesin, two sequence homology groups have been described with either mannotriose specific or monomannose specific binding (Sokurenko et al., 2001). The reported differences in the FimH of *E. coli* strains can explain the differences observed in the agglutination patterns between *E. coli* O157, O2, and O1 as seen in Figure 3 B, D, and F.

Research by various groups has demonstrated that YCWM can reduce the prevalence of *E. coli in vivo*. A study by Yalcin et al. (2013) with Ross 308 male broiler chicks concluded that the dietary supplementation of yeast cell wall material increased growth performance, immunocompetence, and reduced *E. coli* colonization in the intestine. It was also shown that YCWM inhibited *E. coli* O157:H7 binding to both mucosal explants from the jejunum and to bovine colonic cell line cells (Baines et al., 2011). In addition, another study found that YCWM reduced the amount of *E. coli* and *Salmonella* in Ross 308 broilers (Ghosh et al., 2012).

As previously mentioned the type 1 fimbriae of *Salmonella* and *E. coli* are structurally and functionally similar while lacking almost any sequence homology (Kisiela et al., 2013). The difference in sequence homology between *Salmonella* and *E. coli* could account for the variation observed in agglutination between these two strains based on the differences of FimH alleles. In addition to the impact of heterologous FimH on mannose binding affinity, the affect of the shaft protein FimA on which each FimH protein is presented may also impose conformational constraints on FimH, due to steric interference, altering its binding capacity (Duncan et al., 2005).

The agglutination to YCWM by the bacterial cells was evident in Figures 3.2 and 3.3, however, the results are quite subjective and qualitative. The diminutive size of the bacterial cells used made it difficult to reliably count how many cells were bound to YCWM or remained unbound on the slide. With this in mind an effort was made to transfer the agglutination to YCWM by *Salmonella* and *E. coli* from slide format to a 96 well plate format in order to assess if it was possible to make this method uniform and quantitative.

3.3 Yeast cell wall material binding activity assay development.

In vitro assay techniques are of paramount importance for biotechnological and pharmaceutical research. Compared to *in vivo* assays, *in vitro* techniques have the advantage of simplifying the system under investigation focusing on a small number of components (Vignais and Vignais, 2010). They allow for determination of potential mode(s) of action, higher throughput screening of product candidates, and are not influenced by environmental factors that may mask *in vivo* results (temperature, disease, pressure, etc.) (Applegate et al., 2010). Development of an assay system to quantify the amount of bacteria agglutinating to YCWM will help to further build and maintain confidence in the product, monitor batch variation, and identify new products with increased bacteria agglutination ability.

The goal of this section was to develop an assay to quantify the amount of specific bacteria bound by YCWM *in vitro*. Bacteria used in this study included *Salmonella enteritidis, typhimurium, gallinarum,* and *dublin*, and *E. coli* O157:H7, O2:K1:H-, and O1:K1:H7. Assay conditions had to be optimised for each individual strain. A known quantity of bacteria (*Salmonella* or *E. coli* spp.) was incubated with YCWM in a microtiter plate. The plate was washed; the YCWM and bacteria bound to the YCWM were removed. The bacteria remaining on the microtiter plate were quantified by enzyme linked immunosorbent assay (ELISA). As a known quantity of bacteria was added to the plate initially, the quantity of bacteria bound to YCWM was calculated by subtracting what was left on the plate from what was added to the plate initially.

Prior to this, a standard curve was created in an effort to equate bacterial numbers to optical density readings at 650 nm and ultimately to quantify bacterial numbers in growing cultures. The ELISA antibody concentrations were also optimised for each strain of bacteria, a standard curve was then generated to calculate bacterial numbers bound to a microtiter plate. Bacterial numbers (CFU mL^{-1}) were titrated against a fixed YCWM concentration to determine the optimum quantity of which YCWM could bind; this was carried out for each bacteria strain. Confirmation of the bacterial numbers bound to YCWM was assessed by comparing the amount of bacteria removed from the microtiter plate by plate count to the numbers determined by the calculated method for each strain. Method performance testing was then carried out to evaluate the reproducibility of the assays.

3.3.1 Generation of standard curve of optical densities versus plate count.

Optical density readings were taken for a number of different dilutions of growing cultures. An aliquot of each of these dilutions was also plate counted as outlined in Section 2.2.4.1. The bacterial numbers were plotted against the optical density at 650 nm (OD_{650nm}) to generate a standard curve. The relationships between OD_{650nm} and CFU mL^{-1} of each of the bacteria strains are shown in Figure 3.4 for *Salmonella* strains and in Figure 3.5 for *E. coli* strains.

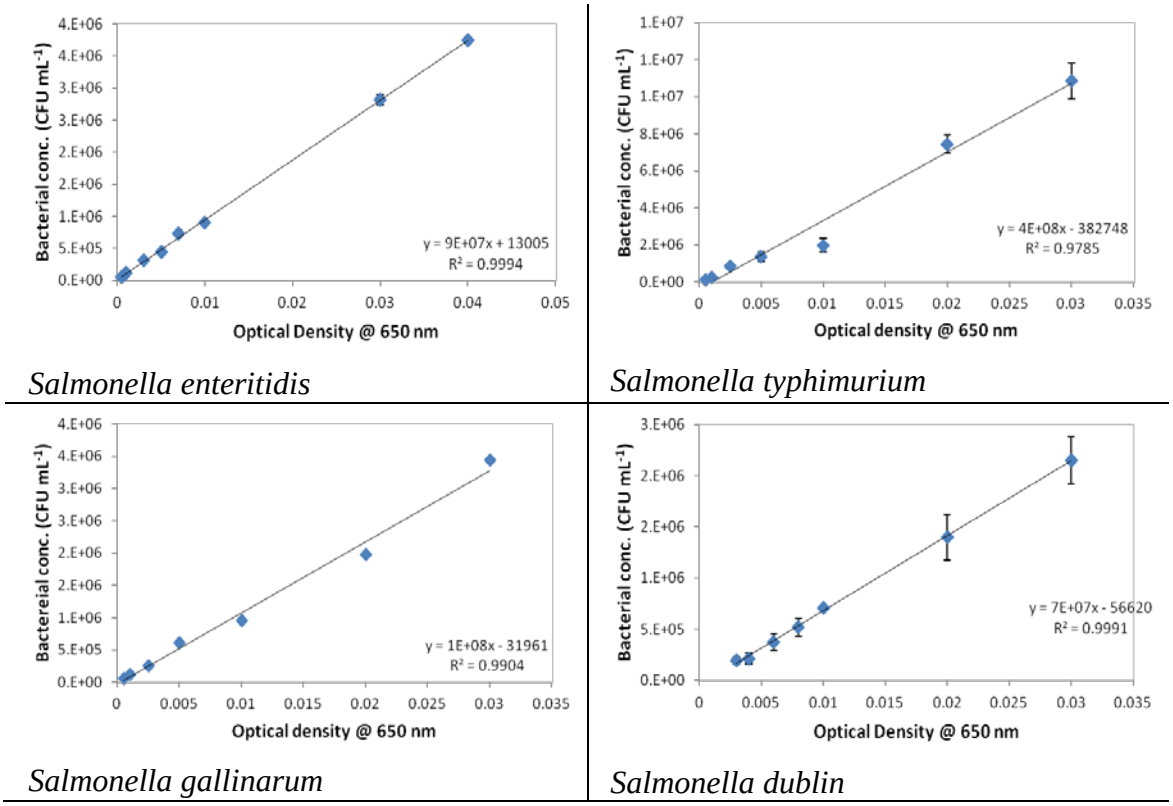
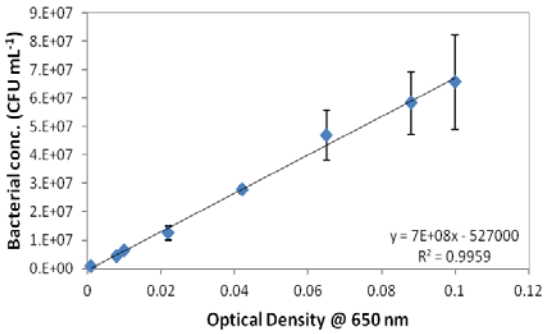
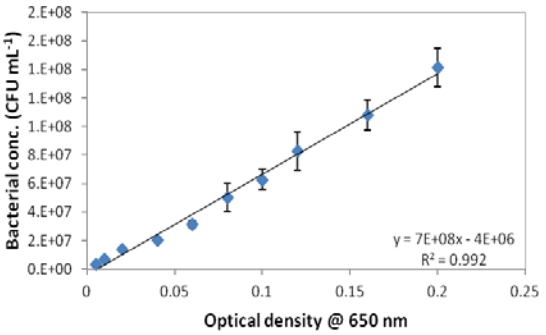


Figure 3.4. Standard curve of OD_{650nm} readings for *Salmonella* versus plate count CFU mL^{-1} values for each reading.

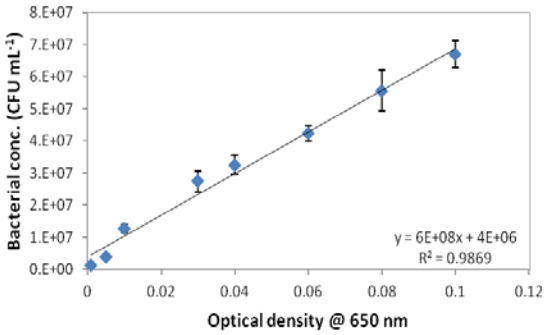
n = 3 for each point.



E. coli O157:H7



E. coli O2:K1:H-



E. coli O1:K1:H7

Figure 3.5. Standard curve of OD_{650nm} readings for *E. coli* versus plate count CFU mL^{-1} values for each reading.

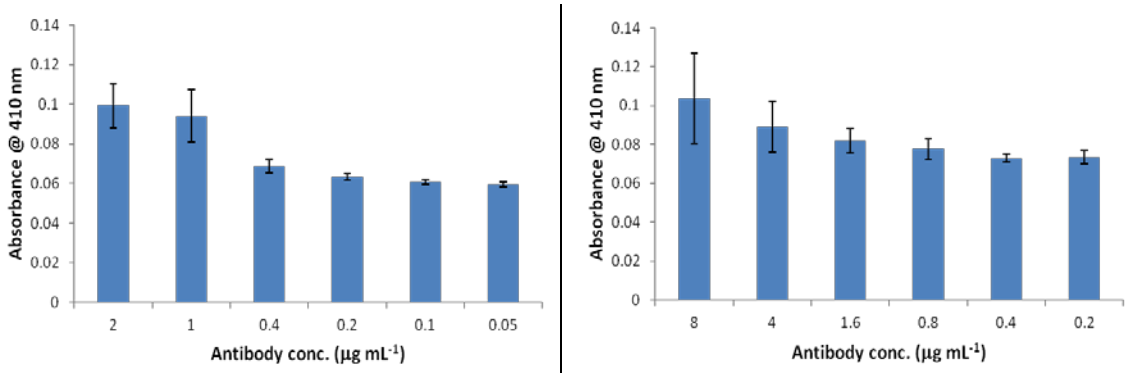
n = 3 for each point.

Linear standard curves were generated for each strain of bacteria with correlation coefficient (r^2) values of close to 1 for each strain. Indeed, r^2 values for each of the strains were shown to be greater than 0.978, indicating that there was a linear relationship between the optical density and the bacterial concentration for all strains of bacteria tested (Dong-ju, 2012). With this relationship established, OD_{650nm} values were used to determine the quantity of bacteria in growing bacterial cultures.

3.3.2 Optimisation of *Salmonella* and *E. coli* detection by Enzyme Linked Immunosorbent Assay (ELISA).

A horseradish peroxidise (HRP) labelled rabbit polyclonal antibody (ab53299) with specific activity against a mixture of *Salmonella* serotypes, and a biotin labelled rabbit polyclonal antibody (ab20640) with specific activity against a mixture of *E. coli*

serotypes were chosen for this study. The anti *Salmonella* antibody was tested against the four strains of *Salmonella* and the anti *E. coli* antibody was tested against the three strains of *E. coli* by ELISA. HRP conjugated streptavidin was used as the label for the detection of the biotin conjugated anti *E. coli* antibody. All bacterial strains were immobilised to the microtiter plate at pH 7.4 overnight at 37 °C using 10 mM PBS and the assays were performed as described in Section 2.2.4.2. Bovine serum albumin (BSA) was used as a blocking agent in the assays. The antibodies were diluted and a number of different antibody dilutions were assessed using the method outlined in Section 2.2.4.2. Absorbance values for blanks, i.e. no bacteria present, were initially assessed to determine the background noise of each antibody dilution. The absorbance at 410 nm for each concentration of anti *Salmonella* and anti *E. coli* antibody against the blank (10 mM PBS, pH 7.4) is shown in Figure 3.6. A blank sample should have an absorbance at 410 nm of less than 0.1, to confirm no non specific binding (Crowther, 2009).



Anti *Salmonella* antibody | Anti *E. coli* antibody

Figure 3.6. Determination of absorbance values for blanks for the anti *Salmonella* and anti *E. coli* antibodies.

n = 9 for each conc.

The antibody dilutions were also tested against a fixed concentration of bacteria, $10^{\wedge 6.5}$ CFU mL^{-1} in the case of *Salmonella* and $10^{\wedge 7.5}$ CFU mL^{-1} for *E. coli*. Work carried out by various groups found that the concentrations of *Salmonella* in chickens was approximately $10^{\wedge 6\text{-}7}$ CFU mL^{-1} while the concentration of coliforms was higher at approximately $10^{\wedge 6\text{-}9}$ CFU mL^{-1} (Jahanian and Ashnagar, 2015, Guard et al., 2015,

Ribeiro et al., 2007, Sims et al., 2004, Spring et al., 2000). Antibody dilutions with absorbance readings close to 1 were selected as the signal to blank was also taken into consideration. Results for antibody optimisation are presented in Figures 3.7 and 3.8.

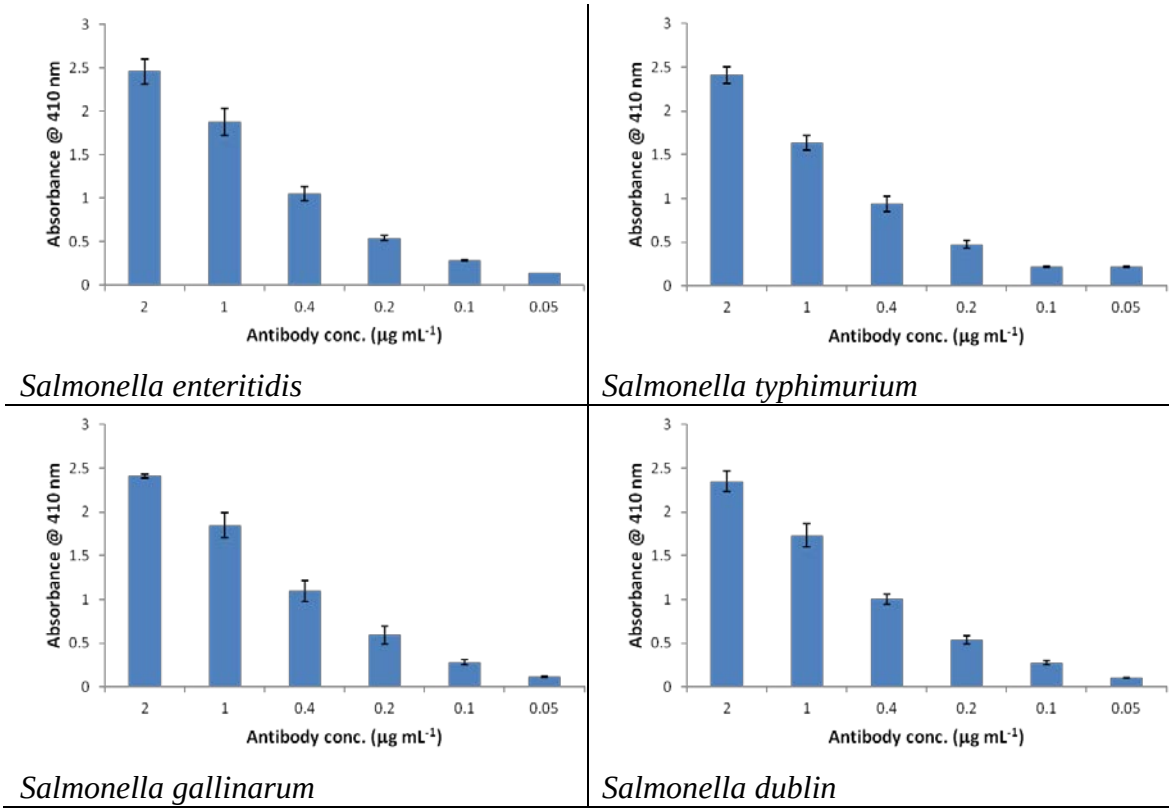
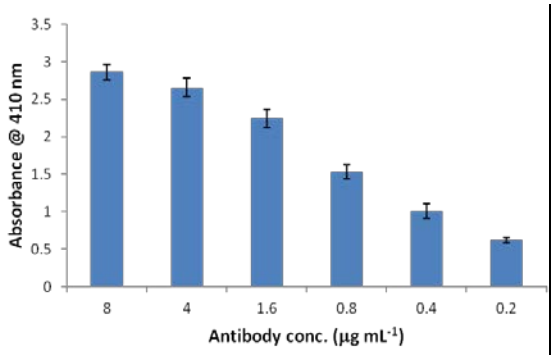
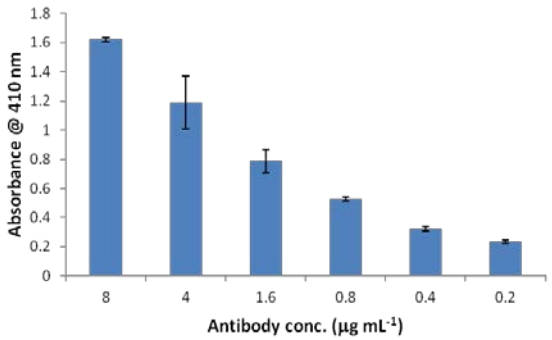


Figure 3.7. Optimisation of anti *Salmonella* antibody concentration for *Salmonella* quantification.

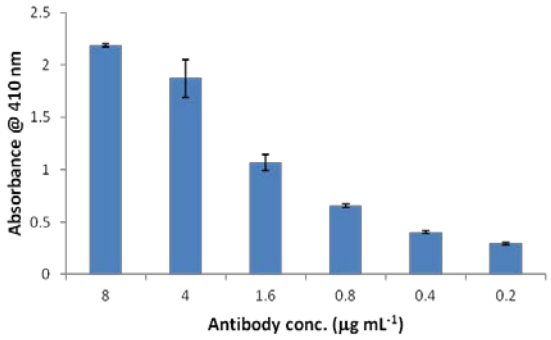
n = 3 for each point. Concentration of all *Salmonella* spp. = $10^{\wedge 6.5}$ CFU mL^{-1}.



E. coli O157:H7



E. coli O2:K1:H-



E. coli O1:K1:H7

Figure 3.8. Optimisation of anti *E. coli* antibody concentration for *E. coli* quantification.

n = 3 for each point. Concentration of all *E. coli* spp. = $10^{\wedge 7.5}$ CFU mL^{-1}

Comparison of absorbance values at 410 nm which were obtained for *Salmonella* spp. (Figure 3.7), an anti *Salmonella* antibody concentration of 0.4 µg mL^{-1} gave an optimum absorbance of approximately 1.0 at 410 nm for *Salmonella enteritidis*. The same antibody concentration was chosen for *Salmonella typhimurium, Salmonella gallinarum,* and *Salmonella dublin* as it produced similar absorbance readings of 1.0. This antibody concentration also produced a low background signal (Figure 3.6). A comparison of the absorbance values obtained for the *E. coli* spp. (Figure 3.8) showed that an anti *E. coli* antibody concentration of 0.4 µg mL^{-1} for *E. coli* O157:H7 produced an absorbance of approximately 1.0 at 410 nm. A concentration of 4 µg mL^{-1} for the antibody for *E. coli* O2:K1:H- yielded an absorbance of 1.0. A concentration of 1.6 µg mL^{-1} for *E. coli* O1:K1:H7 antibody gave an optimum absorbance for this strain of 1.0. The antibody concentrations for each strain of bacteria described here were chosen as

there was a loss of sensitivity with the lower antibody concentrations and the absorbance signal was too strong at the higher concentrations of the antibodies.

Once an antibody concentration was selected for each bacterial strain they were tested to determine if there was any cross reactivity with YCWM. The assay was performed as described in Section 2.2.4.2 with a modification, YCWM (10 μg 100 μL^{-1}) was incubated on the microtiter plate in place of bacteria. NUNC Maxisorp plates were chosen for this work as they only loosely adsorb hydrophobic macromolecules such as yeast cell wall mannan oligosaccharides (Masuoka and Hazen, 2004, Cole-Parmer, 1997). The absorbance values at 410 nm obtained using the anti *Salmonella* and anti *E. coli* antibodies at their optimised concentrations are shown in Table 3.7. Low absorbance values at 410 nm were obtained for all antibodies tested against YCWM. From these data it can be seen that neither the anti *Salmonella* antibody nor the anti *E. coli* antibodies reacted with YCWM, with absorbance values similar to the blank (10 mM PBS, pH 7.4). Due to the antibodies' selectivity for the bacteria strains *Salmonella* and *E. coli*, low blank values, and a lack of selectivity for YCWM these antibodies were used in the further development of the assays.

Table 3.7. Assessment of cross reactivity of antibodies with YCWM.

Antibody type	Antibody code	Antibody concentration (μg mL^{-1})	OD_{410nm} YCWM 10 μg 100 μL^{-1}	OD_{410nm} Blank (10 mM PBS)
Anti *E. coli*	ab20640	0.4	0.078 ± 0.018	0.079 ± 0.004
Anti *E. coli*	ab20640	1.6	0.070 ± 0.021	0.087 ± 0.005
Anti *E. coli*	ab20640	4.0	0.084 ± 0.012	0.076 ± 0.003
Anti *Salmonella*	ab53299	0.4	0.077 ± 0.006	0.066 ± 0.005

The optical density data for each antibody are represented as the mean ± standard deviation of the absorbance at 410 nm. n = 9 for all absorbance values.

3.3.3 Quantification of bacteria using an enzyme linked immunosorbent assay (ELISA).

Following the optimisation of the anti *Salmonella* and anti *E. coli* antibodies concentration, the ELISA's were carried out to determine the detection limits of the optimised antibody concentrations for each strain of bacteria. A standard curve of absorbance at 410 nm versus the $\log_{10}$ CFU mL^{-1} was generated. The results are shown in Figure 3.9 for the *Salmonella* strains and in Figure 3.10 for *E. coli* strains.

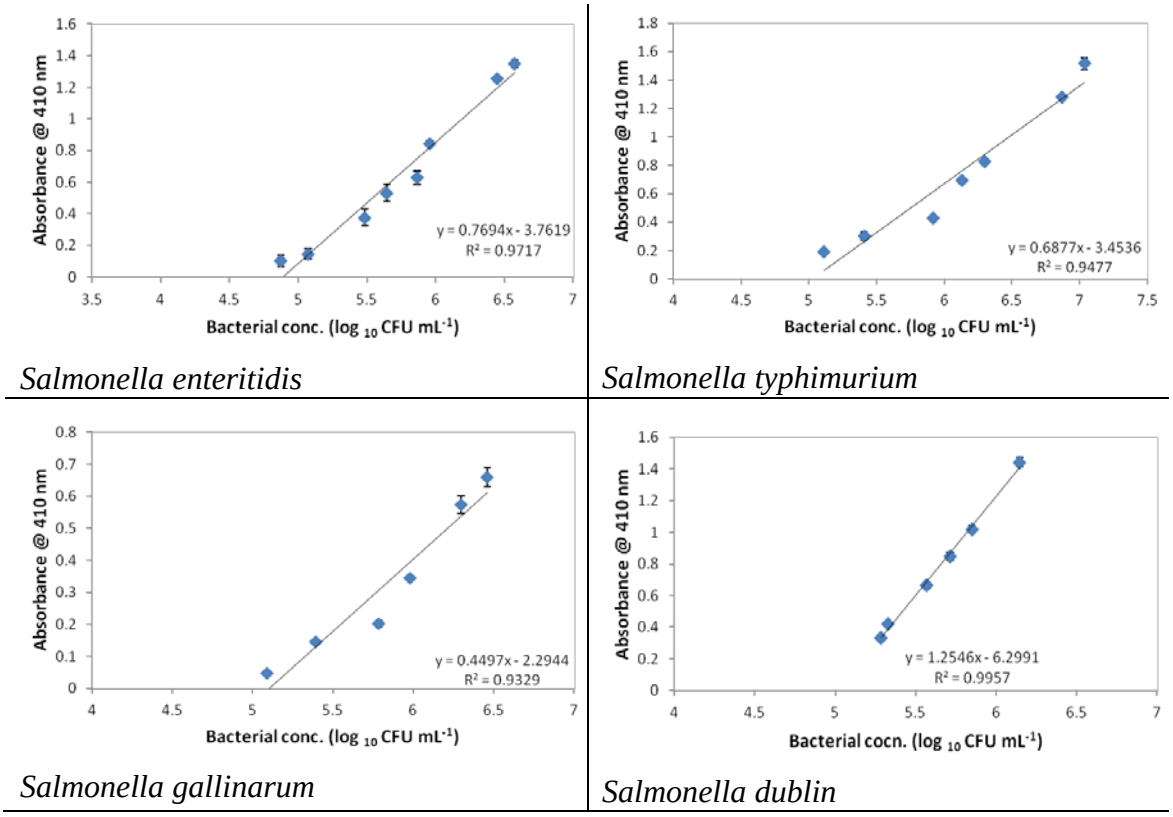
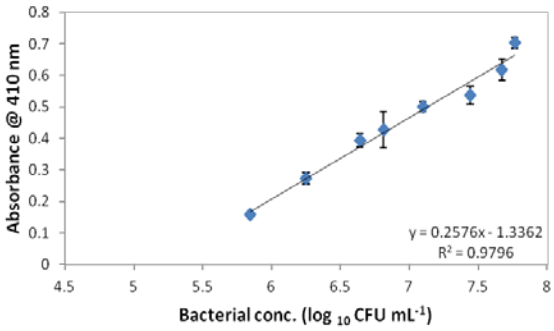
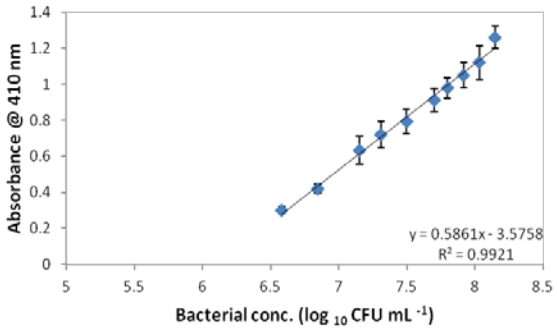


Figure 3.9. Standard curve for detection of *Salmonella* by ELISA.

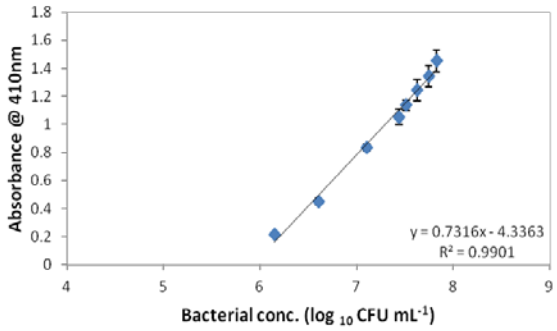
n = 6 for all values



E. coli O157:H7



E. coli O2:K1:H-



E. coli O1:K1:H7

Figure 3.10. Standard curve for detection of *E. coli* by ELISA.

n = 6 for all values.

Analysis of the standard curves in Figure 3.9 indicated that the relationship between the absorbance at 410 nm and the bacterial concentration was linear for all concentrations of bacteria. The r^2 values for each of the strains of *Salmonella* were greater than 0.933, confirming the linear relationship for all the strains of *Salmonella*. The linear range for each strain of *Salmonella* was between 4.68 – 6.57 $\log_{10}$ CFU mL^{-1} for *enteritidis*, 5.11 – 7.03 $\log_{10}$ CFU mL^{-1} for *typhimurium*, 5.08 – 6.45 $\log_{10}$ CFU mL^{-1} for *gallinarum*, and 5.28 – 6.14 $\log_{10}$ CFU mL^{-1} for *dublin*. The anti *Salmonella* antibody was raised against O and H antigens from a mixture of *Salmonella* strains. As each strain of *Salmonella* used in this study had a unique mixture of O and H antigens, for example, *Salmonella enteritidis* D 1, 9, 12 : g, m : -, *typhimurium* B 1, 4, 5, 12 : i : 1, 2, *gallinarum* D 1, 9, 12 : -, and *dublin* D 1, 9, 12 : g, p, the specificity of the antibody varied between the strains. The H antigen is known to have highly variable alleles between strains (Yue and Schifferli, 2014).

The standard curves for *E. coli* spp. are shown in Figure 3.10. The *E. coli* spp. standard curves also had a linear relationship with all concentrations of *E. coli* used; the r^2 values for each of the strains of *E. coli* were greater than 0.976, confirming the linear relationship for each strain of *E. coli.* The linear range for each strain of *E. coli* was between 5.84 – 7.76 $\log_{10}$ CFU mL^{-1} for *E. coli* O157:H7, 6.58 – 8.15 $\log_{10}$ CFU mL^{-1} for *E. coli* O2:K1:H-, and 6.14 – 7.82 $\log_{10}$ CFU mL^{-1} for *E. coli* O1:K1:H7. The numbers of bacteria differ from strain to strain as a result of the specificity of the polyclonal antibodies. The polyclonal anti *E. coli* antibody was similarly raised against O and H antigens from a mixture of *E. coli* strains. The serotypes of the *E. coli* strains used in this study also differed from each other, O157:H7, O2:H-, and O1:H7, possibly affecting the specificity of the anti *E. coli* antibody. These standard curves provided a means for the quantification of bacteria bound to a microtiter plate and were used in further development of the binding assays.

3.3.4 Determination of the bacterial concentration required for the yeast cell wall material binding assay.

Having optimised the method to quantify bacteria bound to a microtiter plate it was necessary to determine what concentration of bacteria was optimal for the cell wall material binding assay. The microscope work in the previous section confirmed that both *Salmonella* and *E. coli* could agglutinate to YCWM. However, this method was crude and the amount of bacteria bound to the YCWM could not be determined. To optimise the quantity of bacteria which could be bound by a fixed concentration of YCWM, a range of bacterial concentrations for each strain of bacteria were incubated with 10 μg YCWM, as described in Section 2.2.6. The concentration of the YCWM chosen was based on the inclusion level of Actigen® in feed (0.01 % - 0.08 %, w/w).

YCWM plus the bacteria bound to it were removed from the microtiter plate during the washing steps of the assays. The excess bacterial cells not bound to the YCWM remaining in the microtiter plate, were quantified by ELISA as outlined in Section 2.2.4.2. This value was subtracted from the initial amount of bacteria added to the plate to determine the quantity of bacteria agglutinated to YCWM. The number of bacteria bound to YCWM was expressed as the percent of bacteria removed and was calculated by dividing the amount of bacteria removed by the initial quantity of bacteria added to

the plate multiplied by 100. The percent bacteria removed from the plate for each concentration of bacteria was plotted against the $\log_{10}$ CFU mL^{-1} and are shown in Figures 3.11 and 3.12.

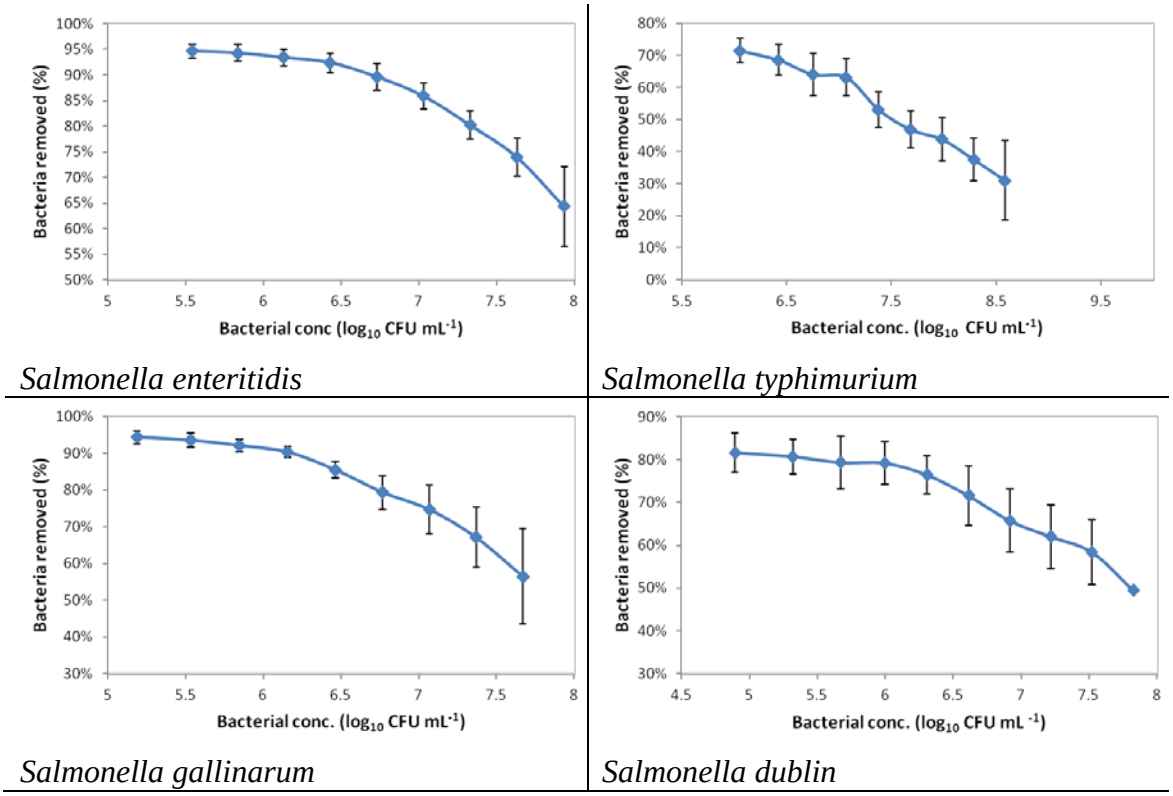
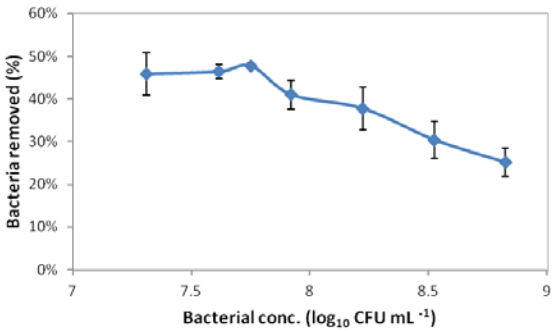


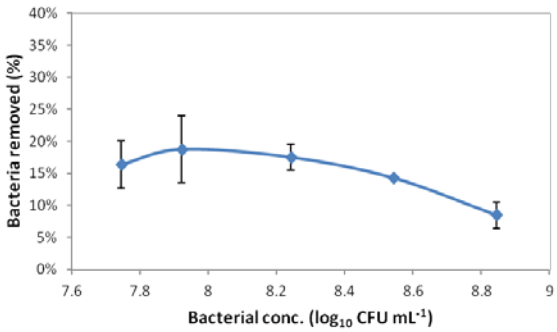
Figure 3.11. Determination of *Salmonella* concentration for YCWM binding assay.

n = 9 for all values. YCWM concentration = 10 μg 100 μL^{-1}.

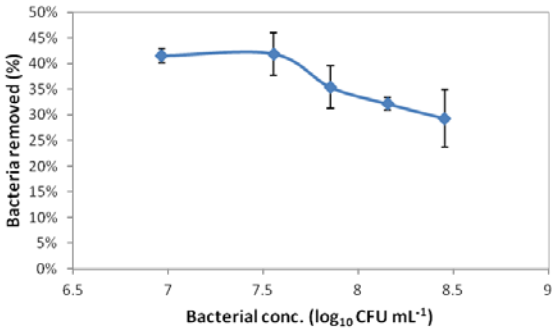
The point at which the graphs begin to decline was the maximum saturation point of the YCWM. Concentrations of bacteria greater than this resulted in the excess bacteria not bound to the YCWM, binding to the microtiter plate. The concentrations at which the YCWM became saturated for each *Salmonella* strain were as follows: *enteritidis* = 6.4 $\log_{10}$ CFU mL^{-1}, *typhimurium* = 7.0 $\log_{10}$ CFU mL^{-1}, *gallinarum* = 6.4 $\log_{10}$ CFU mL^{-1} and *dublin* = 6.0 $\log_{10}$ CFU mL^{-1}. These concentrations of bacteria are the concentrations used in all subsequent YCWM binding activity assays.



E. coli O157:H7



E. coli O2:K1:H-



E. coli O1:K1:H7

Figure 3.12. Determination of *E. coli* concentration for YCWM binding assay.

n = 9 for all values. YCWM concentration = 10 μg 100 μL^{-1}.

The concentrations at which the YCWM became saturated for each of the *E. coli* strains were *E. coli* O157:H7 = 7.6 $\log_{10}$ CFU mL^{-1}, *E. coli* O2:K1:H- = 7.7 $\log_{10}$ CFU mL^{-1}, and *E. coli* O1:K1:H7 = 6.7 $\log_{10}$ CFU mL^{-1}. As with the *Salmonella* strains, these bacterial concentrations were the concentrations of *E. coli* used in all subsequent binding activity assays. The low percentage of bacteria removed of *E. coli* O2:K1:H- may be due to the FimH alleles of this strain and the degree of specificity of the FimH lectin for mannan oligosaccharides present in YCWM (Weissman et al., 2006). Optimisation of the bacteria concentration allowed for repeat analysis with consistent results.

3.3.5 Confirmation of bacteria concentration bound to YCWM.

To corroborate that the calculated bacterial numbers agglutinated to the YCWM were correct, the bacteria removed from the microtiter plate was physically counted by plate count method (Section 2.2.4.1). The contents of the wells containing the mixture of YCWM and the bacteria were aseptically aspirated into sterile buffer, serially diluted, plated on agar plates, incubated overnight, and counted. Calculated removed was obtained by subtracting the quantity of bacteria remaining on the plate, determined by ELISA, from the quantity of bacteria added to the plate initially. The percent recovery of the bacteria was determined using the method outlined by the American Association for Laboratory Accreditation (A2LA, 2014); the results for the percent recovery are outlined in Table 3.8.

Table 3.8. Percent recovery of bacteria bound by yeast cell wall material.

Bacteria strain	**Removed bacteria (Plate count)**	**Removed bacteria (ELISA)**	**% Recovery**
	Log10 CFU mL^{-1} ± SD		Actual/ Calculated (%)
S. enteritidis	6.479 ± 0.86	6.435 ± 0.01	100.68 ± 0.86
S. typhimurium	7.050 ± 0.56	7.142 ± 0.02	98.71 ± 0.49
S. gallinarum	6.417 ± 0.80	6.497 ± 0.01	98.77 ± 0.83
S. dublin	6.048 ± 0.47	6.052 ± 0.01	99.93 ± 0.55
***E. coli* O157:H7**	7.274 ± 0.63	7.744 ± 0.01	93.93 ± 0.49
***E. coli* O2:K1:H-**	7.524 ± 0.62	7.576 ± 0.3	99.31 ± 0.49
***E. coli* O1:K1:H7**	7.275 ± 0.33	7.478 ± 0.02	97.29 ± 0.25

n = 3 for all values. SD = Standard deviation. The concentration of YCWM was 10 μg 100 μL^{-1}.

Comparisons of the bacterial number bound to YCWM (Calculated removed) to those determined by plate count are very similar. These results indicate that the subtraction

method is a suitable alternative to plate counting bacteria bound to YCWM each time the assay is run. The percent recovery of the bacterial strains bound to YCWM was between 93 % and 101 % for all strains of bacteria examined. The variations between the calculated and removed bacteria may be explained by variation in the plate count method. The plate count method has the ability to quantify only live bacteria as distinct from dead cells, whereas the ELISA method will calculate both live and dead cells as it is specific for cell surface antigens. Furthermore, the ELISA takes 2 days to generate results whereas the plate count method can take up to 4 days for colonies to appear in addition to being more labour intensive, and have lower throughput.

3.3.6 Method performance testing of YCWM activity assay

Before proceeding to sample analysis using the assays, the reliability of the assays was determined for each strain of bacteria. This involved ensuring that results obtained did not deviate greatly from day to day and that the method performance could be relied upon. The reproducibility and repeatability for each assay was assessed following the IUPAC guidelines for single laboratory validations (Thompson et al., 2002). A known quantity of bacteria was tested against the standard YCWM (10 μg), as described in Section 2.2.5. Two individual replicates of YCWM were prepared and run each day (inter assay repeatability) for a minimum of five days (intra assay reproducibility). Six replicate values were taken for each individual sample of the YCWM. A known quantity of bacteria was added to the plate at the beginning of the experiment. The quantity of bacteria bound to YCWM was calculated by subtracting what was left on the plate from what was added to the plate initially. The binding activity of the YCWM was calculated as follows:

$$\%\, Binding = \frac{CFU\ bound\ to\ YCWM}{CFU\ added\ initially} \times 100$$

Reliability was determined as the relative standard deviation (RSD) of the percent bacteria bound to YCWM on a minimum of five separate occasions. The RSD intra assay and inter assay were calculated and the results are shown in Table 3.9.

Table 3.9. Repeatability and reproducibility for the yeast cell wall material activity assay

Pathogen Strain	*S. enteritidis*	*S. typhimurium*	*S. gallinarum*	*S. dublin*	O157:H7	O2:K1:H-	O1:K1:H7
% Binding	93.27 %	52.27 %	86.45 %	77.87 %	52.42 %	29.47 %	41.36 %
SD	2.65	3.98	2.30	7.46	6.00	3.44	4.59
RSD Intra assay	2.84	7.62	2.66	9.59	11.44	11.69	11.15
RSD Inter assay	0.5	2.5	0.9	0.6	2.9	2.9	4.7
n	60	60	60	60	60	60	60

Results presented are a representation of mean ± standard deviations of a minimum of 60 replicates. Relative standard deviation (RSD) was calculated standard deviation/mean x 100. n = number of replicates. SD = standard deviation

The inter assay RSDs' varied between 0.5 % and 4.7 % and the intra assay RSDs' for these strains varied between 2.66 % and 11.69 %. The level of precision observed for each individual investigation was below the acceptable level of 15 % (Crowther, 2009, Huber, 2007). The increased intra assay RSD values between the *E. coli* and *Salmonella* strains may have been caused by the additional incubation step in the assay with the labelled avidin.

These results show that YCWM binds a specific quantity of *Salmonella* and *E. coli* depending on the strain. While it was not possible to count the numbers of bacteria bound to the YCWM with the microscope work, it is possible to draw some comparisons between the percent of bacteria removed (Table 3.9) and the images in Figure 3.2 and 3.3. YCWM was shown to remove approximately 90 % *Salmonella enteritidis*, 50 % *typhimurium*, 85 % *gallinarum*, and 75 % *dublin*. The images in Figure 3.2 show that the amount of bacteria bound to YCWM on the slide after agglutination follows a similar trend to the percent bacteria removed, with *Salmonella typhimurium* and *dublin* showing more free bacteria cells on the slide. Agglutination of *E. coli* O157:H7 to YCWM was determined to be approximately 50 % and this corresponded to the image of bound and free cells in Figure 3.3 B. A similar trend may be observed for *E. coli* O1:K1:H7 (Figure 3.3 F), which was approximately 45 % bound to YCWM. However, for *E. coli* O2:K1:H- agglutination to YCWM there were very few free cells visible on the slide after agglutination in Figure 3.3 D indicating high agglutination while the actual value determined was approximately 30 %. The discrepancies between the determined values and the images of agglutination are due to the subjective nature of the agglutination on slides. Uniform binding is not seen across the slide and is difficult to achieve as the YCWM and bacteria are quickly mixed on the slide. This highlights the necessity of a quantitative assay.

As the YCWM standard material was the same for each experiment, the variation in the amount of bacteria agglutinated to the YCWM was strain specific. The variation in binding activity between the strains of bacteria may be due to differences in the FimH lectin of type 1 fimbriae between these strains. The difference in sequence homology of the FimH adhesin between *Salmonella* and *E. coli* reported by Kisiela et al. (2013) may account for the variation in agglutination seen between these two species. YCWM had higher binding activity for *Salmonella* than for *E. coli* strains in this study. Furthermore, the difference in the binding activity of YCWM was observed in this study between

strains of the same species. Significant heterogeneity among FimH adhesins from strains of the same serovar exists and are reported to be responsible for the selective binding to different mannosylated oligosaccharides. Differences in FimH proteins and binding phenotypes have been described between strains of *Salmonella enteritidis*, *typhimurium*, *gallinarum* and *dublin* (Grzymajlo et al., 2013, Kuzminska-Bajor et al., 2012, Kisiela et al., 2006). Variants of FimH, found on different strains of *E. coli*, bind with varying affinity to different mannose oligosaccharides such as mono- or tri-mannose residues. The FimH adhesin's from *E. coli* strains O2 and O1 have different receptor specificities (Kisiela et al., 2006, Weissman et al., 2006, Sokurenko et al., 1995). The variance in FimH between *E. coli* O1 and O2 is reflected in the differences in the binding activity of these two strains to YCWM seen in this study. Additionally, FimH receptors of *E. coli* bind di- and tri-mannose residues 10 fold higher than D-mannose (Bouckaert et al., 2006) suggesting that these mannose residues may be less prevalent in YCWM resulting in lower binding of *E. coli* compared to the *Salmonella* strains. The presence of certain mannan oligosaccharides in YCWM may cause increased binding of specific strains used in this study to the YCWM. It also confirms the requirement for a broader spectrum YCWM product with higher affinity for these specific bacterial strains.

These results demonstrated that the proposed assays can reliably estimate the binding activity of YCWM for bacteria. These assays are suitable for screening multiple yeast cell wall material samples and will be useful for monitoring lot-to-lot consistency and stability of YCWM product, as well as identifying novel products with a high capacity to agglutinate bacteria. Based on these findings, these assays were used to determine the activity of a number of yeast cell wall samples prepared from various yeast strains characterised in Section 3.1.

3.4 Binding activity of yeast cell wall preparations

S. cerevisiae cell wall material (YCWM) is sold worldwide under the trade name Actigen® as an animal feed supplement, which can bind enteropathogenic bacteria such as *Salmonella* and *E. coli* spp. and reduce colonisation (Luquetti et al., 2012, Biggs et al., 2007). Bacteria bound to yeast cell wall material do not bind to the mucous membranes of the gut *in vivo* and are swept away by the flow of secretions (Gedek, 1999). YCWM is rich in mannan oligosaccharides and these polymers in the cell wall are responsible for the binding of bacteria via mannose specific type 1 fimbriae (Sharon and Ofek, 2000). Quantification of the bacteria agglutinated by YCWM is a major advantage in the search for new products for the animal health industry. Following development and method performance testing of the yeast cell wall material binding assays, a selection of the cell wall samples generated and analysed in Section 3.1 of this thesis were assessed for their capacity to bind the bacteria listed previously. Cell wall samples from yeast grown in medium containing glucose as a sole carbon source were screened because glucose is an inexpensive carbon source (Liu, 2012) in the event of scale up. Samples grown in medium containing mannose as the sole carbon source were also analysed as they were shown to slightly increase the cell wall mannose content of a number of yeast cell wall samples compared to the controls in Section 3.1. The relationship between the cell wall monosaccharide content and the binding activity of the cell wall samples was also investigated. All samples were prepared as described in Section 2.2.8 and subjected to the yeast cell wall material binding activity assays as described in Section 2.2.5 using YCWM as the control. The binding activity of each sample for a number of bacterial strains is presented in Figures 3.13 – 3.26.

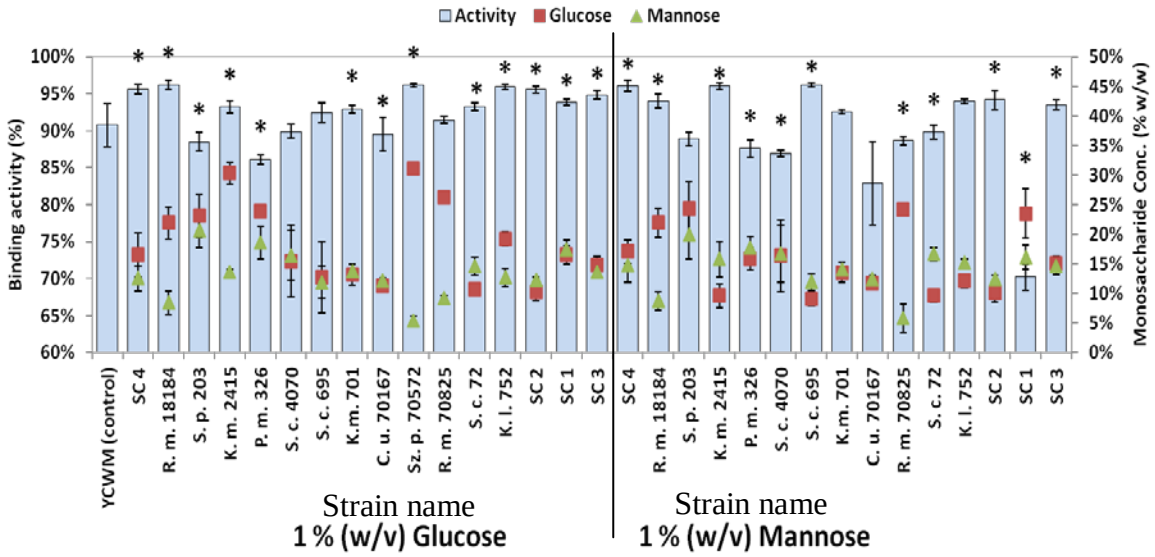


Figure 3.13. Binding activity of *Salmonella enteritidis* by yeast YCWM samples grown in medium containing 1 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

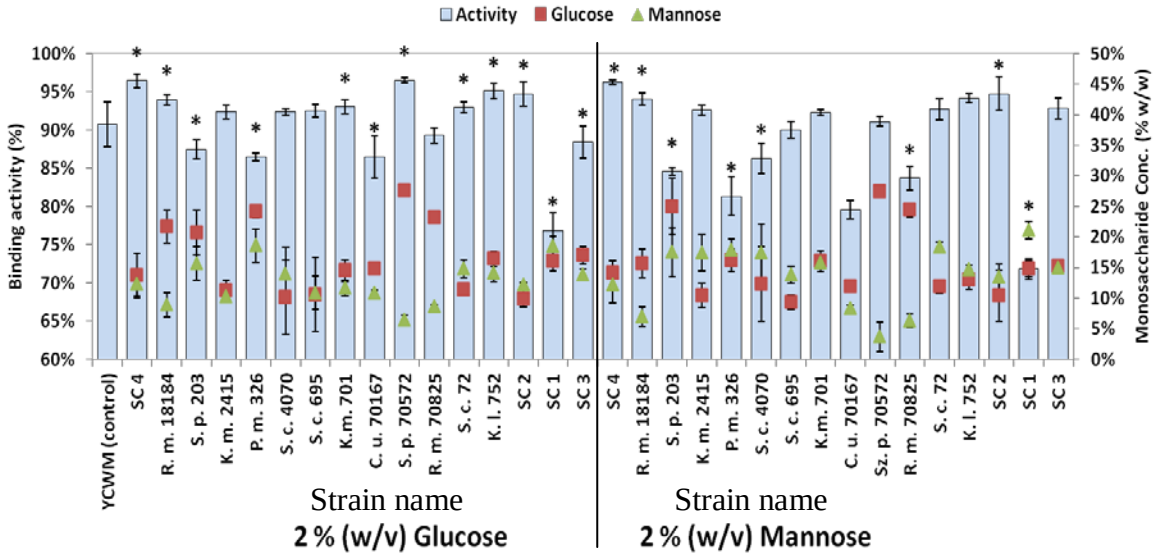


Figure 3.14. Binding activity of *Salmonella enteritidis* by yeast YCWM samples grown in medium containing 2 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

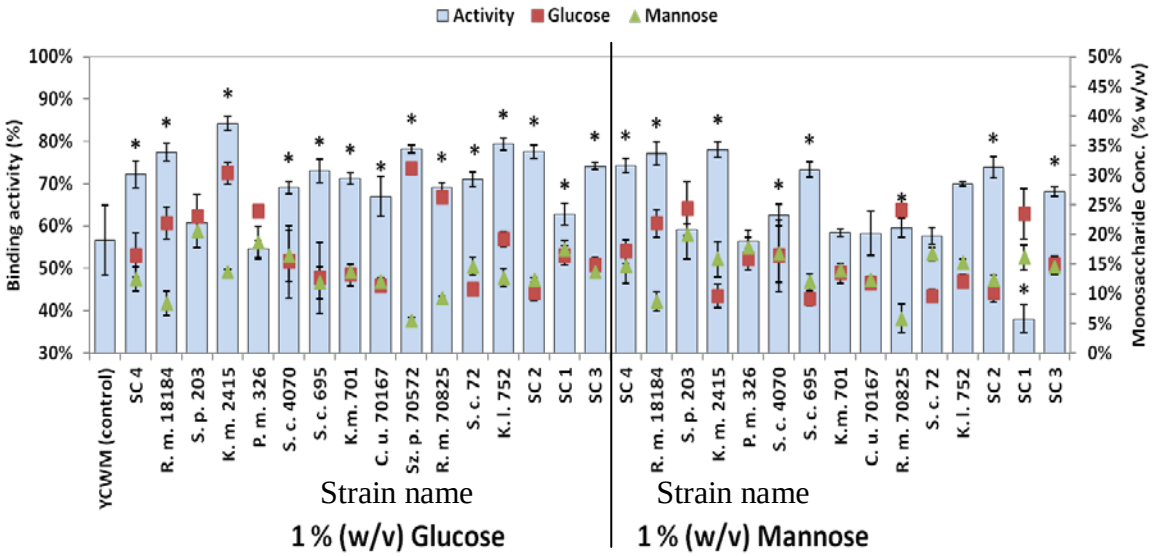


Figure 3.15. Binding activity of *Salmonella typhimurium* by yeast YCWM samples grown in medium containing 1 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

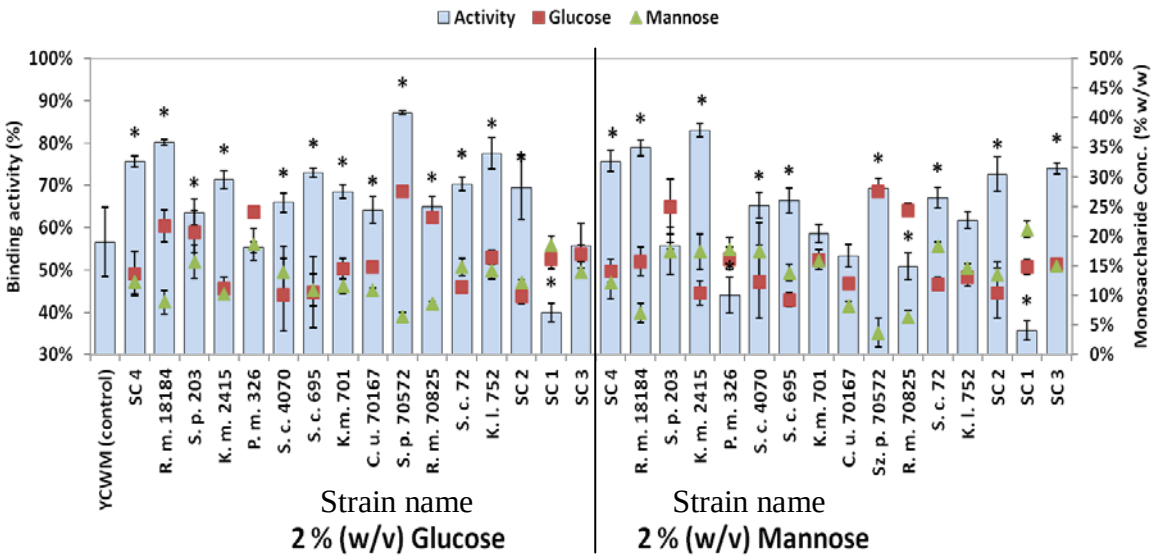


Figure 3.16. Binding activity of *Salmonella typhimurium* by yeast YCWM samples grown in medium containing 2 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

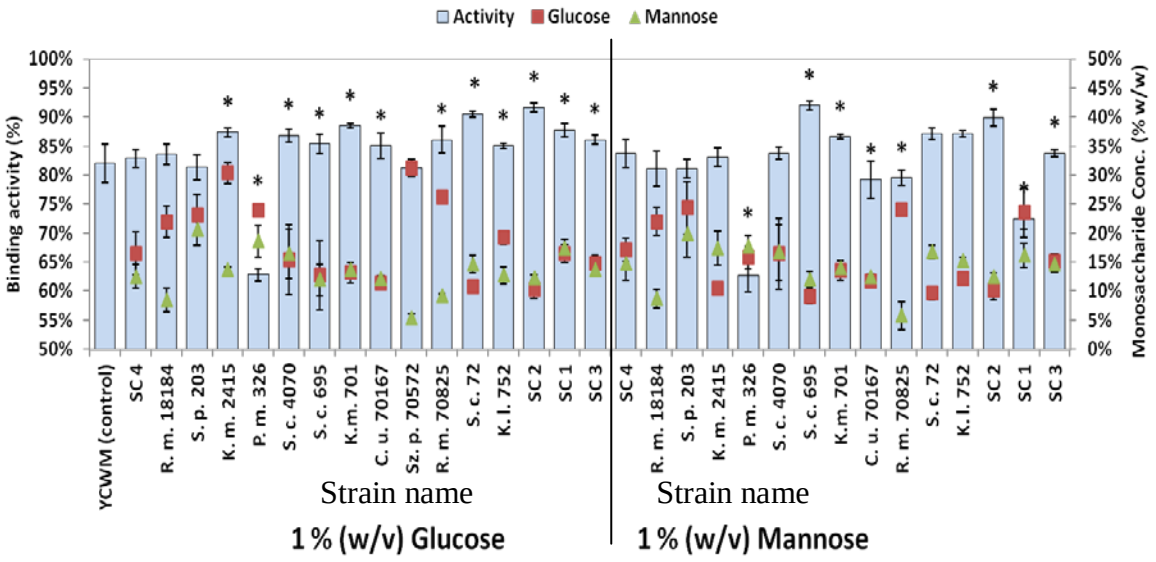


Figure 3.17. Binding activity of *Salmonella gallinarum* by yeast YCWM samples grown in medium containing 1 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

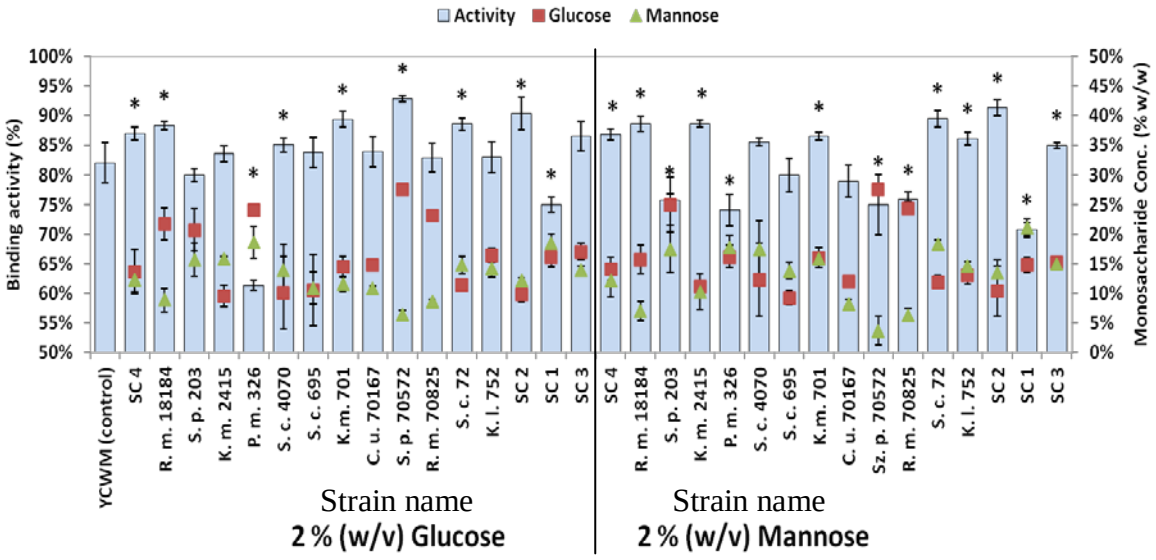


Figure 3.18. Binding activity of *Salmonella gallinarum* by yeast YCWM samples grown in medium containing 2 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

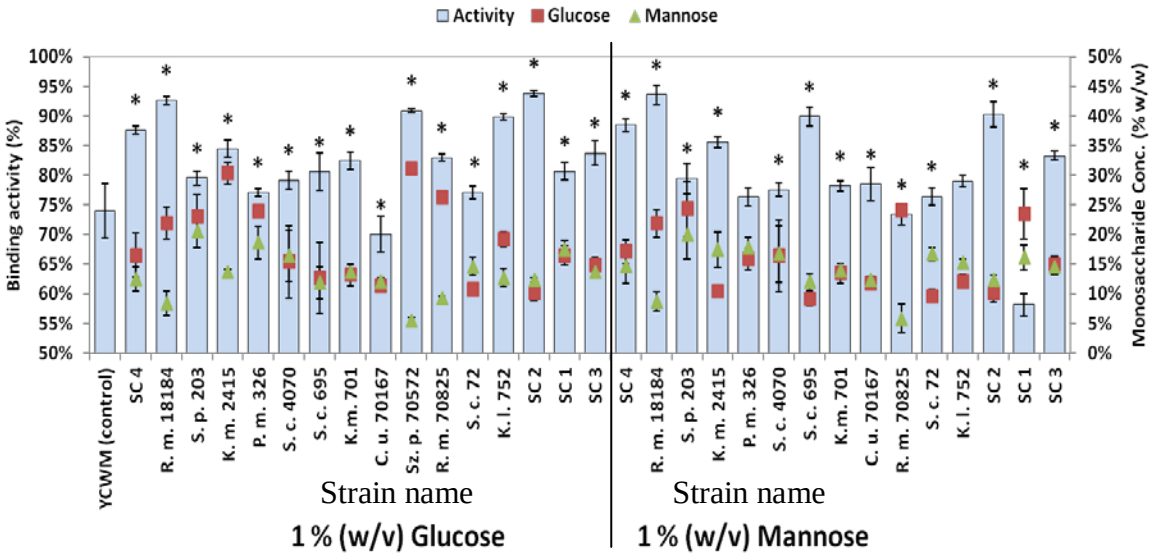


Figure 3.19. Binding activity of *Salmonella dublin* by yeast YCWM samples grown in medium containing 1 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

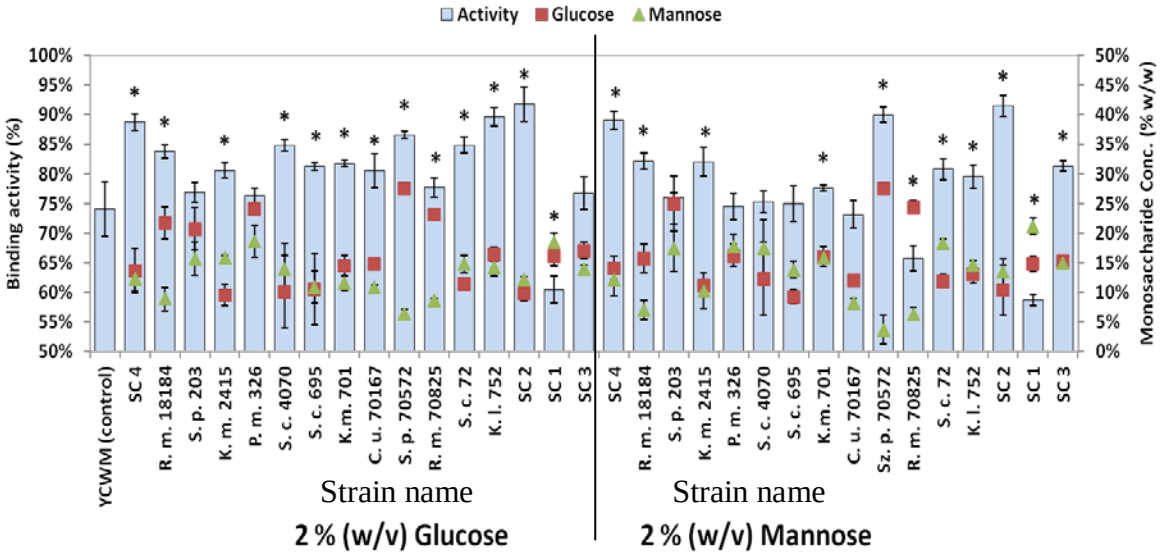


Figure 3.20. Binding activity of *Salmonella dublin* by yeast YCWM samples grown in medium containing 2 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

The percent binding activity of the cell wall samples ranged between 70.2 and 96.5 % for *S. enteritidis*, 35.7 to 87.2 % for *S. typhimurium*, 61.1 to 93.7 % for *S. gallinarum*, and 58.1 to 93.8 % for *S. dublin*, shown in Figures 3.12-3.20. The percent glucose and mannose content (%, w/w) of the yeast cell wall samples were also presented: mannose content ranged between 3.7 and 21.2 % (w/w), and glucose content ranged between 8.2 and 34.3 % (w/w) (Tables 3.1-3.6). One-way analysis of variance (ANOVA) and Dunnett's multiple comparisons were carried out to identify any significant differences among percent binding activity compared to the control sample (YCWM), where the confidence level was set at 95 %. The YCWM standard was prepared through an industrial autolytic process, outlined in Section 1.7, whereas the yeast samples were prepared under lab conditions (Section 2.2.3.2).

With respect to *Salmonella enteritidis*, the binding activity for the majority of yeast cell wall samples was significantly different to the YCWM control sample. Twenty eight samples were identified with significantly higher binding activity than the control and 18 with lower binding activity, while 17 samples had similar binding activity to the control. The sample with the highest activity (96.5 %) was identified as cell wall from *S. pombe* 70572 grown in 2 % (w/v) glucose containing medium; while cell wall sample from SC 1 grown in 1 % (w/v) mannose containing medium had the lowest binding activity with *Salmonella enteritidis*. *S. cerevisiae* 695 grown in 1 % (w/v) mannose containing medium also had high binding activity (96.2 %), as did *S. cerevisiae* SC 4 grown in 2 % (w/v) glucose and mannose as the sole carbon source in the growth medium. Of the remaining samples *K. lactis* 752 grown in 1 % (w/v) glucose as the sole carbon source was shown to have high binding activity (95.9 %).

For *Salmonella typhimurium*, the majority of the samples were again shown to have higher binding activity than the control. Forty eight samples had a higher binding activity for *Salmonella typhimurium*, 14 samples had the same activity, while only 5 samples were found to have lower activity than the control. *S. pombe* 70572 sample, grown in medium containing 2 % (w/v) glucose as the sole carbon source, was identified as having the highest binding activity (87.2 %) for *Salmonella typhimurium*. Again, SC 1 grown in medium containing 1 or 2 % (w/v) mannose had the lowest binding activity for *Salmonella typhimurium* (35 and 38 %). Samples from *K. lactis* 752 and *K. marxianus* 2415 grown in 1 % (w/v) glucose containing growth medium demonstrated high binding activity similar to *S. pombe* 70572. Of the *Saccharomyces*

strains tested for binding activity against *Salmonella typhimurium*, SC 4 and 695 samples grown in medium containing 1 % (w/v) medium had high binding activity. Cell wall from SC 2 and SC 3, grown in 1 % glucose containing medium also had high activity.

With respect to *Salmonella gallinarum*, thirty eight samples were found to have significantly higher binding activity than the control, 18 samples with the same binding activity, and 12 samples with lower binding activity than the control. Cell wall from *S. pombe* 70572 grown in growth medium containing 2 % (w/v) glucose was identified as having the highest binding activity (93.2 %). The sample with the lowest binding activity for *Salmonella gallinarum* was from *P. membranifaciens* 326 grown in each of the 4 carbon source combinations. *S. cerevisiae* 695 grown in growth medium containing 1 % mannose as the sole carbon source was shown to have the highest binding activity (91.9 %) for *Salmonella gallinarum* of all the stains of *Saccharomyces* used. *K. marxianus* 2415 had the highest binding activity of the *Kluyveromyces* strains and its binding activity was significantly higher than the control.

Of all the samples tested against *Salmonella dublin*, 54 samples had significantly higher activity than the control, with 12 samples having similar binding activity to the control, while only 4 samples had significantly lower binding activity than the control. Interestingly, *S. cerevisiae* SC 2 cell wall, grown in growth medium containing 1 % (w/v) glucose as the sole carbon source, had the highest binding activity (93.8 %) of all the cell wall samples for *Salmonella dublin*. Cell wall from *S. pombe* 70572 grown in growth medium containing 2 % (w/v) glucose as the sole carbon source also had a significantly high binding activity of 86.6 % for *Salmonella dublin*. *R. mucilaginosa* 18184 cell wall, grown in 1 % (w/v) glucose and 1 % (w/v) mannose containing medium, had very high binding activity (93.5 and 92.6 %) against *Salmonella dublin* compared to the control. As with *Salmonella enteritidis* and *typhimurium*, SC 1 had the lowest binding activity with *Salmonella dublin* of 58 %. *S. cerevisiae* 695 grown in growth medium containing 1 % (w/v) mannose as the sole carbon source also had significantly high binding activity compared to the control for *Salmonella dublin*. Similarly, *K. lactis* 752 grown in growth medium containing 1 % glucose had the highest binding activity (89.8 %) of all the *Kluyveromyces* strains used for *Salmonella dublin*.

From these results a number of cell wall samples ranked higher in binding activity for all strains of *Salmonella* compared to the other samples. The cell wall samples with high binding activity for all *Salmonella* strains included cell wall from *S. pombe* 70572 grown in growth medium containing 2 % (w/v) glucose, *S. cerevisiae* 695 grown in growth medium containing 1 % (w/v) mannose, and *K. lactis* 752 grown in growth medium containing 1 % (w/v) glucose; indicating that the cell wall of certain yeast strains contain fractions that are involved in increased binding activity for type 1 fimbriae containing *Salmonella*. Indeed the cell wall content of yeast is known to vary between strains (Jaehrig et al., 2008, Klis et al., 2002). Each cell wall sample was shown to contain various amounts of cell wall glucose and mannose. Nevertheless, it may be that the structure and not the content of the cell wall saccharides is important in binding bacteria (Shoaf-Sweeney and Hutkins, 2009).

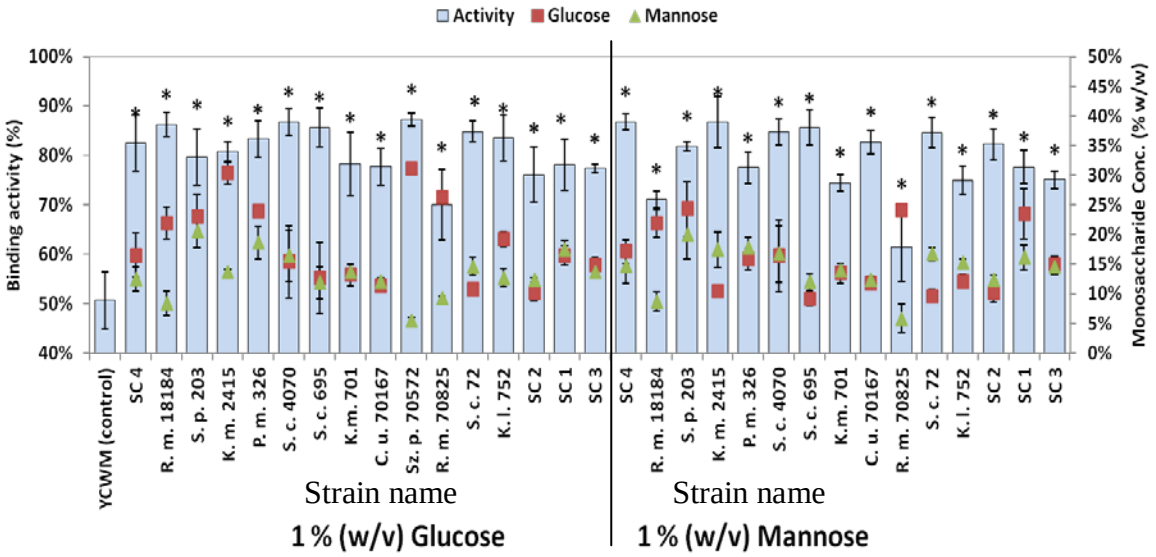


Figure 3.21. Binding activity of *E. coli* O157:H7 by yeast YCWM samples grown in medium containing 1 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

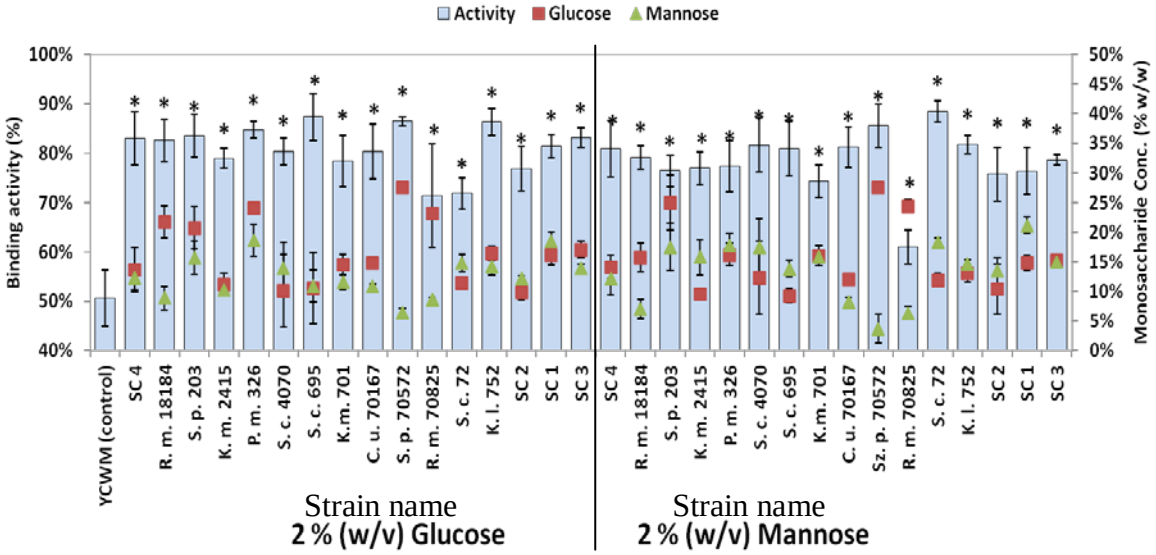


Figure 3.22. Binding activity of *E. coli* O157:H7 by yeast YCWM samples grown in medium containing 2 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

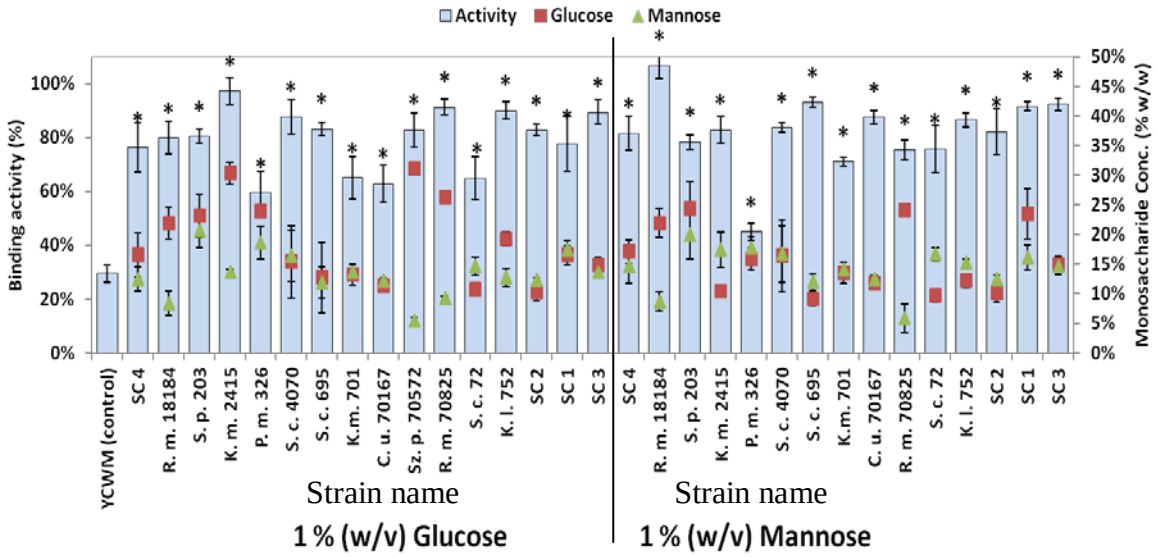


Figure 3.23. Binding activity of *E. coli* O2:K1:H- by yeast YCWM samples grown in medium containing 1 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

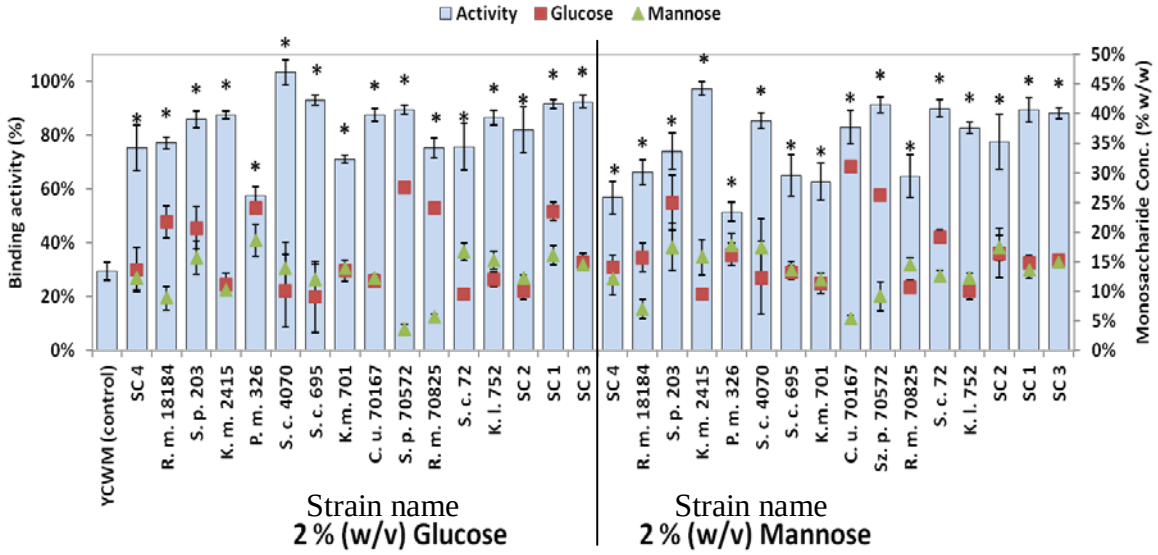


Figure 3.24. Binding activity of *E. coli* O2:K1:H- by yeast YCWM samples grown in medium containing 2 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

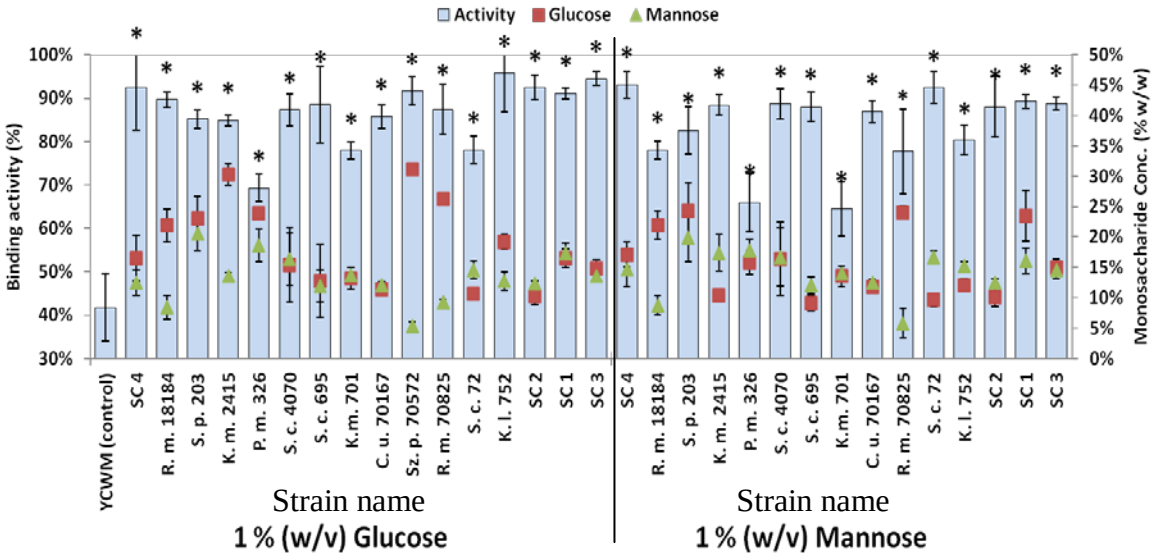


Figure 3.25. Binding activity of *E. coli* O1:K1:H7 by yeast YCWM samples grown in medium containing 1 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

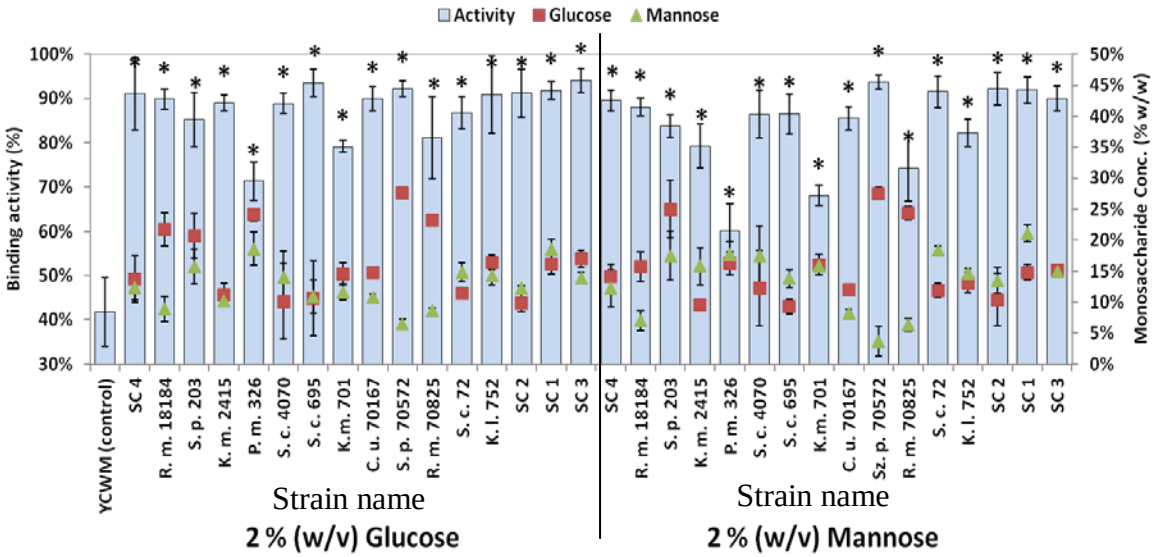


Figure 3.26. Binding activity of *E. coli* O1:K1:H7 by yeast YCWM samples grown in medium containing 2 % (w/v) glucose or mannose as the sole carbon source.

Means labelled with an asterisk [*] are significantly different to the control, $p \leq 0.05$. n = 3 for monosaccharide concentration. n = 9 for activity measurements.

The binding activity of the cell wall samples for the *E. coli* strains are shown in Figures 3.21-3.26. In contrast to the activity results of the *Salmonella* strains, all samples were found to have significantly higher binding activity than the control. With respect to *E. coli* O157:H7, the cell wall of *S. cerevisiae* 72 grown in growth medium containing 2 % (w/v) mannose had the highest activity (88.5 %). Interestingly, *S. cerevisiae* 72 cell wall, grown in growth medium containing 2 % (w/v) glucose, had the lowest percent activity (71.8 %) of all the samples. *S. pombe* 70572 cell wall, grown in medium containing 2 % (w/v) glucose as the sole carbon source, also had high activity (87.3 %). *K. lactis* 752 grown in 1 % (w/v) mannose was found to have high binding activity with this strain of *E. coli.* As with strains of *Salmonella*, cell wall samples from *S. cerevisiae* 695 grown in growth medium containing 1 and 2 % (w/v) glucose and 1 % (w/v) mannose had high binding activity for *E. coli* O157:H7 of above 85 %. As with *E. coli* O157:H7, all samples had significantly higher binding activity than the control sample for *E. coli* O2:K1:H-. *R. mucilaginosa* 18184 cell wall grown in 1 % (w/v) mannose had the highest binding activity. This sample also had high activity for *Salmonella dublin*. In contrast to *R. mucilaginosa* 18184, *R. mucilaginosa* 70825 cell wall was shown to have very low percent activity for this strain of *E. coli* (51.5 %); confirming that the cell wall of *Rhodotorula* spp. is a highly varied structure (Klimek-Ochab et al., 2011). As with *Salmonella gallinarum*, *P. membranifaciens* 326 cell wall samples had the lowest binding activity with this strain of *E. coli* (48.3 %). *S. pombe* 70572 cell wall, grown in growth medium containing 2 % (w/v) glucose as the sole carbon source, was shown to have high activity for binding *E. coli* O2:K1:H- (89.4 %). Samples from *K. lactis* 752 and *K. marxianus* 2415 were also shown to have high binding activity for this strain. A number of *S. cerevisiae* samples including 695, SC 3, and SC 1 also demonstrated high binding activity. The binding activities of all samples were significantly higher than the control for *E. coli* O1:K1:H7. *K. lactis* 752 cell wall, grown in growth medium containing 1 % (w/v) glucose, was shown to have the highest percent activity (95.7 %) for *E. coli* O1:K1:H7. Once more *P. membranifaciens* 326 grown in a number of carbon sources was shown to have the lowest binding activity of all the samples (60.1 – 70 %). *S. pombe* cell wall grown in growth medium containing 2 % (w/v) glucose also had high binding activity (92.1 %) for *E. coli* O1:K1:H7. A number of *S. cerevisiae* samples also demonstrated high binding activity for this strain of bacteria.

While the samples with the highest binding activity vary between strains of bacteria, a number of cell wall samples were found to have high binding activity with the majority of bacterial strains. These samples include *S. pombe* 70572 grown in 2 % (w/v) glucose as the sole carbon source in the growth medium, *S. cerevisiae* 695 grown in 1 % (w/v) mannose as the sole carbon source in the medium, and *K. lactis* 752 grown in 1 % (w/v) glucose as the sole carbon source in the growth medium. In addition, the binding activities of these samples were significantly higher than the control for all strains of bacteria used in this study. Cell wall samples that can bind more bacteria than the control sample are of interest for the development and production of new or next generation products for the control of type 1 fimbriae containing *Salmonella* and *E. coli*.

From the binding activity results of the samples, it is difficult to draw conclusions between the binding activity and the saccharide content without carrying out correlation analysis on the data to determine if there is a relationship between the two. A Spearman-Rho test was used to determine any positive or negative correlations in the glucose or mannose content and the binding activity of the samples for each strain of bacteria. A positive (+) correlation, when an increase in monosaccharide content correlated with an increase in binding activity, or a negative correlation (-), when an increase in monosaccharide content correlating with a decrease in binding activity, are presented in Table 3.10 and 3.11. There were numerous statistically significant correlations ($p \leq 0.05$) between the glucose or mannose content (% w/w) and the binding activity of the yeast samples. One limitation of this type of statistical approach is that it only measures the relationship between two variables and does not allow for determination of cause and effect (Elliott and Woodward, 2006).

Table 3.10. Summary of correlations observed for the relationship between binding of *Salmonella* and the monosaccharide content.

Yeast YCWM		***Salmonella enteritidis***		***Salmonella typhimurium***		***Salmonella gallinarum***		***Salmonella dublin***	
		Glucose (% w/w)	Mannose (% w/w)	Glucose (% w/w)	Mannose (% w/w)	Glucose (% w/w)	Mannose (% w/w)	Glucose (% w/w)	Mannose (% w/w)
Saccharomyces cerevisiae	72	+	-	+	-		-	+	
Saccharomyces pastorianus	203	-	+	-		-	+		+
Saccharomyces cerevisiae	4070	-			+		+	-	-
Saccharomyces cerevisiae	SC 4						+		
Saccharomyces cerevisiae	695				-	-		-	
Saccharomyces cerevisiae	SC 3	-		-	+		-	-	
Saccharomyces cerevisiae	SC 1			+	-				
Saccharomyces cerevisiae	SC 2								
Kluyveromyces lactis	752	+	-	+	-	-	+	+	-
Kluyveromyces marxianus	701		-	-	-		-	-	-
Kluyveromyces marxianus	2415	-		+	-				+
Schizosaccharomyces pombe	70572	+	+	-	+	+	+		-
Candida utilis	70167		+					+	
Rhodotorula mucilaginosa	70825	-	+	-	+	-	+	-	+
Pichia membranifaciens	326						-		
Rhodotorula mucilaginosa	18184	+				-		+	

Significant Spearman Rho correlations are represented here ($p \leq 0.05$). Positive correlations are denoted with (+) and negative correlations with (-).

Table 3.11. Summary of correlations observed for the relationship between binding of *E. coli* and the monosaccharide content.

Yeast YCWM		***E. coli* O157:H7**		***E. coli* O2:K1:H-**		***E. coli* O1:K1:H7**	
		Glucose (% w/w)	Mannose (% w/w)	Glucose (% w/w)	Mannose (% w/w)	Glucose (% w/w)	Mannose (% w/w)
Saccharomyces cerevisiae	72		+		+	+	+
Saccharomyces pastorianus	203	-		-		-	
Saccharomyces cerevisiae	4070	+	+	-	-		
Saccharomyces cerevisiae	SC 4	+	+				
Saccharomyces cerevisiae	695		-	+	-	+	-
Saccharomyces cerevisiae	SC 3	+		+			+
Saccharomyces cerevisiae	SC 1			-	+	+	-
Saccharomyces cerevisiae	SC 2						
Kluyveromyces lactis	752	+	-	+	-	+	-
Kluyveromyces marxianus	701		-		-		-
Kluyveromyces marxianus	2415		+	+	-	-	+
Schizosaccharomyces pombe	70572			-		-	
Candida utilis	70167			+	-	+	
Rhodotorula mucilaginosa	70825	-	+		+	-	+
Pichia membranifaciens	326	+	+	+	+	+	+
Rhodotorula mucilaginosa	18184			+	+		

Significant Spearman Rho correlations are represented here ($p \leq 0.05$). Positive correlations are denoted with (+) and negative correlations with (-).

The results in Table 3.10 and 3.11 underscore the considerable variation in the correlation data between the yeast cell wall sample's glucose and mannose content and the binding activity for the strains of bacteria. Of the yeast samples shown to have high binding activity with the majority of bacterial strains *S. pombe* 70572 was found to have varying correlations between its cell wall monosaccharide content and its binding activity for each strain of bacteria. A negative correlation was found between the cell wall glucose content of *S. pombe* 70572 and the binding activity for *E. coli* O2:K1:H-, O1:K1:H7, and *Salmonella typhimurium*. A positive correlation between the cell wall glucose content and the binding activity existed for *Salmonella enteritidis* and *gallinarum*. The cell wall mannose content of *S. pombe* was positively correlated with *Salmonella enteritidis, typhimurium,* and *gallinarum,* and negatively correlated with *Salmonella dublin*. *K. lactis* 752 was found to have a significant positive correlation between the cell wall glucose content and the binding activity for each strain of *E. coli* and for *Salmonella enteritidis, typhimurium,* and *dublin,* while a significant negative correlation was shown for the cell wall mannose content and the binding activity for the same strains. Similar to *K. lactis* 752, *S. cerevisiae* 695 was found to have a negative correlation between the cell wall mannose content and the binding activity for all strains of *E. coli* and with *Salmonella typhimurium*. Furthermore, a positive correlation between the cell wall glucose content of *S. cerevisiae* 695 and the binding activity was found for *E. coli* O2:K1:H- and O1:K1:H7, while a negative correlation between cell wall glucose content and the binding activity of *Salmonella gallinarum* and *dublin* was observed. It is evident that there is no clear pattern between the yeast cell wall monosaccharide content and the binding activity of these strains.

The relationship between the monosaccharide content and the binding activity was unclear from these results. It suggests that other factors are involved in the binding activity of samples and not necessarily the glucose and mannose content of the samples. The differences observed may be dependent on the strain of yeast and the structure of the mannan oligosaccharides. Again differences in the cell wall material structure and content between different strains of yeast within the same species have been reported (Lane et al., 2011, Nguyen et al., 1998). Differences in the binding activity between cell wall samples derived from different strains of same species were observed with all strains of bacteria in this study. For example, cell wall samples from the various strains within the same species of *Saccharomyces, Kluyveromyces,* or *Rhodotorula* displayed

different binding activities, and correlations between cell wall saccharide content and binding activity, from other cell wall samples from the same species. Tiago et al. (2012) also found that certain strains of *Saccharomyces* could agglutinate *Salmonella* and *E. coli* quicker than others *in vitro*. *K. marxianus* 2415 displayed a different set of correlations between binding activity and cell wall glucose or mannose content when compared to the other two strains of *Kluyveromyces*. This may be due to reported differences between various strains of *Kluyveromyces* (Belloch et al., 2002).

The correlation between the cell wall glucose or mannose content of the samples and binding activity for bacteria did not follow an obvious pattern, with several samples having positive correlations between the glucose or mannose content and the binding activity of various strains of *Salmonella* or *E. coli* and negative correlations between the glucose and mannose content and the binding activity with others. Differences in the composition or structure of the cell wall mannans of various yeast strains exist. For example, the mannose segment of *S. cerevisiae* cell wall is predominately composed of α-(1, 2), α-(1, 3) and α-(1, 6) mannan linkages. *Candida* spp. produce poly β-(1, 2) linked mannose chains attached by linkages to their mannans. *Pichia* spp. do not contain the α-(1, 3) mannan caps of *S. cerevisiae* mannan chains (Gemmill and Trimble, 1999). *S. pombe* contain α-(1, 2) linked galactose side chains (Perez and Ribas, 2004). And *Kluyveromyces* species contain GlcNAc residues on their mannan oligosaccharides (Park et al., 2011). *Rhodotorula* species have mannose side chains containing alternating β-(1, 3) and β-(1, 4) linked residues (McGinnis, 2012). According to Badia et al. (2013) it is the structure of the cell wall mannans that are important in the binding activity of type 1 fimbriae containing bacteria. For example, different strains of *E. coli*, bind with varying affinity to different mannose oligosaccharides such as mono or trimannose residues (Shoaf-Sweeney and Hutkins, 2009, Weissman et al., 2006).

The search for cell wall samples as potential new products with high binding activity for specific pathogens led to the discovery of a number of candidate samples. These include *S. pombe* 70572, *S. cerevisiae* 695, and *K. lactis* 752. Each yeast strain is currently employed in an industrial or commercial application and are GRAS (Benito et al., 2014, van Ooyen et al., 2006, Parker et al., 1980). *S. pombe* 70572 cell wall, grown in 2 % (w/v) glucose as the sole carbon source in the growth medium, was found to have significantly higher binding activity than the control for each strain of *Salmonella* and

E. coli. The cell wall of this fission yeast is known to differ from budding yeast such as *S. cerevisiae* in a number of ways. Acid hydrolysis and subsequent analysis of the hydrolysate revealed it to have relatively low cell wall mannose content compared to other yeast strains. Moreover, *S. pombe* contains galactomannans in its cell wall as opposed to the more common yeast mannans (Ballou et al., 1994). Galactomannans are a yeast mannan with galactose units bound at terminal positions, mainly by α-(1, 2) and β-(1, 2)-linkages (Hamilton and Gerngross, 2007, Perez and Ribas, 2004). This yeast also contains unconventional α-(1, 3) glucan residues located on the surface of the cell wall. Interestingly, while traces of GlcNAc, the monomer from which the polymer chitin is composed, have been found in the cell wall of this yeast no chitin has ever been identified in the cell wall (Perez and Ribas, 2004). These unique properties of *S. pombe* may explain why this strain of yeast had high binding activity with all strains of bacteria. A study using β-galactomannan oligosaccharides in pig feed showed a significant reduction in the *Salmonella* populations, including *Salmonella typhimurium*, compared to an untreated control group (Andres-Barranco et al., 2015). *In vitro* investigations also found that galactomannans reduced the adhesion of *Salmonella typhimurium* and *E. coli* to pig intestinal cell line IPI-2I (Badia et al., 2013). Interestingly, *E. coli* K88 which is mannose resistant, as it contains type 4 (F4) and not type 1 fimbriae, was also sensitive to galactomannan residues (Gonzalez-Ortiz et al., 2014, Badia et al., 2013). This indicates that galactomannans may have a wider range of target binding sites other than the mannose sensitive type 1 lectins.

S. cerevisiae 695 cell wall sample, grown in medium containing 1 % (w/v) mannose as the sole carbon source, had significantly higher binding activity than the control for all strains of bacteria. Interestingly, this cell wall sample had relatively low levels of cell wall glucose (~ 9 %, w/w) and mannose (~ 12 %, w/w). This suggests that further investigation into the structure of the mannan oligosaccharides fraction is necessary. The high activity of this sample may be due to the hypermannosylated residues of cell wall glycoproteins of *S. cerevisiae* spp. *S. cerevisiae* mannoproteins contain long α-(1, 6) linked mannose backbones linked with shorter α-(1, 2) and α-(1, 3) mannose side chains (Orlean, 2012). However, the cell wall samples from other strains of *Saccharomyces* used in this study had different binding activities to each other. A review by Klis et al. (2006) states that the cell wall content of different strains of *S.*

cerevisiae can vary between strains. This supports the observation that binding activity may be strain dependent.

K. lactis 752 has a cell wall structure similar to *S. cerevisiae* (Backhaus et al., 2011). However, different carbon sources were shown to influence the cell wall architecture and content of *Kluyveromyces* compared to *Saccharomyces* species, signifying fundamental differences between the cell walls of the various stains of yeast (Backhaus et al., 2010). Additionally, *Kluyveromyces* species are known to add GlcNAc, a common modification of mammalian oligosaccharides (Park et al., 2011), during glycosylation and this might contribute to the increased activity seen with a number of bacteria stains. GlcNAc residues are part of a number of pathogen oligosaccharide adherence sites on host mucosal surfaces, including endothelial, respiratory, stomach, and genital, for a variety of species (Shoaf-Sweeney and Hutkins, 2009, Firon et al., 1984).

In contrast to the samples with high binding activity, *P. membranifaciens* had the lowest binding activity with a number of strains of bacteria regardless of the carbon source. Hypermannosylation occurs less frequently and to a lesser extent in *Pichia* spp. (Liu et al., 2009a). *Pichia* spp. are known to have shorter N-glycans than *Saccharomyces* spp., in addition they lack α-(1, 3) linked mannan caps on their mannan oligosaccharides (Gemmill and Trimble, 1999) due to the absence of an α-(1, 3) mannosyltransferase (Wildt and Gerngross, 2005). These differences may be contributing factors to the decreased binding activity of this species as type 1 fimbriae containing bacteria have been reported to bind α-(1, 3) mannanoligosaccharides (Shoaf-Sweeney and Hutkins, 2009).

Interestingly, no significant difference in the cell wall monosaccharide content was observed for samples grown in growth medium containing any of the carbon sources for *S. pombe* 70572, *S. cerevisiae* 695, and *K. lactis* 752. Different concentrations of carbon source have been shown to alter the cell wall composition of *Saccharomyces* (Liu et al., 2009b, Aguilar-Uscanga and Francois, 2003) but these studies looked at only one specific strain of *Saccharomyces,* expanding their study to include other strains may have shown different results similar to the observations in this study. It is difficult to explain the variation in binding activity between the samples based on the cell wall glucose and mannose content; it may be due instead to the structure of the N-linked

mannans of the cell wall proteins (Schierholt et al., 2011). N-linked glycoproteins of yeast cell wall are hypermannosylated structures extending out from the cell (Lipke and Ovalle, 1998).

As the bacterial binding mechanisms are only partially understood, it may be that cell wall structure, yeast strain diversity, and non specific interactions might also play important roles in bacterial agglutination. The structure of the carbohydrate, specifically the mannan fraction, of the yeast cell wall material samples may be critical in the binding activity of the samples. Fragmentation of the complex sugar moieties of the high activity yeast samples was undertaken. Comparison of the profiles generated for each sample was carried out to try and explain the differences in activity observed for each sample.

3.5 N-glycan profiling of yeast cell wall samples.

The role of complex oligosaccharides and bacteria binding has been well documented (Shoaf-Sweeney and Hutkins, 2009, Yang et al., 2008, Terré et al., 2007, Mourao et al., 2006, Sharon and Ofek, 2000, Spring et al., 2000, Oyofo et al., 1989). Some strains of bacteria were found to vary as much as 100 fold with their affinity to various mannan oligosaccharides (Sharon, 2006). With this in mind, analysis of the N-glycan profile of yeast cell wall material samples with high binding activity for type 1 fimbriae containing bacteria was carried out.

The majority of the proteins found on the cell surfaces of most organisms are glycosylated (Bowman and Free, 2006, Andreishcheva et al., 2004). Glycosylation is the most common and most diverse protein modification in eukaryotic cells (Zhou et al., 2007). N- and O-linked glycosylation of *S. cerevisiae*, *C. albicans*, *P. pastrious*, *K. lactis*, and *S. pombe* glycoproteins have been studied in depth (De Groot et al., 2005, Wildt and Gerngross, 2005). Glycans are attached at the reducing terminus through N-acetylglucosamine (GlcNAc) to the amide group of certain asparagine (Asn) residues and are typically comprised of seven or more component monosaccharides (Haltiwanger and Lowe, 2004). Yeast N-glycans have a common core type oligosaccharide with 9-13 mannose residues, which may be extended with an α-(1, 6) mannosyl backbone heavily substituted with short mannosyl side chains, to produce hypermannosylated glycans. These long hypermannosylated structures attached to cell wall proteins extend out from the cell (Jigami, 2008, Lipke and Ovalle, 1998). The variation in binding activity of YCWM may be attributed to more than just the mannose or glucose content but rather to the complexity of how these sugars are linked together (Sharon, 2006). The aim of this section was to examine the glycan profile for a number of yeast cell wall material samples, to shed light on whether differences between their profiles corresponded to differences in their binding activities.

Yeast cell wall samples were chosen for N-glycan analysis based on their high binding activity for type 1 fimbriae containing bacteria. The samples chosen for further analysis are listed in Table 3.12. The control YCWM was also subjected to N-linked glycan analysis.

Table 3.12. Yeast cell wall samples for glycan profiling.

Yeast strain	Carbon source (% w/v)
***S. cerevisiae* (695)**	1 % (w/v) Mannose
***S. Pombe* (70572)**	2 % (w/v) Glucose
***K. lactis* (752)**	1 % (w/v) Glucose

3.5.1 N-glycan analysis of yeast cell wall samples.

The cell wall proteins were extracted from the samples using CHAPS extraction buffer as described in Section 2.2.10.1. In order to profile the N-glycans, they were first released from the polypeptide backbone (Triguero et al., 2010) by PNGase F digestion (Section 2.2.10.2). The glycan samples were fluorescently labelled with 2-aminobenzamide (2-AB) and analysed by hydrophilic interaction chromatography (HILIC-UPLC) (Section 2.2.10.3-4). This is a variation of normal phase chromatography that allows detection at femtomole levels (Royle et al., 2008). Polar analytes partition between an immobilized water layer surrounding a polar stationary phase in a high solvent environment. Weak interactions, such as van der Waal forces and hydrogen bonding, become significantly enhanced. Oligosaccharide retention is based on the interaction of hydrophilic surfaces on sugar molecules with the polar HILIC phase. The larger oligosaccharides contain more hydrophilic surfaces and have increased retention times. Very large polysaccharides contain too many hydrophilic surfaces to be resolved on the column and are eluted in the wash step. However, digestion of the N-glycan samples with specific glycosidases reduce the larger oligosaccharide chains that are outside the range of the method to shorter chain saccharides or monomers within the range of the method (Royle et al., 2006). Once the oligosaccharides have been enzymatically digested the UPLC profile can be re-analysed and peak shifts will identify peaks that originally contained glycans with the monosaccharides of interest. As YCWM was used as the control in the yeast cell wall material binding assays its glycan profile was analysed as a reference. The results are shown in Figure 3.27.

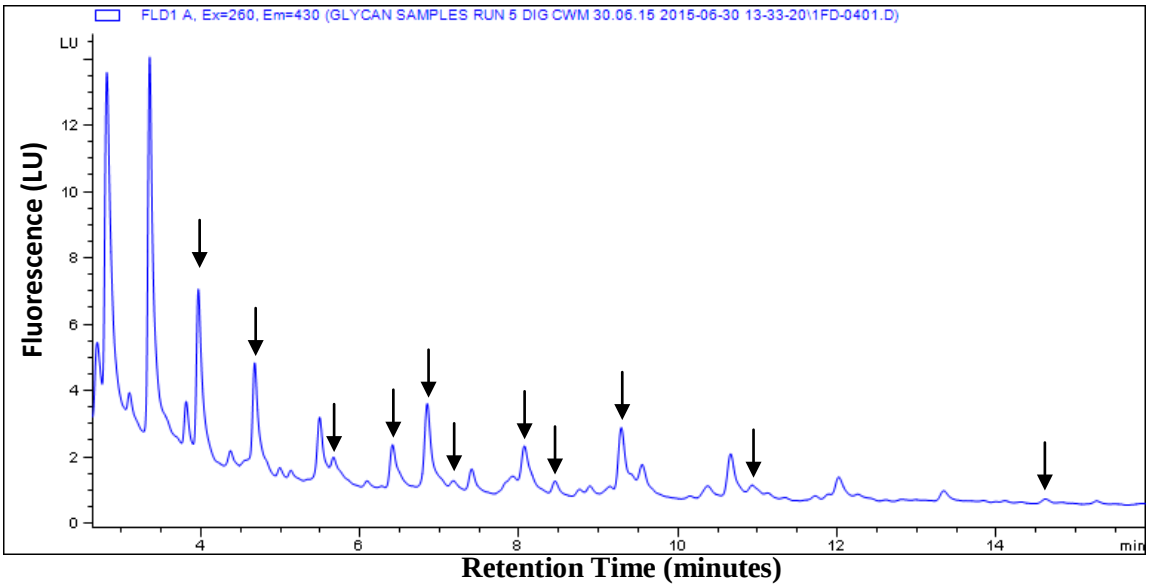


Figure 3.27. HILIC chromatogram of 2-AB labelled YCWM PNGase F digested sample.

Samples were run on equal weight basis and in duplicate. ↓ arrows indicate peaks that resolved at the same time in the glycan profile of *S. cerevisiae* 695, *K. lactis* 752, and *S. pombe* 70572.

YCWM is produced from *S. cerevisiae* var. *boulardii*, the PNGase F digest of this sample had a glycan retention profile between 3 and 16 minutes. The chromatogram was cropped between 3 and 16 minutes to exclude the excess 2-AB label peak which elutes at 1 minute, a large monosaccharide peak that eluted between 2 and 3 minutes, and material that came off in the wash step above 16 minutes. The saccharides elute based on the size of the glycan polymers. Monomers elute first followed by oligosaccharides of increasing size.

In *S. cerevisiae* the N-glycans consist of mannose residues elongated from the GlcNAc core. Yeast glycans are hypermannosylated (Wildt and Gerngross, 2005) and can contain more than 150 mannose residues (Liu et al., 2009a). The N-glycan profile of YCWM (Figure 3.27) contains many peaks which represent oligosaccharides of different lengths and complexity. The large peaks at the beginning of the glycan profile between 3 and 5 minutes indicate an abundance of short chain sugar residues. This chromatogram also indicates that there are numerous oligosaccharides of different lengths present in this sample, eluting between 5 and 15 minutes. In addition, the solid arrows in Figure 3.27 indicate peaks that resolved at the same time in the glycan profile of the other samples analysed (Figure 3.28-3.30). Analysis of the glycan profiles

allowed for comparison between YCWM and samples with increased binding activity. Both common and sample specific peaks were identified.

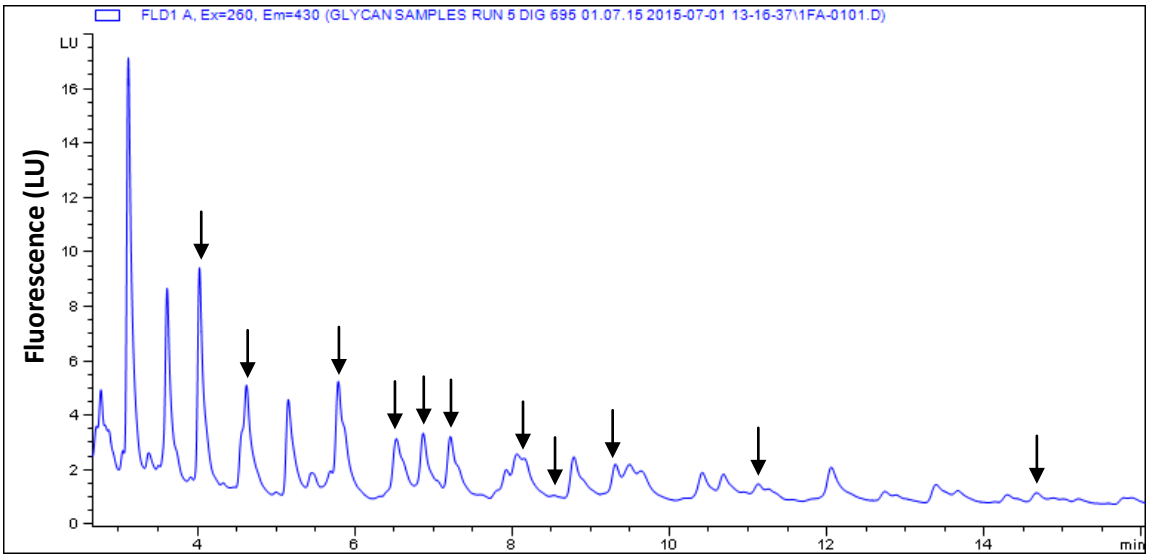


Figure 3.28. HILIC chromatogram of 2-AB labelled *S. cerevisiae* 695 PNGase F digested sample.

Samples were run on equal weight basis and in duplicate. ↓arrows indicate peaks that resolved at the same time in the glycan profile of YCWM, *K. lactis* 752, and *S. pombe* 70572.

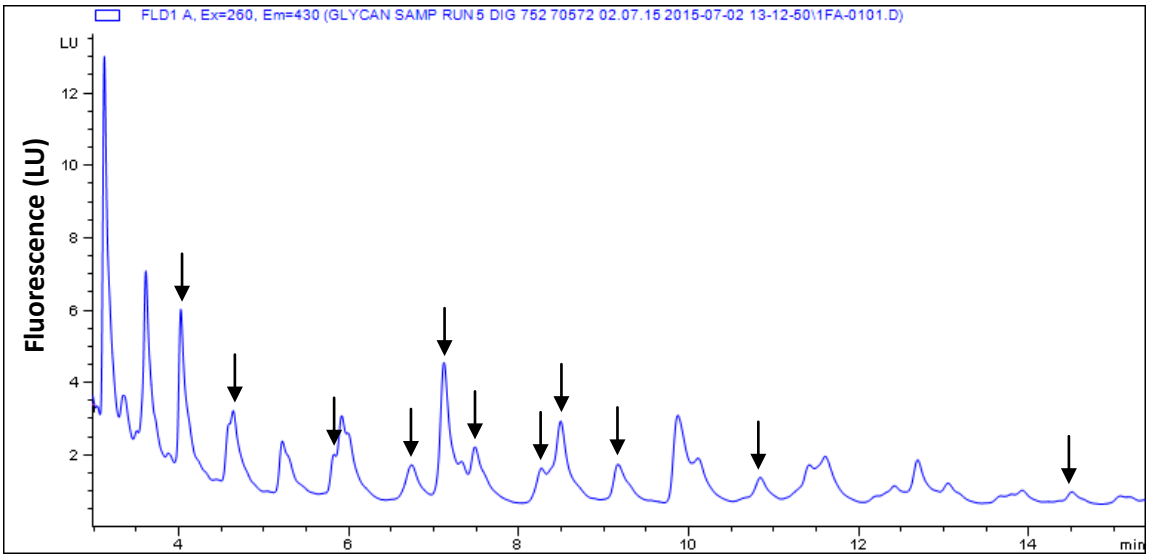


Figure 3.29. HILIC chromatogram of 2-AB labelled *K. lactis* 752 PNGase F digested sample.

Samples were run on equal weight basis and in duplicate. ↓ arrows indicate peaks that resolved at the same time in the glycan profile of YCWM, *S. cerevisiae* 695, and *S. pombe* 70572.

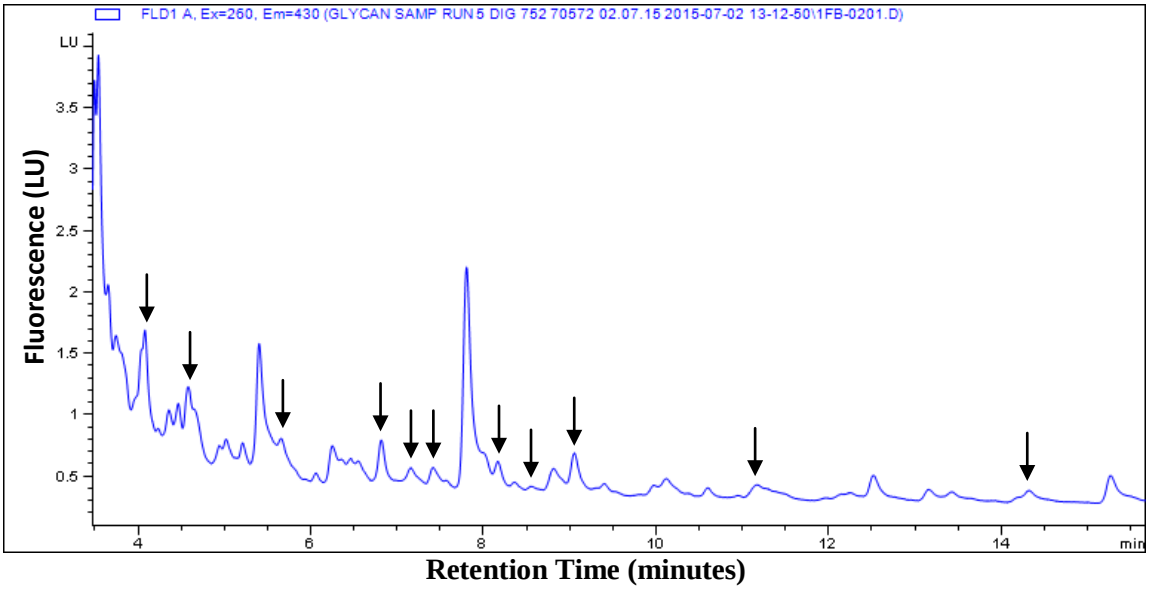


Figure 3.30. HILIC chromatogram of 2-AB labelled *S. pombe* 70572 PNGase F digested sample.

Samples were run on equal weight basis and in duplicate. ↓ arrows indicate peaks that resolved at the same time in the glycan profile of YCWM, *S. cerevisiae* 695, and *S. pombe* 70572.

The common peaks in each of the profiles had retention times of approximately 4.1, 4.6, 5.9, 6.7, 7.1, 7.6, 8.3, 8.5, 9.2, 10.8, and 14.5 minutes. These samples shared eleven peaks with the same retention time in the glycan region, indicating that oligosaccharides of the same length were present in each of the samples. In addition to the common peaks, each of the profiles contained peaks that were sample specific. For example, the YCWM profile contained a peak at 6.1 and at 7.4 minutes that were not present in the other samples. There were four peaks unique to *S. cerevisiae* 695's glycan profile at 9.5, 12.8, 13.8, and 14.4 minutes shown in Figure 3.28. Interestingly, *S. cerevisiae* 695 and YCWM contained peaks common to each other, but absent in the other samples, at 8.7, 8.8, 9.6, 10.4, 10.7, 12.1 and 13.4 minutes. These two profiles demonstrated a degree of similarity between the two strains of *Saccharomyces*. However, the differences in the peak profiles mentioned above between the two samples allude to differences in the structures or complexity of the oligosaccharides which may in part contribute to the higher binding activity of *S. cerevisiae* 695 for type 1 fimbriae containing bacteria compared to YCWM. *K. lactis* 752 contained unique peaks at 9.9, 10.1, 11.4, 11.6, 13.1, and 13.9 minutes. This strain was identified to have the most unique peaks (6)

compared to the other sample analysed. *S. pombe* 70572 also had a number of unique peaks at 5.1, 7.9, 12.5, and 15.2 minutes.

It may be speculated that the common peaks identified between the samples, described previously and displayed with solid black arrows in Figure 3.27-3.30, contain oligosaccharides that may be involved in binding activity. Each sample was also found to have its own glycan profile containing oligosaccharide residues specific to the individual sample. The presence of unique peaks in each sample is in agreement with reports that have identified different N-glycan structures of various yeast strains (McGinnis, 2012, Park et al., 2011, Perez and Ribas, 2004, Gemmill and Trimble, 1999).

As mannose and mannose linked oligosaccharides are associated with binding to the FimH lectin of type 1 fimbriae it was necessary to ascertain if the N-glycan oligosaccharides present in each of the profiles were indeed mannan oligosaccharide chains. Each of the glycan samples were digested with the exoglycosidase Jack Bean Mannosidase (JBM). JBM hydrolyses α-(1, 2), α-(1, 3), and α-(1, 6) linked mannose residues back to a single mannose residue linked to a GlcNAc core, this β-(1, 4) linkage is not hydrolyzed by JBM (Mulloy et al., 2009). The subsequent JBM digested glycan profiles for each of the samples are shown in Figures 3.31-3.34. Disappearance of a peak after JBM digestion confirmed the presence of α-(1, 2), α-(1, 3), and/or α-(1, 6) linked mannose in a peak. New peaks are the product of larger oligosaccharides reduced to shorter oligosaccharides. Liberated monosaccharides eluted in the peak at 2-3 minutes were not shown in the chromatograms as the peaks were too large and skewed the chromatograms, making it difficult to identify changes in the smaller peaks.

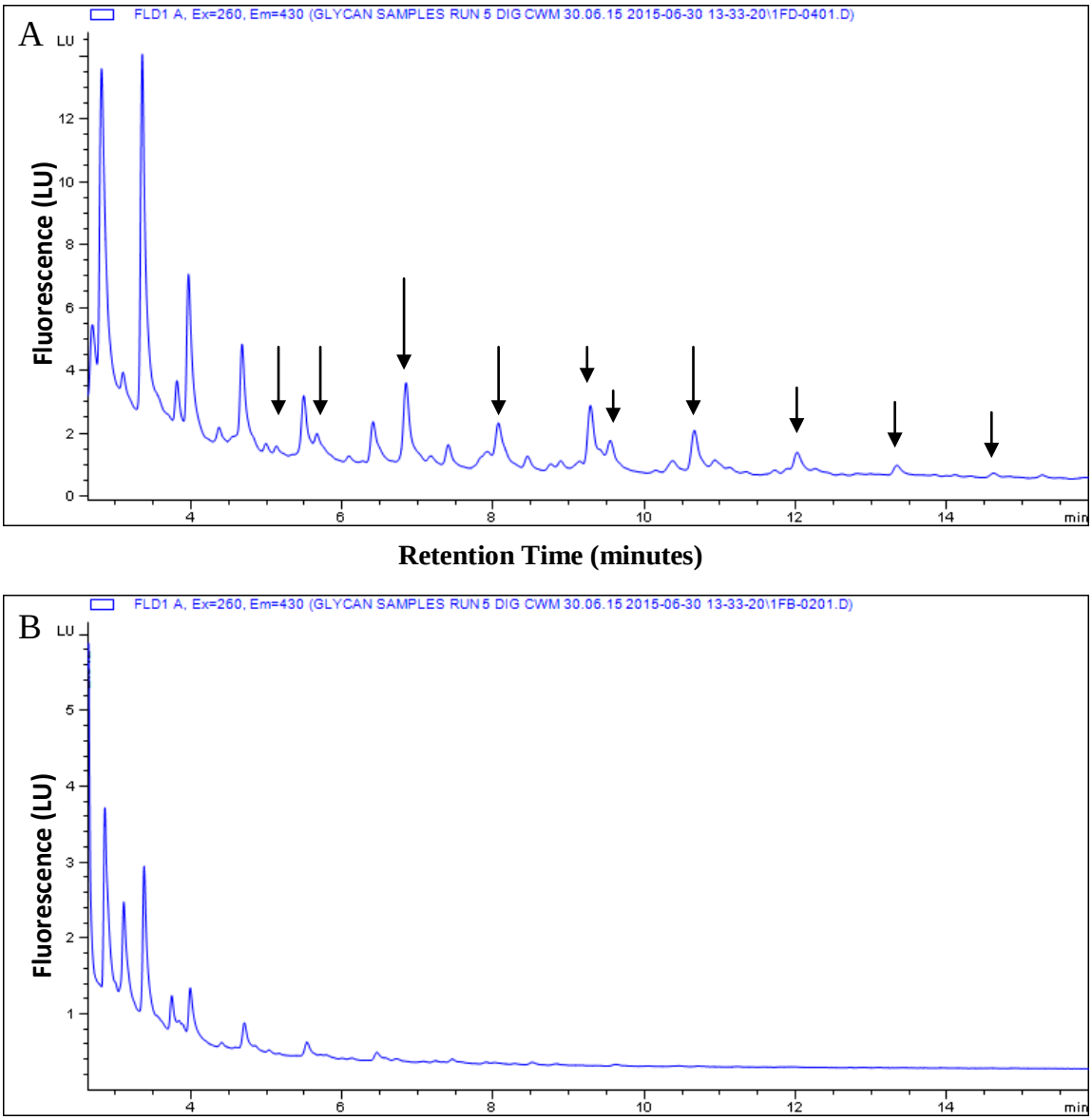


Figure 3.31. HILIC chromatogram of (A) YCWM PNGase F digested and (B) YCWM PNGase F and JBM digested samples.

Samples were run on equal weight basis and in duplicate. ↓ arrows indicate the peaks in A that disappeared in B.

A number of peaks disappeared from the YCWM chromatogram when the glycan sample was digested with JBM compared to the PNGase F digested sample, confirming the peaks were indeed mannose chains. Ten peaks disappeared in the YCWM samples upon digestion with JBM, as indicated with solid arrows in Figure 3.31 A. These peaks eluted at 5.1, 5.7, 6.8, 7.4, 8.1, 9.3, 9.6, 10.7, 12.0, 13.4, and 14.6 minutes in the PNGase F digested sample but were absent in the JBM digested sample. The liberated

mannose residues from these mannan oligosaccharides were eluted in the large monosaccharide peak resolved between 2 and 3 minutes.

A number of undigested peaks remain in the chromatogram, these may be either mannose oligosaccharides that were not completely digested due to the ability of JBM to release α-(1, 2) and α-(1, 6) linkages more efficiently than α-(1, 3) linked mannans or due to the presence of β-glucan residues. β-glucans are a large part of the yeast cell wall (Klis et al., 2002) but are not involved in the binding of type 1 fimbriae and were not investigated further.

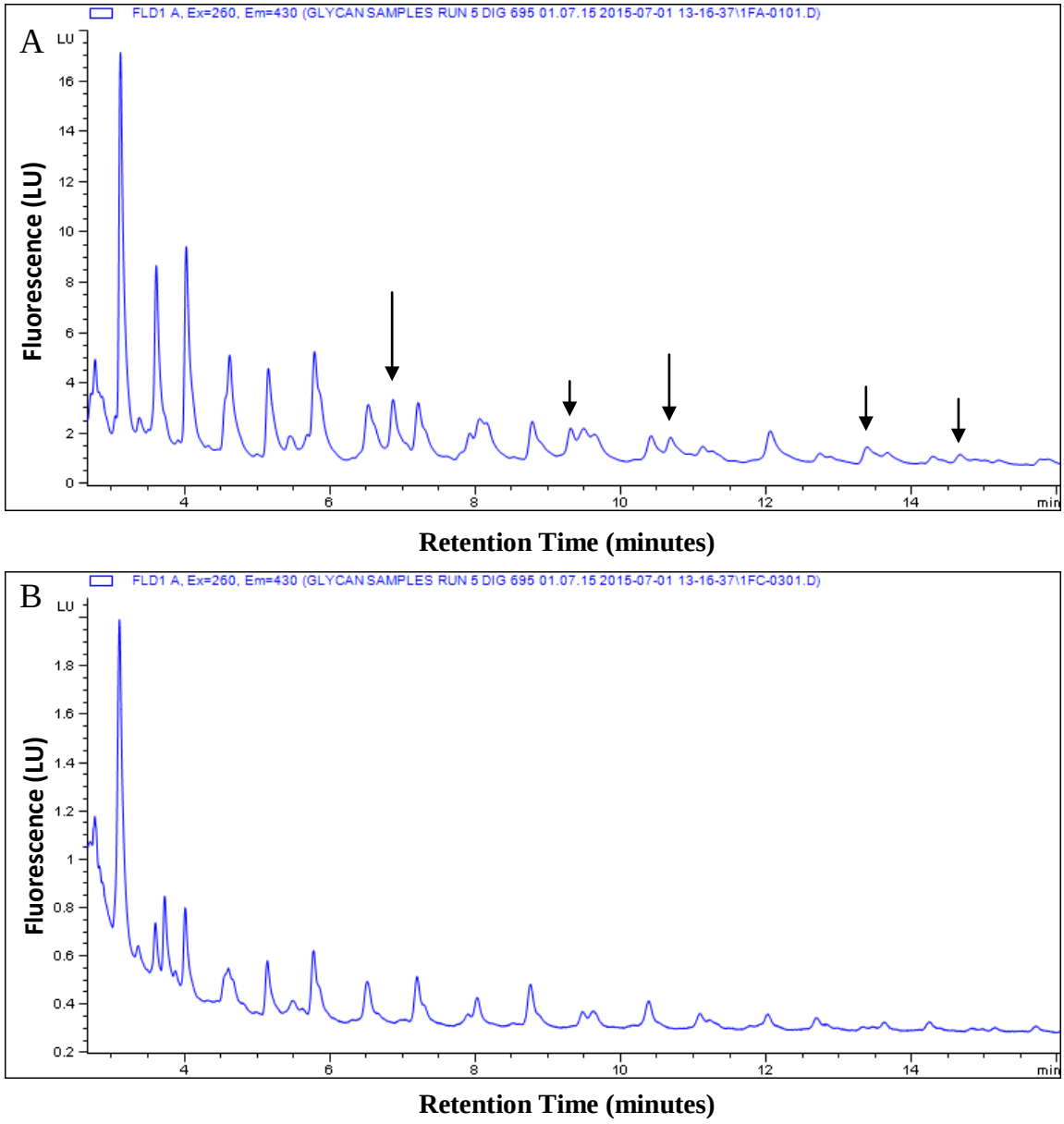


Figure 3.32. HILIC chromatogram of (A) *S. cerevisiae* 695 PNGase F digested and (B) *S. cerevisiae* 695 PNGase F and JBM digested.

Samples were run on equal weight basis and in duplicate. ↓ arrows indicate the peaks in A that disappeared in B.

Similarly the N-glycans of *S. cerevisiae* 695 were subjected to enzymatic digestion with JBM. A number of peaks were digested back to a single peak at the beginning of the chromatogram between 2 and 3 minutes indicating the presence of mannose linked residues. Peaks that disappeared in Figure 3.32 B are shown with solid arrows in Figure 3.32 A. A triple peak between 6.5 and 7.5 minutes was reduced to a double peak with enzymatic digestion, where the middle peak at 6.8 minutes was removed. Similarly, a

triple peak between 9 and 10 minutes was reduced to a double peak, and single peaks at 10.7, 13.4 and 14.6 minutes were also removed. The disappearance of these peaks indicated the presence of mannan oligosaccharides. A number of peaks that were digested by JBM in *S. cerevisiae* 695 were also digested in the YCWM sample, and included the peaks at 6.8, 10.7, 13.4, and 14.6 minutes. This implied that these two samples contained similar sized mannan oligosaccharides based on retention times. The mannan oligosaccharide at 9.4 minutes was a unique peak to *S. cerevisiae* 695 compared to the YCWM sample demonstrating diversity between the samples.

A number of peaks remained undigested by JBM indicating that these peaks did not contain mannose. These remaining peaks may have been protected from digestion by a greater number of α-(1, 3) linked mannose residues present in the sample compared to the YCWM. As mentioned previously, hypermannosylated oligosaccharides are too large to be resolved on the column. However, digestion with JBM may shorten these residues which were then resolved on the column and detected. The peaks undigested by JBM may also contain β-glucan residues as was seen in the YCWM sample.

The difference in the JBM digested profiles of *S. cerevisiae* 695 cell wall and YCWM seen here may be due to the difference in the parent strains, *S. cerevisiae* and *S. cerevisiae* var. *boulardii*. From these results there were more short chain mannan oligosaccharides present in the YCWM sample. These results agree with Bzducha-Wrobel et al. (2013) who demonstrated a difference in the cell wall composition between *S. cerevisiae* R9 (brewer's yeast) and *S. cerevisiae* var *boulardii*. *S. cerevisiae* var. *boulardii* was found to have a higher cell wall mannoprotein content compared to *S. cerevisiae* R9, while *S. cerevisiae* R9 cell wall was characterised by an increased β-glucan content compared to *S. cerevisiae* var. *boulardii*.

As *S. cerevisiae* 695 had increased binding activity for all strains of bacteria compared to YCWM, the structure of the mannan oligosaccharides may be important in binding activity. The presence of a number of unique peaks in *S. cerevisiae* 695, including a mannan oligosaccharide peak at 9.4 minutes, correlated with increased binding activity of type 1 fimbriae containing bacteria.

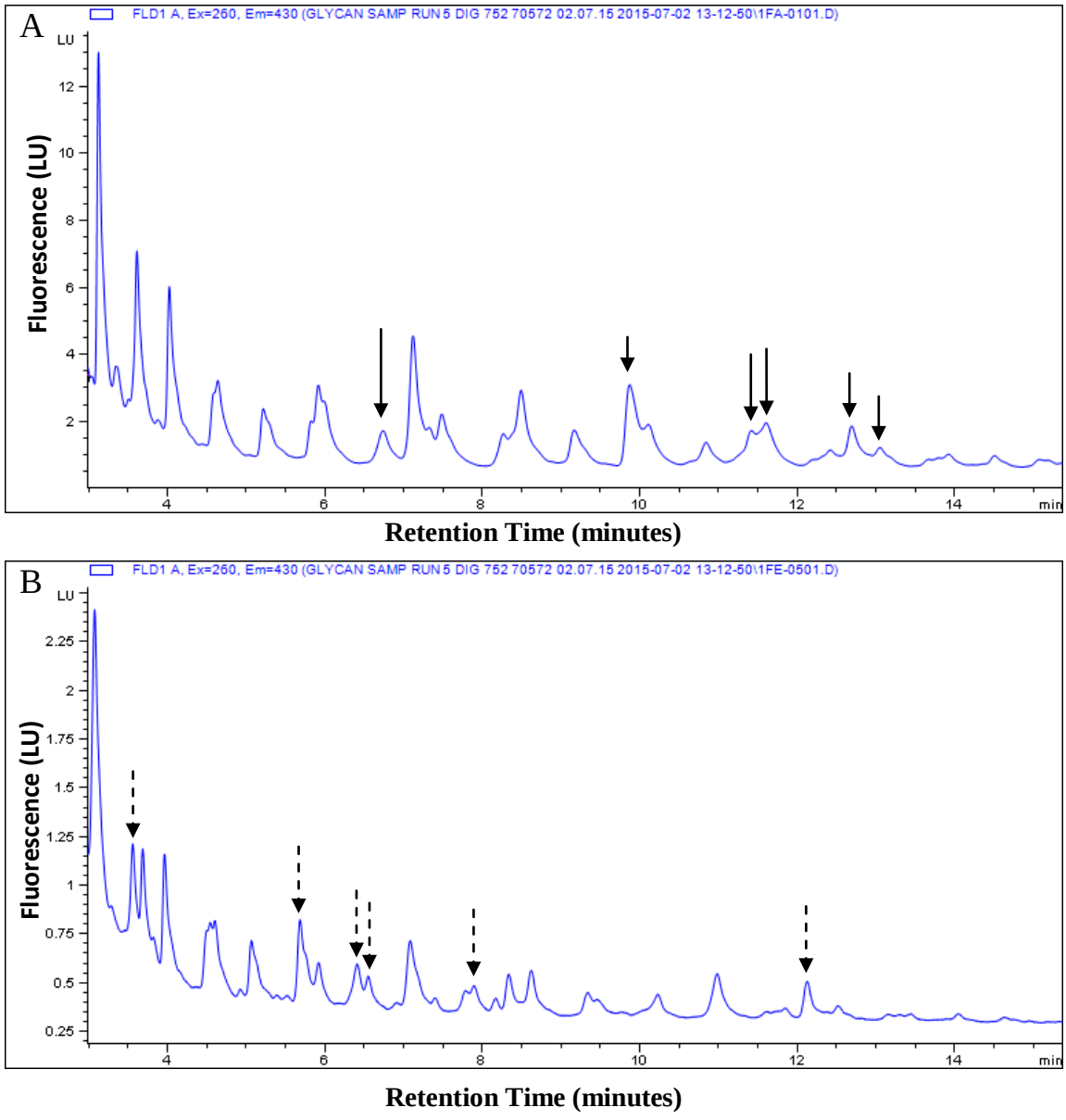


Figure 3.33. HILIC chromatogram of (A) *K. lactis 752* PNGase F digested and (B) *K. lactis 752* PNGase F and JBM digested samples.

Samples were run on equal weight basis and in duplicate. ↓ arrows indicate the peaks in A that disappeared in B after JBM digestion. ⇣ arrows in B indicate the presence of new peaks not present in A.

K. lactis 752, grown in 1 % (w/v) glucose as the sole carbon source, had high binding activity for all bacterial strains used in this study. *K. lactis* is both scientifically and biotechnologically one of the most relevant non-*Saccharomyces* yeasts. The vast use of *K. lactis* as a model eukaryote can be ascribed to its GRAS status and to the availability of molecular tools for genetic manipulation (Rodicio and Heinisch, 2013). *K. lactis* is

known to contain long chain mannan oligosaccharides in its cell wall (Zanni et al., 2009). The N-glycans of *K. lactis* are high mannose structures and resemble those produced in *S. cerevisiae* but are typically smaller (Wildt and Gerngross, 2005). Hydrolysis of the N-glycan sample with JBM removed a number of peaks in the PNGase F digested sample confirming the presence of mannose oligosaccharides in the sample.

There are clear observable differences in the glycan profile of the PNGase F digested *K. lactis* 752 sample (Figure 3.33 A) and the sample subsequently digested with JBM (Figure 3.33 B). A number of peaks were removed from the PNGase F digested sample, indicated by black arrows in Figure 3.33 A, at 6.8, 9.9, 11.4, 11.6, 12.7 and 13.1 minutes. These peaks were shown by enzymatic digestion to contain mannan oligosaccharides. The peaks with retention times of 9.9, 11.4, and 13.1 minutes were identified as unique peaks for this sample and absent in the other samples. Interestingly, only one of the mannan oligosaccharide peaks (6.8 minutes) of *K. lactis* was also present in the YCWM sample. In fact, *K. lactis* 752 had the least number of common mannan oligosaccharides peaks to that of YCWM of all the samples analysed. These unique mannan oligosaccharides may be responsible for the increased binding activity seen with this sample.

In addition to the disappearance of peaks, a number of new peaks were also resolved in the JBM digested sample. These new peaks are represented with dashed arrows in the chromatogram (Figure 3.33 B) and were resolved at 3.5, 5.8, 5.9, 6.4, 6.6, 7.9, and 12.1 minutes. In contrast to *S. cerevisiae*, *K. lactis* contains GlcNAc residues on the outer chain part of its N-glycans (Abeijon et al., 1996). GlcNAc is not reported to bind type 1 fimbriae containing bacteria and was not investigated further. Furthermore, *Kluyveromyces* glycans are rich in α-(1,3) mannose side chains (Liu et al., 2009a) which are not as readily digested by JBM.

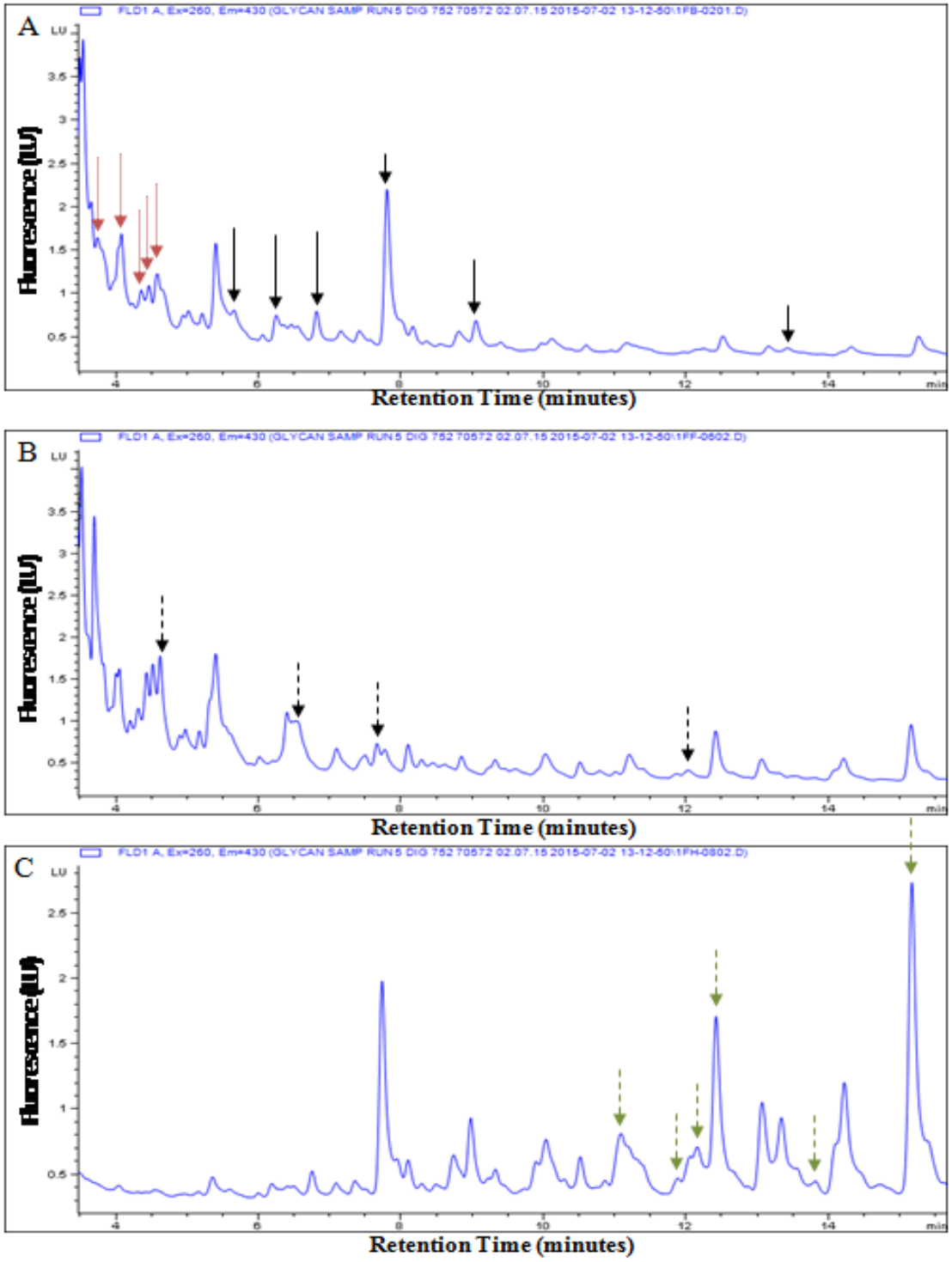


Figure 3.34. HILIC chromatogram of (A) *S. pombe* 70572 PNGase F digested, (B) *S. pombe* 70572 PNGase F and JBM digested, and (C) *S. pombe* PGNase F and galactosidase digested samples.

Samples were run on equal weight basis and in duplicate. ↓ arrows indicate the peaks in A that disappeared in B after JBM digestion. ↓ arrows in B indicate the presence of new peaks not present in A. ↓ arrows in A indicate the peaks that disappeared in C after galactosidase digestion; ↓ arrows in C indicate the presence of new peaks not present in A.

The final sample chosen for glycan analysis was *S. pombe* 70572, grown in 2 % (w/v) glucose as the sole carbon source. *S. pombe* 70572 was the only fission yeast used in this study, it is important in the production of wine (Benito et al., 2014), heterologous proteins, and as a model eukaryotic (Nkeze et al., 2015, Magnelli et al., 2005). The cell wall material of this yeast is unusual compared to budding yeast as it contains α-(1, 3) glucan and galactomannan (Konomi et al., 2003). Galactomannans are yeast mannan oligosaccharides with galactose units bound at terminal positions, mainly by α-(1, 2) linkages (Hamilton and Gerngross, 2007, Perez and Ribas, 2004).

A number of peaks were shown to contain mannan oligosaccharides as they were digested with JBM and absent from Figure 3.34 B, as shown by the solid arrows in Figure 3.34 A. These mannan oligosaccharides were resolved at 5.6, 6.3, 6.8, 7.9, 9.1, and 13.4 minutes. Of these digested peaks, the peaks resolved at 5.6, 6.3, 7.9, and 9.1 minutes in the PNGase F sample were unique to *S. pombe* 70572 compared to the other samples analysed. These unique mannan oligosaccharides may be involved in the increased binding activity of this sample for type 1 fimbriae containing bacteria. Two of the mannan oligosaccharides of *S. pombe* 70572, resolved at 6.8 and 13.4 minutes, were also present in both *S. cerevisiae* 695 and YCWM. Furthermore, new peaks appeared in the JBM digested sample at 4.6, 6.6, 7.6, and 12.1 minutes and are represented by dashed arrows in Figure 3.34 B. These new peaks suggest that some of the mannan oligosaccharides and the hypermannosylated chains were hydrolysed to even shorter oligosaccharide chains. As *S. pombe* contains galactomannans, these new peaks may contain galactose residues. Galacomannans have been shown to reduce *Salmonella* infection *in vivo* (Andres-Barranco et al., 2015) and to reduce *Salmonella* binding to porcine intestinal epithelial cells *in vitro* (Badia et al., 2013), and were investigated further.

To determine the presence of galactose linked mannan oligosaccharides, the PNGase F digested sample of *S. pombe* 70527 was digested with galactosidase and the results are shown in Figure 3.34 C. The results from the digestion of the sample with galactosidase demonstrated that a number of peaks were reduced to monomers of galactose and short chain mannose residues, as the galactose residues are terminally linked to the mannose side chains (Perez and Ribas, 2004). The peaks containing galactose were at the beginning of the glycan region with retention times of 3.7, 4.0, 4.2, 4.5, and 4.6 minutes, shown with dotted arrows in Figure 3.34 A. These released monomers were

eluted in the peak between 2-3 minutes. In addition there were a number of new peaks present in the galactosidase digested sample at retention times of 11.0, 11.9, 12.1, 12.5, and 13.8 minutes, shown with dashed arrows in Figure 3.34 C. It may be hypothesized that these peaks represent galactomannans that were hydrolysed to mannan chains of different length from the hypermannosylated polysaccharides too large to be resolved before galactosidase digestion. It was evident that the glycan profile for the JBM digested and the galactosidase digested sample of *S. pombe* 70572 contained different peak profiles. The large peak at 7.9 minutes in Figure 3.34 A and C was definitely shown to be a mannan oligosaccharide as it was not digested by galactosidase. This mannan oligosaccharide peak at 7.9 minutes was unique to *S. pombe* and may be involved in the increased binding activity of this sample for type 1 fimbriae containing bacteria. *S. pombe* 70572 has been shown to contain both mannan and galactomannan residues in its N-glycans by enzymatic digestion and HILIC analysis. Mannan and galactomannan oligosaccharides in the N-glycan profile of this sample may be involved in the increased binding activity of this sample.

N-glycan analysis of the cell wall from three samples (*S. cerevisiae*, *K. lactis*, and *S. pombe*), revealed the presence of both short and long chain mannan oligosaccharides. While there were a number of common peaks in the PNGase F digested fraction of all the samples, the N-glycan profiles did differ between the samples. The JBM digestion of the samples produced different profiles for each cell wall sample indicating that the structure of the oligosaccharides differ between the samples. Unique mannan oligosaccharide peaks were identified in each of the samples. These unique peaks may be involved in the increased binding activity of these samples compared to the YCWM sample.

Other groups have proposed the importance of the oligosaccharide structure in the binding activity to type 1 fimbriae lectin (Badia et al., 2013, Schierholt et al., 2011, Shoaf-Sweeney and Hutkins, 2009, Sharon, 2006). Some of the common peaks between the strains may represent mannan oligosaccharide structures that have specific binding activity for type 1 fimbriae containing bacteria. However, further work is necessary to characterise the N-glycans present in these samples to elucidate their structure and role in the binding activity for type 1 fimbriae containing bacteria. The galactomannan residues of *S. pombe* 70572 may also have a significant role in binding activity.

This method of glycan analysis was limited as it only allowed for the identification of glycans with a relatively low number of monosaccharide units (up to 20). Due to the increased number of hydrophilic residues of long chain oligosaccharides and polysaccharides of yeast cell wall these residues were beyond the range of the column. This method was originally developed for the determination of mammalian protein N-glycans which are considerably shorter than yeast N-glycans and contain many different glycan residues including glucose, N-acetylglucosamine, galactose, mannose, sialic acid, fructose, xylose, and N-acetylgalactosamine. In addition, there are several possible mannose glycoforms due to linkage isomerism (Tao et al., 2014) and it is not possible to distinguish between them using this method of exoglycosidase digestion with JBM and HILIC analysis. Characterisation would require more specific enzymes (endoglycosidases) such as α-(1, 2) mannosidase, α-(1, 3) mannosidase, or α-(1, 6) mannosidase with subsequent LC-MS analysis (Mulloy et al., 2009).

The variety of oligosaccharide chains in both length and complexity between samples may be the reason for variation in the binding activity of the cell wall material for different strains of bacteria. As the FimH lectins are heterogenous between strains of bacteria (Kisiela et al., 2013) they may be more or less specific for certain oligosaccharides present in the cell wall material sample. The binding mechanisms involved are still not fully understood and the results of this study underline the complexity of this. Biological variation in cell wall structure and natural diversity make predictions even more difficult. Macromolecular structure of polymers isolated from cell walls of various yeasts may be characterized by various degrees of polymerization and branching. Further in depth research is necessary to definitively characterise cell wall mannan components, the relationship between bacterial agglutination, and their structure.

Section 4

Conclusion

4. Conclusion

The initial aim of this study was the production and characterisation of cell wall samples from a variety of yeast species in search of a new or next generation MOS product. A collection of yeast strains were cultured in growth medium containing different concentrations of various simple carbon sources to assess the effect of carbon source on the cell wall saccharide content. The cell wall glucose content of the strains ranged from 8.2 – 34.3 % (w/w) and the cell wall mannose content was between 3.7 – 21.2 % (w/w), showing that the total glucose and mannose content of the cell wall for each of the strains varied, depending on both the species and the strain. The influence of the carbon source and its concentration on the cell wall saccharide composition of yeast species was also found to be strain specific. Only a few strains of *Saccharomyces* were observed to be significantly different from the 2 % (w/v) glucose as sole carbon source control in terms of cell wall glucose and mannose content when the growth medium contained different carbon sources. For example, *S. cerevisiae* 4070 had a 2 fold increase in cell wall glucose content when grown in growth medium containing 1 % (w/v) fructose (21.1 %, w/w) compared to the 2 % (w/v) glucose control (10.1 %, w/w). The cell wall glucose content of *S. cerevisiae* SC 1 was significantly increased (23.5 %, w/w) compared to the control (16.1 %, w/w) with 1 % (w/v) mannose as the sole carbon source in the growth medium. No significant changes were observed in the cell wall saccharide content of the seven remaining strains of *Saccharomyces* used in this study compared to the 2 % (w/v) glucose control at 1 or 2 % (w/v) carbon source in the growth medium. There were a number of significant effects with the strains of *Kluyveromyces* depending on the carbon source. For example, the majority of carbon sources at both 1 and 2 % (w/v) in the growth medium caused a significant increase in cell wall mannose content of *K. marxianus* 701 and 2415 when compared to the 2 % (w/v) glucose control; while fewer significant changes were observed in the glucose content of the cell wall samples. In contrast to this with *K. lactis* 752, fewer significant differences in cell wall saccharide content versus the control were seen. Carbon source also had significant effects on the cell wall saccharide content of *Rhodotorula* strains. The cell wall glucose content of both strains of *Rhodotorula* was significantly reduced when grown in 1 % (w/v) fructose containing growth medium compared to the 2 % (w/v) glucose containing control medium. A number of other carbon sources including mannose had a significant effect on the cell wall glucose content of these strains,

however, no significant changes were observed for the cell wall mannose content of these strains when the growth medium contained 1 % (w/v) of any of the carbon sources used. *C. utilis* 70167 also had a number of significant changes in the cell wall glucose content, but not the mannose content, when grown in medium containing different carbon sources. No significant changes were observed in the cell wall saccharide content of *S. pombe* 70572 or *P. membranifaciens* 326 when grown in medium containing 1 or 2 % (w/v) carbon sources. A clear trend was not observed between the carbon source and the cell wall saccharide content of the yeast samples, the effect of carbon source on the cell wall saccharide content was shown to be strain specific.

Increases in the concentration of the carbon sources from 1 to 2 % (w/v) were shown to have significant effects on the cell wall saccharide content of certain samples. Medium containing 2 % (w/v) mannose significantly decreased the cell wall glucose content of *S. cerevisiae* SC 1 compared to medium containing 1 % (w/v) mannose from 23.5 to 14.8 % (w/w). An increase in fructose concentration in the growth medium from 1 to 2 % also significantly decreased the cell wall glucose content of *S. cerevisiae* 4070 from 21.1 to 15.0 % (w/w). Fructose at 2 % (w/v) in the growth medium caused a 3 fold decrease in the cell wall glucose content of *K. marxianus* 2415 compared to medium containing 1 % (w/v) fructose, from 34.3 to 10.6 % (w/w). There were significant differences in the cell wall glucose and mannose content of *K. marxianus* 2415 observed with a change in the concentration of a number of carbon sources. An increase in sucrose concentration from 1 to 2 % (w/v) caused significant changes in the cell wall glucose content of both *R. mucilaginosa* 70825 and 18184. The overall effects of the concentration of carbon source on the yeast cell wall saccharide content were strain specific and thought to be related to catabolite repression where an increase in carbon source concentration caused a decrease in cell wall glucose or mannose content.

In the search for a second generation MOS product, it was necessary to assess whether the yeast cell wall samples generated in this study were effective at agglutinating bacteria. Prior to assessing these samples, the binding ability of the existing product, yeast cell wall material (YCWM), was first investigated. Initially, seven strains of type 1 fimbriae containing bacteria were assessed for their ability to agglutinate to a sample of YCWM derived from *S. cerevisiae* var. *boulardii*. Four strains of *Salmonella* (*enteritidis, typhimurium, gallinarum* and *dublin*) and three strains of *E. coli* (O157:H7, O2:K1:H-, and O1:K1:H7), were chosen for their pathogenicity to either domestic food

animals or as a zoonotic agent of human infection. Analysis of the agglutination of the bacteria to YCWM was performed initially by light microscopy. YCWM had the capacity to bind each of the bacterial strains, though variation seemed to exist in the agglutination between the bacterial strains, although this was difficult to clearly identify. This method was subjective and uniform binding was not shown across the microscope slide as the YCWM and bacteria were mixed on the slide. It was also difficult to reliably count the amount of bacteria bound to the YCWM based on the size of the bacterial cells. This clearly indicated the need for a more reliable quantifiable method of determining YCWM's capacity to bind different pathogenic species. A quantitative *in vitro* assay was developed for each strain of bacteria, listed previously.

In an effort to make the microscope agglutination analysis quantitative, the method was transferred to a 96 well plate format. To determine the number of bacterial cells bound to YCWM a known amount of bacteria was incubated with a specific concentration of YCWM in a 96 well plate. YCWM and bacteria agglutinated to YCWM were removed from the plate by washing. The excess bacteria, not bound to YCWM, remained bound to the microtiter plate and were quantified by ELISA. The quantity of bacteria bound to YCWM was calculated by subtracting what was left on the plate from what was added to the plate initially. The binding activity was calculated by dividing the quantity of bacteria bound to YCWM by the initial amount of bacteria added and multiplied by 100, this was expressed as percent binding activity. Each step in the assay was optimised and the assay was subjected to precision and accuracy testing prior to analysing any samples. The assay was also optimised for each bacterial strain used in this thesis. The binding activity of YCWM was different for each strain of bacteria. For example, YCWM bound 93 % of the *Salmonella enteritidis* added to the plate intially, 52 % *typhimurium*, 86 % *gallinarum*, 78 % *dublin*, and 52 % *E. coli* O157:H7, 29 % O2:K1:H-, and 41 % O1:K1:H7. The low binding of *E. coli* O2:K1:H- to YCWM highlights the need for second generation MOS products with increased binding activity for such strains. These assays were used to assess the binding activity of yeast samples generated in the first part of this thesis.

Although the assay has high throughput, due to its 96 well format, it was not possible to analyse all the yeast samples as over 192 samples were generated. The binding activity of samples grown in growth medium containing glucose as the sole carbon source because of its low cost, and samples grown in growth medium containing mannose as

the sole carbon source as they were shown to slightly increase the cell wall mannose content of a number of samples, were assessed. Interestingly, the binding activity of the cell wall samples were found to be significantly higher than the YCWM for all strains of *E. coli*, while there was greater variation in the range of binding activity for the strains of *Salmonella*. A number of cell wall samples were identified with significantly higher binding activity than the YCWM control for all strains of bacteria. These samples included cell wall material derived from *S. pombe* 70572, grown in medium containing 2 % (w/v) glucose, *K. lactis* 752, grown in medium containing 1 % (w/v) glucose, and *S. cerevisiae* 695, grown in medium containing 1 % (w/v) mannose. The increased binding activity of the cell wall samples compared to the YCWM was thought to be related to the differences in the mannan oligosaccharide content of the samples and the specificity of the FimH lectin for these different mannose residues. As the cell wall saccharide content of all the samples was previously determined in this study a correlation between the saccharide content and the activity was carried out.

A number of significant correlations were found between each of the monosaccharide contents and the binding activity of the cell wall samples; however, no clear pattern was evident. Cell wall samples derived from different strains of yeast, even within the same species, did not share many similar correlations between cell wall saccharide content and binding activity for bacteria. Although correlations do not imply causation, the variation in the correlations between the monosaccharide content and the binding activity for the different strains of bacteria indicated that the quantity of cell wall mannose may not be critical in relation to the binding activity of the samples. In addition, strains such as *S. pombe* and *Rhodotorula*, with low cell wall mannose content, were found to have high binding activity for a number of the bacterial strains. Factors such as the structure are conceivably more important in terms of the binding of bacteria by these samples. With this in mind, comparison of the glycan structure of these samples was performed to determine if a link existed between high binding activity and the structure of the mannan oligosaccharides present in the samples.

In-depth analysis of the N-glycan profile of cell wall samples with significantly high binding activity, e.g. *S. cerevisiae* 695, *K. lactis* 752, and *S. pombe* 70572, was carried out, the control YCWM was also analysed. Mannosidase digestion confirmed the presence of mannan oligosaccharides in each of the samples. Each cell wall sample had a number of peaks in common with the YCWM control; in addition, unique mannan

oligosaccharides were also identified in each of the samples. These unique peaks may be responsible for the increased binding activity of these samples compared to the control. Further examination of the *S. pombe* 70572 profiles by galactosidase digestion confirmed the presence of galactomannan residues. This was exclusive to this strain and galactomannans have been reported to reduce the adhesion of *Salmonella* and *E. coli in vitro* (Badia et al., 2013, Searle et al., 2010) and *in vivo* (Andres-Barranco et al., 2015).

A significant outcome of this study was the development of a quantitative assay to determine the amount of different bacterial strains that YCWM can agglutinate. This assay can be used to screen samples and help in new product development. It can also be adapted for other type 1 fimbriated strains of bacteria. Binding activity of each batch of MOS type products could also be monitored using the developed assays thus adding to the existing quality assurance process. Full assay validation studies will be carried out on each of the assays to ensure that the assays meet international standards set at the International Conference of Harmonisation (ICH, 1996).

S. pombe 70572 was found to have the highest binding activity for all strains of bacteria. Galactomannans have been reported to reduce type 1 fimbriae containing *Salmonella* and *E. coli* colonisation *in vitro* and *in vivo* (Andres-Barranco et al., 2015, Badia et al., 2013). In addition, galactomannans have also been shown to reduce mannose resistant enterotoxigenic *E. coli* K88, which contains type 4 fimbriae (F4) *in vitro* (Badia et al., 2012). An *in vivo* challenge type study would be required to confirm the efficacy of *S. pombe* cell wall at reducing pathogen load in animals.

Appendix

Table A 1. Spearman-Rho correlations for *Salmonella* Spp.

Yeast strain		*S. enteritidis*		*S. typhimurium*		*S. gallinarum*		*S. dublin*	
		Cell wall saccharide content							
		Glucose	Mannose	Glucose	Mannose	Glucose	Mannose	Glucose	Mannose
***S. cerevisiae* 72**	Binding Activity	0.491	-0.347	0.342	-0.574	0.328	-0.33	0.691	0.146
	P value	0.003	0.041	0.041	0	0.051	0.049	0	0.396
***S. pastorianus* 203**	Binding Activity	-0.397	0.43	-0.83	-0.074	-0.445	0.44	-0.155	0.572
	P value	0.017	0.009	0	0.667	0.007	0.007	0.365	0
***S. cerevisiae* 4070**	Binding Activity	-0.545	-0.309	-0.155	0.457	-0.12	0.567	-0.397	-0.462
	P value	0.001	0.067	0.365	0.005	0.487	0	0.017	0.005
***S. cerevisiae* SC 4**	Binding Activity	-0.301	-0.194	-0.312	-0.402	0.276	0.467	-0.206	-0.206
	P value	0.074	0.258	0.064	0.015	0.103	0.004	0.229	0.229
***S. cerevisiae* 695**	Binding Activity	-0.306	-0.258	0.141	-0.555	-0.385	-0.163	-0.344	-0.199
	P value	0.069	0.128	0.412	0	0.02	0.343	0.04	0.246
***S. cerevisiae* SC 3**	Binding Activity	-0.868	-0.301	-0.612	0.05	0.062	-0.416	-0.847	-0.208
	P value	0	0.074	0	0.771	0.719	0.012	0	0.223
***S. cerevisiae* SC 1**	Binding Activity	-0.048	0.048	0.344	-0.344	0.306	-0.306	0.079	-0.079
	P value	0.782	0.782	0.04	0.04	0.069	0.069	0.647	0.647
***S. cerevisiae* SC 2**	Binding Activity	0.203	-0.045	0.196	-0.06	0.273	0.036	0.112	-0.234
	P value	0.234	0.792	0.252	0.729	0.108	0.835	0.514	0.169

Table A 2. Spearman-Rho correlations for *E. coli* Spp.

Yeast strain		O157:H7		O2:K1:H-		O1:K1:H7	
		Cell wall saccharide content					
		Glucose	Mannose	Glucose	Mannose	Glucose	Mannose
***S. cerevisiae* 72**	Activity	0.182	0.538	0.289	0.61	0.057	0.78
	P value	0.289	0.001	0.087	0	0.739	0
***S. pastorianus* 203**	Activity	-0.529	-0.108	-0.78	-0.325	-0.344	-0.12
	P value	0.001	0.532	0	0.053	0.04	0.487
***S. cerevisiae* 4070**	Activity	0.517	0.49	-0.682	-0.337	0.031	-0.148
	P value	0.001	0.002	0	0.044	0.857	0.388
***S. cerevisiae* SC 4**	Activity	0.407	0.394	0.101	0.261	0.072	0.112
	P value	0.014	0.017	0.589	0.155	0.677	0.516
***S. cerevisiae* 695**	Activity	0.311	-0.486	0.755	-0.916	0.478	-0.524
	P value	0.065	0.003	0	0	0.003	0.001
***S. cerevisiae* SC 3**	Activity	0.725	-0.062	0.488	-0.28	0.136	0.863
	P value	0	0.719	0.003	0.098	0.428	0
***S. cerevisiae* SC 1**	Activity	-0.088	0.088	-0.514	0.514	0.366	-0.366
	P value	0.608	0.608	0.001	0.001	0.028	0.028
***S. cerevisiae* SC 2**	Activity	-0.201	0.115	-0.169	-0.109	-0.019	-0.053
	P value	0.24	0.505	0.363	0.559	0.912	0.761

Table A 3. Spearman-Rho correlations for *Salmonella* Spp.

Yeast Strains		*S. enteritidis*		*S. typhimurium*		*S. gallinarum*		*S. dublin*	
		Cell wall saccharide content							
		Glucose	Mannose	Glucose	Mannose	Glucose	Mannose	Glucose	Mannose
***K. lactis* 752**	Activity	0.749	-0.749	0.71	-0.71	-0.648	0.648	0.813	-0.813
	P value	0	0	0	0	0	0	0	0
***K. marxianus* 701**	Activity	-0.285	-0.608	-0.541	-0.686	-0.289	-0.806	-0.56	-0.758
	P value	0.092	0	0.001	0	0.087	0	0	0
***K. marxianus* 2415**	Activity	-0.469	0.258	0.713	-0.837	-0.203	0.007	0.098	0.605
	P value	0.004	0.128	0	0	0.234	0.967	0.569	0
***R. mucilaginosa* 70825**	Activity	-0.914	0.426	-0.952	0.55	-0.848	0.702	-0.872	0.678
	P value	0	0.01	0	0.001	0	0	0	0
***S. pombe* 70572**	Activity	0.553	0.862	-0.472	0.943	0.631	0.582	0.121	-0.553
	P value	0.003	0	0.013	0	0	0	0.483	0
***P. membranifaciens* 326**	Activity	-0.179	-0.079	-0.017	0.057	-0.22	-0.395	0.27	0.151
	P value	0.295	0.647	0.923	0.739	0.197	0.017	0.111	0.38
***C. utilis* 70167**	Activity	-0.297	0.383	-0.191	0.277	-0.124	0.086	0.6	0.151
	P value	0.079	0.021	0.264	0.101	0.47	0.618	0	0.38
***R. mucilaginosa* 18184**	Activity	0.41	-0.227	-0.313	0.189	-0.841	-0.122	0.889	0.234
	P value	0.013	0.183	0.063	0.27	0	0.479	0	0.169

Table A 4. Spearman-Rho correlations for *E. coli* Spp.

Yeast Strains		O157:H7		O2:K1:H7		O1:K1:H7	
		Cell wall saccharide content					
		Glucose	Mannose	Glucose	Mannose	Glucose	Mannose
***K. lactis* 752**	Activity	0.665	-0.665	0.705	-0.705	0.725	-0.725
	P value	0	0	0	0	0	0
***K. marxianus* 701**	Activity	-0.242	-0.486	-0.283	-0.515	-0.23	-0.761
	P value	0.156	0.003	0.124	0.003	0.178	0
***K. marxianus* 2415**	Activity	0.01	0.545	0.682	-0.868	-0.624	0.706
	P value	0.956	0.001	0	0	0	0
***R. mucilaginosa* 70825**	Activity	-0.547	0.583	-0.26	0.442	-0.568	0.408
	P value	0.001	0	0.2	0.024	0	0.014
***S. pombe* 70572**	Activity	0.13	-0.141	-0.714	-0.031	-0.341	-0.227
	P value	0.448	0.411	0	0.857	0.042	0.182
***P. membranifaciens* 326**	Activity	0.596	0.627	0.423	0.488	0.749	0.687
	P value	0.00	0.00	0.01	0.003	0.000	0.000
***C. utilis* 70167**	Activity	0.213	0.041	0.82	-0.385	0.44	0.01
	P value	0.213	0.814	0	0.02	0.007	0.956
***R. mucilaginosa* 18184**	Activity	-0.039	-0.06	0.83	0.42	-0.243	0.012
	P value	0.821	0.729	0	0.014	0.153	0.945

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