In Vitro Assessment of Saccharomyces cerevisiae Derivatives using Bovine Epithelial Cells, Macrophages, and Mycobacterium avium spp. paratuberculosis

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ABSTRACT

*In Vitro Assessment of Saccharomyces cerevisiae Derivatives using Bovine Epithelial Cells, Macrophones, and Mycobacterium avium spp. paratuberculosis*

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Since yeast *Saccharomyces cerevisiae* and its cell wall components (CWCs) are being used for the prevention and treatment of enteric diseases in different species, they may also be useful for preventing Johne’s disease (JD), a chronic inflammatory bowel disease of ruminants caused by *Mycobacterium avium* spp. *paratuberculosis* (MAP). Therefore, the purpose of this thesis was to assess potential anti-MAP adhesive and immunomodulatory properties of certain *S. cerevisiae* derivatives. The adherence of mCherry-labeled MAP to bovine mammary epithelial cells (MAC-T) and bovine primary epithelial cells (BECs) co-cultured with yeast CWCs from two different yeast strains A and B, and two forms of dead yeast from strain A were investigated. Several macrophage function-related parameters such as phagocytic activity, reactive oxygen species (ROS) production, and immune-related gene expression were also measured using a bovine macrophage cell line (BOMACs) in the presence of these yeast derivatives. Results demonstrated that these yeast derivatives reduced MAP adhesion to both MAC-T cells and BECs as well as MAP uptake by BOMACs in a concentration-dependent manner after 6-hr exposure. ROS production by BOMACs was increased in response to 6-hr exposure to yeast derivatives. The expression of immune-related genes was also altered after 6 and 24-hr exposure to these yeast derivatives. Overall, this thesis provided insight into various aspects of yeast derivative immunomodulatory bioactivity, and demonstrated *in vitro* evidence that they may help to prevent MAP infection.
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LIST OF ABBREVIATIONS

BEC  Bovine primary intestinal epithelial cell
BOMACs  Bovine macrophage cell line
CFU  Colony Forming Units
CWC  Cell wall component
DC  Dendritic cell
E. coli  Escherichia coli
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GIT  Gastrointestinal tract
IL-6  Interleukin 6
IL-10  Interleukin 10
IL-12p40  Interleukin 12p40
IL-13  Interleukin 13
IL-23  Interleukin 23
JD  Johne’s disease
LPS  Lipopolysaccharide
MAC-T cells  Bovine mammary epithelial cell line
MAP  Mycobacterium avium subspecies paratuberculosis
MOS  Mannan oligosaccharides
ROS  Reactive oxygen species
TGF-β  Transforming growth factor-beta
TLR  Toll-like receptor
YCW  Yeast cell wall
Chapter 1. Literature review

For the past few decades, it has been known that non-processed yeasts can be used as probiotic microorganisms, which when administered in adequate amounts confer a health benefit to the host. Specifically, yeast *Saccharomyces cerevisiae* strain has been used for the prevention and treatment of several types of infectious diarrhea in both children and adults (Kurugöl & Koturoğlu, 2005; Htwe et al., 2008). However, there have been problems associated with increased use of probiotic yeast. The survivability of yeast for example, is questionable in the supplement diet before ingestion, and even more so in the host after ingestion in the presence of gastric acids, and intestinal bile salts and pancreatic enzymes (Jin et al., 1998). Moreover, infection cases associated with *S. cerevisiae* have also been reported due to the extensive use of yeast in food industry, especially within immune-compromised individuals (Enache-Angoulvant & Hennequin, 2005; De Llanos et al., 2006). One solution to these probiotic yeast problems might be to use yeast prebiotics.

Prebiotics were defined by Roberfroid as, “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits to the host well-being and health” (Roberfroid, 2007). Currently, several derived food ingredients are known to have prebiotic properties, such as non-digested carbohydrates (CHO) molecules, resistant starches, sugar polyols, and a range of di-, oligo- and polysaccharides (Al-Sheraji et al., 2013). Among those prebiotic food ingredients, one type of oligosaccharide, the mannan oligosaccharides (MOS), has been commercially produced for human, livestock and companion animals. Many studies have evaluated the benefits of MOS as food ingredients to promote consumer well-being and health (Newman, 1994). Most prebiotic MOS used today are obtained from *S. cerevisiae* by a process that involves enzymatic hydrolysis or chemical synthesis via trans-glycosylation of mono- or disaccharides (Saad et al., 2013).
Similarly, another commonly used polysaccharide prebiotic, β-glucans, can also be extracted from natural sources, especially from brewer’s yeast *S. cerevisiae*. Specifically, alkaline extraction, alcohol precipitation and cell wall-enzymatic degradation have been used for the purification of β-glucans (Lam & Cheung, 2013). In addition, recombinant β-glucans can also be produced by microorganisms (Kearney et al., 2011).

Since MOS and β-glucans are the two major components derived from yeast *S. cerevisiae* cell wall (YCW), this review provides information about the derivatives from YCW with emphasis on their bioactivity which includes anti-adhesive and immunomodulatory properties. Other aspects that will be covered include their health benefits, potential mode of action and background of Johne’s disease.

### 1.1 Yeast cell wall

The YCW is composed of several classes of macromolecules that protect against mechanical injury and unwanted uptake of materials. There are four classes of macromolecules making up the YCW (Fig. 1.1): highly glycosylated glycoproteins ("mannoproteins"), two types of β-glucans, and chitin (Feldmann, 2012). The outer layer of yeast contains mannoproteins that largely determine the surface properties of the YCW (Walker, 1998). Mannoproteins are linked to the inner glucan layer, presenting as an α-1,6 linked inner core with α-1,2- and α-1,3 side chains (Walker, 1998). Extraction of mannoproteins can be obtained using a procedure described by Ganan et al. (Ganan et al., 2009). β-glucans are a major structural polysaccharide component of YCW (Ahmad et al., 2012), and they are also classified as novel prebiotics (Lam & Cheung, 2013). The β-D-glucopyranose units of β-glucans are linked together through β-(1, 3) linkages to form a long backbone, whereas side chains mostly arise through β-(1, 6) linkages (Ahmad et al., 2012). The yield of β-glucans during extraction varies and depends on the botanical sources,
temperature and pH (Lam & Cheung, 2013), and the extraction of β-glucans from *S. cerevisiae* can approach 87% β-glucans (Freimund et al., 2003). Alkaline extraction, alcohol precipitation and cell wall-enzymatic degradation have been used for the purification of β-glucans (Lam & Cheung, 2013). Chitins are structurally diverse polysaccharides composed of chains of monosaccharides bound by glycosidic bonds (Song et al., 2014). Extraction of chitins involves alkali treatment, which simultaneously removes protein and deacetylates chitin (Ravi Kumar, 2000).

1.2 **Health benefits of yeast derivatives**

Derivatives from the yeast have beneficial effects to the host, first of all, by providing nutrients to promote host growth performance. Secondly, they can also alter the biodiversity and efficiency of microbiota in the gastrointestinal tract (GIT). In addition, yeast derivatives may help promote host disease resistance, in part, by interfering with the adherence and invasion of pathogens to the gastrointestinal epithelium. Lastly, supplementing feed with yeast derivatives is also reported to modulate immune responses. Information about how yeast derivatives promote host health will be described as following within four sections.

1.2.1 **Growth performance**

Yeast derivatives have been commercially produced for animal feeding to improve animal growth performance, feed efficiency and milk production in dairy ruminants or carcass quality in beef cattle (Chaucheyras-Durand & Durand, 2010). The explanation for the effect of yeast products on animal performance could be that some yeast products stimulate rumen bacterial yield by providing nutrients such as organic acids, B vitamins, and amino acids (Callaway & Martin, 1997) and to increase mineral retention (Cole et al., 1992) and nutrient digestibility (Wohlt et al., 1991).
Yeast derivatives such as MOS have been extensively applied in aquaculture to promote the growth performance on many species, such as lobster (Huu & Jones, 2014), shrimp (Zhang et al., 2012), and fish (Torrecillas et al., 2013). Besides aquaculture, MOS have also been applied to livestock, such as calves; however, no significant increase in the growth rate of calves supplemented with MOS in milk replacer was observed, but the starter intake tended to be greater (Terre et al., 2007). Moreover, similar unimpressive results were reported on rabbits, where neither their daily weight, nor feed intake was affected by supplementation with MOS (Mourao et al., 2006). However, unlike the findings in calves and rabbits, supplementation with a unique mannose rich fraction, derived from *S. cerevisiae* cell wall, increased grower and finisher pig carcass weights and enhanced the yield of saleable pork (Edwards et al., 2014). In addition, beneficial effects of dietary supplementation with MOS on poultry growth performance have also been reported. For instance, a combination of MOS supplementation and whole millet feed improved growth performance in older age guinea fowl (Oso et al., 2014). In another study conducted with broiler chickens, dietary supplementation with MOS was also shown to have a promoting effect on body weight gain and also feed conversion ratio (Attia et al., 2014).

Similarly, dietary supplementation with β-glucans has beneficial effects on animal growth performance. In fish for example, β-glucan-supplemented diets increased the growth rates of sea cucumber (*Apostichopus japonicus*) after 4-week feeding period; additionally, higher grow rate was found in the treatment group fed combinations of β-glucan and MOS (Gu et al., 2011), which indicates that both of yeast derivatives might have the complementary effects on fish growth performance. However, there is recent evidence that growth performance was not affected in weaned pigs with β-glucan for 28 days (Zhou et al., 2013). Additionally, in rabbits, no significant differences in body weight were observed among treatments with or without β-
glucan supplementation (Wu et al., 2011). A similar result was also found in broilers after a 17-d feeding trial (Chae et al., 2006). These findings suggest that the effect of dietary β-glucan on growth performance is variable among species; however, the feed supplemented with β-glucan might have other beneficial effects on animals.

1.2.2 Gastro-intestinal microflora

Dietary manipulations can play an important role in altering the biodiversity and efficiency of microbiota in the GIT. MOS for example, were shown to increase the bacterial diversity and number in weaned piglet ileal and colonic fluids in vitro (Hang & Zhu, 2012). MOS administration to rabbits also affected the ileal morphology and caecal microflora by inducing the ileal villi growth and reducing caecal pathogenic Lactobacillus count (Oso et al., 2013). In fish, gilthead sea bream specifically, it was reported that dietary supplementation with MOS was able to increase gastrointestinal microbiota species richness and diversity (Dimitroglou et al., 2010). Furthermore, in a study evaluating the effects of dietary supplementation with yeast derivatives containing MOS on lipopolysaccharide (LPS) adsorption, dietary yeast derivatives supplementation decreased free LPS concentration in plasma, feces, and the digesta of the lower gut including the ileum, cecum and colon in beef cattle (Lei et al., 2013).

Besides MOS, β-glucan can also modulate the intestinal microbiota. The porcine colonic digesta for example, has been shown to be affected by the addition of yeast β-glucan in an in vitro study (Luo et al., 2013), in which the diversity of methanogens that are crucial to the efficiency of microbial fermentation in the GIT was significantly increased. The beneficial properties of β-glucan derived from other sources have also been reported. Cereal-derived β-glucan diets for example, had a positive influence on Bifidobacterium counts in the porcine GIT.
(Murphy et al., 2012). This is in agreement with work carried out by another group (Reilly et al., 2010).

1.2.3 Disease resistance

In addition to acting as food supplementation affecting on growth performance and gastro-intestinal microflora in consumers, yeast and its derivatives have been also reported to have a beneficial effect on disease resistance and immune response. In fish for example, MOS significantly lowered Tilapia mortality rate after challenge with Streptococcus agalactia (Samrongpan et al., 2008). Moreover, MOS administration also lowered infected European sea bass number after challenge with Vibrio alginolyticus (Torrecillas et al., 2007). Lastly, MOS are also reported to be effective adherents for some pathogens such as Campylobacter jejuni (Ganan et al., 2009), Escherichia coli (Baurhoo et al., 2007), and Salmonella (Fernandez et al., 2002; Posadas et al., 2010) to reduce these bacterial infection within hosts.

Similar to the properties of MOS, β-glucans derived from yeast S. cerevisiae also have the potency to promote disease resistance. For instance, in humans, a double-blind, randomized, placebo-controlled, multi-centric study demonstrated that a yeast β-glucan preparation increased the potential to defend against invading pathogens (Auinger et al., 2013). The study showed that number of symptomatic common cold infections was reduced by 25 % in participants receiving insoluble yeast (1, 3)-(1, 6)-β-glucan as compared to placebo group (Auinger et al., 2013). Similar observations have also been reported in zebrafish, where an injection of β-glucan before Aeromonas hydrophila challenge significantly enhanced zebrafish survival rate against infection (Rodríguez et al., 2009). Furthermore, in a preliminary study for the potential treatment of arthritis, a substantial decline of the level of plasmatic carbonyls, indicating oxidative tissue damage during the progress of arthritis diseases was observed in the rats that were administered
with carboxymethyl (1→3)-β-D-glucan derived from *S. cerevisiae* and were pre-induced with adjuvant arthritis (Kogan et al., 2005).

### 1.2.4 Immunomodulation effects

Supplementing feed with yeast derivatives has also been reported to modulate immune responses. For example, supplementation with yeast derivatives induced concentrations of circulating IgG and IgM antibody to sheep red blood cells in weaning pigs (Molist et al., 2014). Oral administration of MOS also enhanced specific immunity in cows, including increasing serum anti-rotavirus immunoglobulin titers at calving, and serum protein concentrations from birth to 24 h, the latter implying enhanced passive immunity (Franklin et al., 2005).

Similar to the properties of MOS, β-glucans derived from yeast *S. cerevisiae* also have immunomodulatory properties (Marques et al., 2006) and anti-inflammatory capacity (Du et al., 2014). For instance, β-glucan-supplemented diets increased phagocytic activity of gilthead sea bream after 2 or 4 weeks of administration, and also the pro-inflammatory cytokine interleukin-1β (IL-1β) and interferon-γ transcripts were up-regulated in the head-kidney (Guzmán-Villanueva et al., 2014). Additionally, β-glucans derived from yeast altered IL-10 and IL-12 expression in murine dendritic cells (DCs) after stimulation with LPS (Mikkelsen et al., 2014). Similarly, a mushroom β-glucan mixture was also reported to activate the innate immune system in fish, as indicated by enhanced lysozyme activity, alternative complement activity, and phagocytic activity of the head kidney leucocytes and respiration burst activity (Chang et al., 2013).
1.3 Potential mechanisms of action of yeast derivatives

Based on the bioactivities above, yeast derivatives appear to be promising alternatives to probiotics, especially with regard to their capacity to bind enteropathogenic bacteria and to beneficially modulate the immune system (Ganner & Schatzmayr, 2012).

1.3.1 Anti-adhesive properties of yeast derivatives

Yeast derivatives are suggested as anti-adhesive agents and are thus proposed to prevent attachment of certain bacteria to the intestinal wall (Ganner & Schatzmayr, 2012). The mechanisms used by yeast derivatives include increasing mucus secretion and binding to enterobacteria. The mucosal surface represents an important protective barrier lining the GIT. Increased mucus secretion for example, flushes invading bacteria through the gut lumen and prevents their adhesion to the underlying epithelium (Torrecillas et al., 2014). Mucus contains anti-microbial peptides, immunoglobulin A and commensal bacteria. A recent study conducted on sea bass showed that dietary supplementation with MOS increased mucus secretion in gut (Torrecillas et al., 2011). Mucus secretion and sloughing is an important defense mechanism utilized at the host epithelial mucosal surface to prevent bacterial adhesion (Bavington et al., 2004). The increased mucus secretion in sea bass may explain why a lower number of infected sea bass were found in an earlier study after direct inoculation of V. alginolyticus into the gut (Torrecillas et al., 2007).

The adherence of pathogenic bacteria at the mucosal surface is also inhibited by pathogen binding to alternative sites presented on yeast derivatives. The initiation of bacterial pathogenesis depends on the adherence of bacterial surface proteins (lectins) to host intestinal mucosal surface carbohydrate moieties (oligosaccharide components). In order for disease to occur, it is necessary for bacteria to first bind to host epithelial cells, colonize the intestinal mucosal surfaces, and then
finally invade the underlying tissues. The surface lectins produced by enterobacteria such as *E. coli* and *Salmonella* spp. (Firon et al., 1984; Ofek & Beachey, 1978) are commonly found as forms of fimbriae that are hair-like proteinaceous appendages on the surface of bacteria (Sharon, 1987). Among these fimbriae, type 1 fimbrial lectins are mannose-specific (Sharon, 1987); they not only appear to mediate bacterial adherence to mucosal epithelial cells, but also act as pathogen-associated molecules that trigger macrophage phagocytosis (Sharon, 1987). Therefore, adherence to the host cell surface plays a key role in the pathogenesis of enterobacteria, and by reducing the adherence of enterobacteria to mucosal surfaces, it is possible to reduce infectious disease occurrence.

The anti-adhesive properties of yeast derivatives have been extensively studied (Ofek et al., 2003). Soluble oligosaccharides derived from yeast for example, have the capability to interrupt the adherence process by resembling or mimicking host oligosaccharide receptors as host receptor analogs or decoys (Shoaf-Sweeney & Hutckins, 2008). This was shown in another study where researchers developed a quantitative assay to assess the binding activity of YCW factions by measuring the optical density as growth parameter of adhering bacteria (Ganner et al., 2010b). The YCW fractions were firstly coated on the bottom of microplates, and then certain bacteria such as *Salmonella, E. coli* and *Campylobacter* were added; the non-adherent bacteria were then washed off after one-hour of incubation, and the adherent bacteria were cultured for 18 hr (Ganner et al., 2010b). The optical density of bacteria culture medium was collected every 15 min during the 18-hr incubation to generate a growth curve of the adhering bacteria, of which the results showed that bacterial growth was concentration-dependent with YCW concentration; in another words, adherence and subsequent growth were YCW concentration-dependent (Ganner et al., 2010b).
1.3.2 Immumomodulation

There are many examples of how yeast derivatives can modulate host immune system.

Yeast β-glucans for example, have been proposed to interact with pathogen recognition receptors on cells of the innate immune system (Ganner & Schatzmayr, 2012). For example, dectin-1, the C-type lectin receptor for β-glucans, is expressed in cells such as macrophages, neutrophils, natural killer (NK) cells, fibroblasts, and DCs (Ariizumi et al., 2000; Kougias et al., 2001; Taylor et al., 2002; Williams, 1997). β-glucans ligation with dectin-1 activated and induced the maturation of a murine DC line (D2SC/1 cells), and increased the expression of mRNA coding the ligand for glucocorticoid–induced tumor necrosis factor receptor (GITRL) on D2SC/1 cells that promoted cytotoxic T lymphocyte responses (Tian et al., 2011). β-glucans binding with dectin-1 induces intracellular signaling and mediates a variety of cellular responses. Zhu et al. (2013) for example, have found that β-glucan-induced cell activation via dectin-1 and spleen tyrosine kinase (Syk), which inhibited LPS-induced inflammation factors in primary rat mammary epithelial cells. It was suggested that the activation of the dectin-1/Syk signaling pathway decreased the expression of Toll-like receptor 4 (TLR4), which is responsible for the recognition of LPS and initiates a signaling cascade resulting in inflammation (Zhu et al., 2013).

Another study showed that yeast-derived zymosan, which is high in β-glucans content (Di Carlo & Fiore, 1958), was recognized by dectin-1 receptors, and this led to increased production of Src family kinases, the Tec kinase Btk, phosphatidylinositol 3-kinase and the Map kinases ERK and p38 (Olsson & Sundler, 2007), all of which contribute to the release of membrane arachidonic acid, which is an important mediator in the initiation of inflammation (Larsen & Henson, 1983).

The immunomodulatory effect of yeast derivatives containing mannan-rich carbohydrate has also been reported (Schaffer et al., 2009). Mannan-rich carbohydrates are ligands for
mannose-binding lectin (MBL). MBL exists in a complex with MBL-associated serine proteases (MASPs), and when activated they trigger the lectin pathway of the complement cascade (Gadjeva et al., 2004). The complement system is of great importance in the innate immune system, acting as a first line of defense against pathogens, and modulating adaptive immune responses. *S. cerevisiae* zymosan has been shown to induce complement components C3 and C4 in a human leukemic cell line (THP-1) (Rajagopalan et al., 2010). Complement system activation is apparently required to initiate the phagocytosis of zymosan (Rajagopalan et al., 2010).

Regarding the anti-adhesive and immunomodulatory activities of yeast derivatives, and the reported benefits of using yeast derivatives to reduce bacterial infection within the GIT (Baurhoo et al., 2007; Fernandez et al., 2002; Posadas et al., 2010), yeast derivatives might be helpful to reduce *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection for the prevention and treatment of Johne’s disease (JD).

### 1.4 Background of JD

JD is a chronic inflammatory bowel disease (IBD) in ruminants, caused by MAP (Chase et al., 2008). The main characteristic of JD is the long latency or asymptomatic sub-clinical stage, which occurs in ruminants between the ages of 2-10 years (Khalifeh & Stable, 2004). During this time, MAP is shed from infected ruminants via the feces into the environment and this increases the risk of infecting their progeny and herd-mates (Tiwari et al., 2009). Furthermore, MAP can infect ruminants during neonatal development via ingestion of milk and colostrum. In cattle, calves are considered the most susceptible subject for infection, due to their undeveloped immune system (Chase et al., 2008). The primary transmission of MAP is via contaminated feces, even though MAP has been isolated from semen, prostate, testes and mammary glands, and the milk of infected cattle (Gilmour et al., 1965). Because a dose of 103
MAP has been proven to be sufficient to cause JD, and one-gram of feces from a clinically infected cow can contain 106-108 MAP colony-forming units, only a few milligrams of contaminated fecal matter is deemed to be infectious dose for a young calf (Chiodini et al., 1984; Jørgensen, 1982; Whittington et al., 2000).

1.4.1 Pathogenesis of JD

Following ingestion, MAP gains into the host by translocating into Peyer’s patches in gut-associated lymphatic tissues (GALT) via specialized intestinal absorptive cells called M cells or villous epithelial cells (Momotani et al., 1988; Sigurðardóttir et al., 2001; Ponnusamy et al., 2013). In addition, MAP can translocate across the intestinal epithelium via migratory dendritic cells that sample the pathogen from intestinal lumen and migrate to draining lymph nodes for antigen presentation (Coussens et al., 2010). Of those cells mentioned above, M cells have been suggested as the target for MAP due to their deficiency of lysosomes and hydrolytic enzymes (Wolf & Bye, 1984), and also richness of β1 integrins on cell membrane (Miller et al., 2007). β1 integrins are well-known type of pattern-recognition receptors on the surface of intestinal epithelial cells that can recognize MAP fibronectin attachment protein (FAP); MAP facilitates binding to the intestinal epithelium by adhering to β1 integrins (Sigurðardóttir et al., 2004; Olsen et al., 2002; Secott et al., 2004). Besides M cells, enterocytes have been suggested as another access cell type that allows MAP to invade the submucosa (Bermudez et al., 2010). Following the colonization and invasion of intestinal epithelium, MAP gains entry into Peyer’s patches where it is taken up by sub-epithelial macrophages. There are several strategies that macrophages employ to recognize pathogen-associated molecular patterns on MAP, such as TLRs 2 and 4 (Ferwerda et al., 2007), mannose receptors (Astarie-Dequeker et al., 1999), β-integrin receptor (Souza et al., 2007), CD14 receptors (Peterson et al., 1995), dectin-1 (Pant et al., 2014) and
complement receptors (Schlesinger et al., 1990). Generally, after being engulfed by macrophages, pathogens are internalized within an early phagosome that matures and undergoes acidification and then fuses with a lysosome forming a phagolysosome (Alonso-Hearn et al., 2008), where the elimination of pathogen is completed. However, instead of killing MAP, the phagosomes containing MAP are unable to proceed to a mature phagolysosome, because MAP blocks this progress (Kuehnel et al., 2001; Pieters, 2001), which enables MAP to survive and replicate within host cells.

1.4.2 Impact of JD

JD is globally widespread and is present in every country with significant livestock industries, with a herd prevalence ranging from 7-60 % (Grant, 2005). Infected cattle that develop clinical symptoms suffer from a chronic untreatable diarrhea that leads to cachexia and ultimately culling or death (Fecteau & Whitlock, 2010). Premature culling and decreased milk production and slaughter value of infected animals are the direct losses for the dairy industry (McKenna et al., 2006). Besides these direct losses for the dairy cattle, there could be huge product economic losses for dairy and beef producers if the potential association between JD in cattle and Crohn’s disease in humans is confirmed (Barkema et al., 2010; Behr, 2010).

1.4.3 Control of JD

JD is a difficult disease to control for several reasons. MAP has a long incubation period within the host, and MAP shedders with in herds are difficult to detect. MAP may be introduced into herds by free ranging birds, wild animals and even insects, which means biosecurity is critical to controlling disease. Diagonosis of JD is critical for the identification of infected animals, however, rapid MAP-specific diagnostic tools are not available and current tests are
often not reliable. Lastly, there is currently no effective vaccine for preventing JD (Salem et al., 2013; Corn et al., 2005; Motiwala et al., 2005).

Probiotics or prebiotics may be useful for preventing JD. They have been used to treat other IBD. For instance, patients with ulcerative colitis (UC), a subcategory of IBD, have been treated using a few licensed probiotics in Europe (Guandalini et al., 2014). A pediatric trial has shown the beneficial effect of probiotics on treating UC, where the appearance of clinical symptoms was decreased within patients treated with probiotics compared with the placebo group (Miele et al., 2009). In addition, clinical improvement was also observed in patients with mild to moderate UC that received a prebiotic during daily feeding (Bamba et al., 2002). These clinical trials support the possibility that this new class of therapeutic agents may be used to help prevent and treat JD. Moreover, there is evidence from a mouse study that other probiotics such as *Lactobacillus animalis* may be useful for reducing JD (Karunasena et al., 2013). Studies that investigate effects of probiotics and prebiotics on JD, however, remain limited. Therefore, there are still many aspects to discover about the bioactivities of probiotics and prebiotics in the context of MAP infection.
Figure 1.1: Composition and structure of the cell wall of *S. cerevisiae* (Ganner & Schatzmayr, 2012).
Chapter 2. Experimental Rationale, Hypotheses and Objectives

2.1 Experimental rationale

Dairy producers have been using commercially available yeast probiotics and their derivatives as feed supplements for nearly two decades based on claims that these products will improve animal production, promote health, and reduce the need for antibiotic use. In addition to having nutritional value, there is evidence that yeast probiotics and their derivatives (i.e. mannanoligosaccharides) can adhere to enteric pathogens thereby reducing their ability to attach to and invade host epithelial cells (Ganner et al., 2010b). Yeast probiotics and their derivatives also have immunomodulatory properties that can affect both the innate and acquired immune system (Ganner & Schatzmayr, 2012). Given these properties, it is possible that dietary supplementation with yeast probiotics and/or their derivatives may help protect calves that are vulnerable MAP infection.

Since considerable variation in the efficacy of different yeast strains and derivatives has been reported (Jawhara et al., 2012), methods need to be developed to identify bioactive yeast derivatives and to monitor product quality. Although in vivo studies are recommended to validate product efficacy before commercialization, in vitro studies also have utility because they are less susceptible to environmental influences, and are useful for high throughput screening of potentially bioactive compounds and for studying their mechanisms of action (Ganner & Schatzmayr 2012).

The present study was designed to 1) develop in vitro assays that will help to assess potential anti-adhesive properties of yeast derivatives using bovine epithelial cells and the target pathogen MAP that causes Johne’s disease in ruminants; and 2) use bovine macrophage cell line (BOMAC) to assess the potential immunomodulatory properties of yeast derivatives.
2.2 Experimental hypotheses

Yeast derivatives will be useful for preventing MAP infection and for stimulating macrophage function. Specifically, yeast derivatives will help reduce MAP adhesion to bovine epithelial cells, and yeast derivatives will affect BOMAC function by altering BOMAC phagocytic activity, reactive oxygen species (ROS) production and immune-related gene expression.

2.3 Experimental objectives

The first objective was to assess potential anti-adhesive properties of specific yeast derivatives provided by Lallemand Inc.. Methodology for this binding assay was adapted from a previously published study using human T84 cells (Czerucka et al., 2000); we modified this methodology to assess mCh-MAP binding to bovine mammary epithelial cell line (MAC-T cells), as well as primary bovine epithelial cells (BECs) from ileum tissues, and carried out concentration-response studies with different yeast derivatives.

The second objective was to assess potential immunomodulatory properties of yeast derivatives (provided by Lallemand Inc.) using bovine macrophage cell line (BOMAC). Concentration-response studies were carried out with different yeast derivatives using BOMAC, and macrophage function was assessed by measuring BOMAC phagocytic activity (i.e. fluorescent intensity of phagocytized MAP), ROS production, and immune-related gene expression following stimulation with yeast derivatives. Immune-related genes included cytokines that steer acquired immunity (i.e. pro-inflammatory cell response-IL-6 and IL-12p40; anti-inflammatory response- IL-10; T helper 2 (Th2) cell response- IL-13; Th17 cell response-IL-23 and TGF-β; and T regulatory response- IL-10 and TGF-β).
Chapter 3. In vitro bio-assessment of the binding activity of S. cerevisiae derivatives using bovine epithelial cells and MAP

3.1 Abstract

Since yeast S. cerevisiae and its derivatives are being used for the prevention and treatment of enteric diseases in different species, they may also be useful for preventing Johne’s disease, a chronic inflammatory bowel disease of ruminants caused by Mycobacterium avium spp. paratuberculosis (MAP). This study aimed to identify potential yeast derivatives that may be used to help prevent MAP infection. The adherence of mCherry-labeled MAP to bovine mammary epithelial cell line (MAC-T cells) and bovine primary epithelial cells (BECs) co-cultured with yeast cell wall components (CWCs) from two different yeast strains (A and B) and two forms of dead yeast from strain A was investigated. Results demonstrated that CWCs from both yeast strains and two forms of dead yeast from strain A reduced MAP adhesion to MAC-T cells and BECs in a concentration-dependent manner after 6-hr exposure. In summary, yeast derivativeness may be useful for preventing MAP infection.

3.2 Introduction

Dairy producers have been using commercially available yeast probiotics and their derivatives as feed supplements for nearly two decades based on claims that these products will improve animal production, promote health, and reduce the need for antibiotic use. Studies demonstrate that supplementing the ruminant diet with specific strains of S. cerevisiae improves feed intake (Williams et al., 1991; Robinson & Garrett, 1999), weight gain (Salama et al., 2002), and fiber digestion (Wohlt et al., 1998; Kamel et al., 2004). It has also been reported that live yeast stabilize rumen pH (Doreau & Jouany, 1998; Jouany et al., 1998), and the number of anaerobic cellulolytic bacteria (Mosoni et al., 2007; Silberberg et al., 2013).
In addition to having nutritional value, there is evidence that yeast probiotics and derivatives (i.e. mannan-oligosaccharides) can adhere to enteric pathogens such as *Campylobacter jejuni* (Ganan et al., 2009), *E. coli* (Baurhoo et al., 2007), and *Salmonella* (Fernandez et al., 2002; Posadas et al., 2010), thereby reducing their ability to attach to and invade host cells (Ganner et al., 2010b). Given these properties, it is possible that dietary supplementation with yeast probiotics and/or their derivatives may help protect calves that are vulnerable to Johne’s disease. Neonatal calves and calves less than six months of age are most vulnerable to MAP infection, the causative agent of Johne’s disease (Hines et al., 1995; Veterinary Record, 2008), in part, due to their under-developed immune system (Windsor & Whittington, 2010). MAP infects the small intestine, causing diarrhea, weight loss and severe dehydration, and also reduces milk production (Whitlock & Buergelt, 1996). Clinical signs of Johne’s disease may take 2 to 5 years to develop (Coussens et al., 2001), and its long subclinical stage facilitates continuous exposure of non-infected animals within an infected herd. Currently, there is no satisfactory treatment for Johne’s disease, since antibiotics only help to contain the disease, and vaccines only help to reduce disease incidence but they do not eliminate the disease completely (Rosseels & Huygen, 2008). Presently, the best way to control Johne’s disease is through good management practices, and early detection that can be performed by using different commercial available diagnostic tests (National Center for Animal Heath Programs-APHIS, 2008), however, these diagnostic tests all have limitations and are costly.

Given these limitations, alternative strategies need to be explored to help reduce calf exposure to MAP and stimulate the host immune system to help combat MAP infection. We hypothesized that yeast derivatives may help reduce MAP adhesion to bovine epithelial cells,
which may help support the further hypothesis that yeast derivatives may help reduce risk of Johne’s disease by preventing MAP adhesion to the gastrointestinal epithelium.

3.3 Materials and methods

3.3.1 Bacterial strains and culture conditions

The mCherry-labeled MAP used in the present study was developed by Mead et al. (2014) using the clinical isolate Gc86 strain previously isolated in the laboratory of Dr. Lucy Mutharia by Melinda Raymond (Department of Molecular and Cellular Biology, University of Guelph).

3.3.2 Preparation of bacterial infection stock

Liquid nitrogen frozen mCherry MAP Gc86 was thawed at 37 °C and was used to inoculate 5 ml of 7H9 broth (Difco laboratories, Franklin Lakes, NJ, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.25% v/v Tyloxopol (Sigma-Aldrich, St. Louis, MO, USA), 50 µg/ml kanamycin and 2 µg/ml of mycobactin J (Allied Laboratories, Wichita, KS, USA). The cultures were incubated at 37 °C, and once they reached a fluorescent intensity (FI) of 45000, equivalent to OD$_{600}$ = 0.8 (Mead, 2013), 5 ml aliquots were sub-cultured into 100 ml of media in a 250 ml sterile culture flask and incubated at 37 °C. When the cultures reached the logarithmic stage of growth (FI = 40000-50000 equivalent to OD$_{600}$ = 0.6-0.9), cells were centrifuged at 2000 × g for 30 min. The cells were resuspended to reach FI = 60000 equivalent to OD$_{600}$ = 1.0 using Fig. 3.1, then to establish Colony Forming Units (CFU/ml) using Fig. 3.2 (Mead, 2013). Quantification of fluorescence was based on the specific emission (587 nm) and excitation wavelengths (610 nm) for mCherry using the Wallac-1420 VICTOR3 Multilabel Counter (Perkin Elmer, Woodbridge, ON, Canada).
3.3.3  Bovine mammary epithelial cell line (MAC-T cells) and culture conditions

The bovine mammary epithelial cell line (MAC-T cells) was cultured according to the reference (Huynh et al., 1991). MAC-T cells were cultured in T75 tissue culture flasks (Corning, Tewksbury, MA, USA) at 37 °C with 5% CO₂, containing Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented with 4.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS; Invitrogen), 25 mM HEPES buffer (Invitrogen), 0.25 µg/ml amphotericin B (Invitrogen), 1% Penicillin/Streptomycin (100 unit/ml of Penicillin and 100 µg/ml Streptomycin; Invitrogen), 1 mM Sodium Pyruvate (Invitrogen), and 5 µg/ml Insulin-Transferrin-Selenium (Invitrogen).

3.3.4  Bovine intestinal epithelial cell (BEC) and culture conditions

Bovine ileum was aseptically harvested from a neonatal Holstein calf that was euthanized under the approval of the University of Guelph Animal Care Committee. The ileum (15 cm) was flushed with tap water and placed in ice cold HBSS (Invitrogen) supplemented with 5x100 U/ml Penicillin/Streptomycin (Invitrogen), 2.5 µg/ml Fugizone (Invitrogen), and 25 µg/ml Gentamicin. After the tissue was washed with HBSS several times, the mucosa was scrapped off the underlying epithelium, which was then cut into small pieces, and washed three times in HBSS by centrifugation at 140 × g for 5 min at 4°C to remove mucus. The tissue pellet (6-10 g) was then digested in 30 ml of DMEM containing ~150 U/ml collagenase I, 100~150 U/ml Penicillin/Streptomycin, 2.5 µg/ml Gentamicin and 2.5 µg/ml Fugizone by shaking for 30 min in a 37°C water bath. Following this, the epithelium was mechanically dispersed using a 25 ml serological pipette by drawing the digested tissue through it several times. DMEM containing 10% FBS (approximately 30 ml) was added to stop enzymatic digestion, and the crypts were concentrated by centrifuged at 80 × g for 5 min. To isolate crypts, the cell pellet was diluted with
DMEM and enriched 3-5 times by centrifugation over a 2% D-sorbitol gradient for 5 min at 50 x g. The isolated crypts were washed with DMEM, and then cultured in the cell culture flask using DMEM supplemented with high glucose, 10% heat inactivated FBS, 2.5% HEPES buffer, 1% Fugizone, 1% Penicillin/Streptomycin, 1% sodium pyruvate, 1% insulin, 1% non-essential amino acid solution, Gentamicin (25 µg/ml) and Epidermal growth factor (30 ng/ml; EGF; Sigma-Aldrich); each crypt consists of 200-300 epithelial cells (Follmann et al., 2000). The cells that grew from the crypts were passed two to three times to remove fibroblasts by controlling the dislodging time (Kaushik et al., 2008). Primary BECs were confirmed by fluorescent microscopy using the monoclonal anti-cytokeratin antibody pan (mixture) (Sigma-Aldrich), which reacts specifically with a wide variety of epithelial tissues, and a secondary antibody is anti-mouse IgG FITC conjugated antibody. The primary BECs were subsequently frozen in cryopreservation media containing 10% DMSO, 40% DMEM and 50% FBS at -80°C for storage.

In preparation for the binding assay, frozen BECs were thawed at 37°C and washed with DMEM centrifuged at 300 × g for 6 min. Following this, the pellet was re-suspended with DMEM supplemented with high glucose, 1% heat inactivated FBS, 2.5% HEPES buffer, 1% Fugizone, 1% Penicillin/Streptomycin, 1% sodium pyruvate, 1% insulin, 1% non-essential amino acid solution, Gentamicin (25 µg/ml) and EGF (30 ng/ml) according to Bridger et al. (2010), and cultured for at least a week to reach confluence.

3.3.5 Assessment of epithelial cell viability

After reaching 80-100% confluence in flask, epithelial cells were washed with warm phosphate buffered saline (PBS; Sigma-Aldrich), dislodged with TrypLE Express (Invitrogen) for 5 min, and counted with 0.4 % trypan blue (Invitrogen) using a hemocytometer chamber slide.
Cells were seeded into black 96-well flat bottom plates (50,000 cells per well), and incubated at 37 °C with 5% CO₂ overnight (17 hr for MAC-T cells and 21 hr for BECs). Cells were then exposed to a range of concentrations (0.25, 0.5, 1, 2, 4, 6, 8 and 16 mg/ml) of yeast CWCs from strains A and B of *S. cerevisiae* and two forms of dead yeast from strain A provided by Lallemand Inc., QC, Canada. The cell culture plate was centrifuged briefly at 200 × g for 3 min to ensure interaction between epithelial cells and yeast derivatives before incubation at 37 °C with 5% CO₂. After a 6-hr period, the epithelial cells were washed with warm PBS, then incubated with calcein AM diluted in culture media (Invitrogen) at room temperature for 30 min to stain live cells. The number of live cells was estimated by measuring the fluorescence of calcein AM (excitation 494/ emission 517 nm) using a 1420 Victor2 Multilabel Counter (Beckman Coulter, Inc. California). The cell viability was calculated by using the formula below.

\[
\text{Percent of cell viability} = \left( \frac{X}{Y} \right) \times 100,
\]

where X is the fluorescence value in each well containing yeast derivative-treated cells and Y is the mean value of the fluorescence reading of all control wells.

3.3.6 Assessment of yeast derivative anti-adhesive properties

The MAC-T cells/BECs were seeded into 96-black well flat bottom plates at 50,000 cells per well, and MAC-T cells and BECs were incubated for 17 and 21 hr, respectively, at 37 °C with 5% CO₂. The cells were then exposed to a range of concentrations of yeast derivatives (Table 3.1) with MAP at a 10:1(CFU: Cell) multiplicity of infection. The cell culture plates were centrifuged at 200 × g for 3 min to ensure interaction between MAP and epithelial cells before incubation at 37 °C with 5% CO₂ for 6 hr. Following this, the plate was washed with warm PBS, and the adhesion of MAP was estimated by measuring the fluorescence intensity of mCherry-MAP (excitation 587/ emission 610 nm) using a 1420 Victor2 Multilabel Counter. The adhesion
of MAP to epithelial cells was calculated for each yeast fraction using the formula, the adhesion of mCherry-MAP = the fluorescence value in each well of treatments with MAP infection (A) - the mean value of the fluorescence of all wells of the corresponding control group with same exposure concentration of yeast derivative (B).

3.3.7 Statistical analysis

The BEC and MAC-T cell viability were all analyzed separately as randomized complete block designs, in which the three independent trials represented the random blocks in each analysis. There were 7 or 8 treatments (one control and 6 and 7 different concentrations of CWCs from strain B and rest of yeast derivatives, respectively) and 6 replicates for each treatment within each block. The model for each of experiments included blocks as a random effect and yeast derivatives and concentration level plus their interactions fixed effects. All data were log-transformed prior to analysis in order to stabilize variances. Separate residual variances for each yeast derivative were incorporated in the model. Linear and quadratic orthogonal polynomial contrasts across concentration level were used to assess changes in viability over concentration using the mixed procedure SAS 9.4 (SAS Institute Inc., 2012).

For the binding assay data, MAP binding to BECs or MAC-T cells were analyzed separately as a randomized complete block design study with three independent trials representing each block. All data were log-transformed prior to analysis in order to stabilize variances. Concentration-dependent evaluations were compared between control and treatments by one-way ANOVA using Dunnett’s test for statistical significance with the mixed procedure from SAS 9.4 (SAS Institute Inc.).

Graphs were generated using the Graphpad Prism version 4.00 (GraphPad Software, 2003, San Diego California, USA), and all data were presented as least squares means of the log
transformed data from the percentage of cell viability and adhesion of MAP. A $p$-value $\leq 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Effect of yeast derivatives on epithelial cell viability

MAC-T cell viability was significantly reduced by CWCs from strain A ($p < 0.01$) and strain B ($p < 0.01$) as indicated by linear contrasts (Fig. 3.3). MAC-T cell viability was significantly increased by inactive yeast from strain A ($p < 0.01$) as indicated by quadratic contrasts, and no cytotoxicity was observed. The autolysed yeast also reduced MAC-T viability at the highest concentration (16 mg/ml) and this resulted in a significant linear contrast ($p < 0.01$) across concentration (Fig. 3.4).

BEC viability was significantly reduced by CWCs from both strain A ($p < 0.01$) and strain B ($p < 0.01$) as indicated by linear contrasts (Fig. 3.5). BEC viability was also significantly increased by inactive yeast from strain A ($p < 0.01$) as indicated by quadratic contrasts, and cytotoxicity was not observed for either form of dead yeast from strain A (Fig. 3.6).

3.4.2 Effect of yeast derivatives on MAP binding to epithelial cells

CWCs from strains A and B concentration-dependently reduced MAP binding to MAC-T cells under co-cultured conditions for 6 hr. There was significant reduction in the number of MAP adherent to MAC-T cells after 6-hr exposure to CWCs from strain A at 0.5-8 mg/ml (Fig. 3.7A). A significant reduction of MAP adhesion was also seen when MAC-T cells were co-cultured with all concentrations of CWCs from strain B (Fig. 3.7B). Similarly, the number of MAP adhering to MAC-T cells appeared to decrease as the concentration of both forms of dead
yeast strain A increased; the significant reduction can be seen from concentrations 2 mg/ml and higher for both inactive and autolyzed yeast (Fig. 3.8).

CWCs from strain A also concentration-dependently reduced MAP binding to BECs during the 6-hr exposure period. A significant reduction in MAP binding was observed at concentrations 1-8 mg/ml (Fig. 3.9A). However, there was no difference in MAP binding in response to CWCs from strain B (Fig. 3.9B). Additionally, a concentration-dependent reduction of MAP binding to BECs was observed with both inactive and autolyzed yeast from strain A with significant differences appearing between 2-16 mg/ml (Fig. 3.10).

3.5 Discussion

Here we report that the adherence of MAP to bovine epithelial cells was reduced by the addition of yeast S. cerevisiae derivatives in cell culture. To our knowledge, this is the first investigation of the anti-adhesive properties of S. cerevisiae derivatives against MAP in vitro. The bovine mammary epithelial cell line (MAC-T cells) was used in this study because of ease of culture and lack of a suitable bovine small intestinal epithelial cell line, and because it is challenging to obtain sufficient numbers of primary intestinal epithelial cells for high throughput in vitro screening of bioactive compounds. One concern with using MAC-T cells is that they may not appropriately model MAP binding to intestinal epithelial cells; however, we found in this study that MAP binding was largely similar between MAC-T cells and primary BECs. Additionally, MAP has previously been detected within mammary gland of sub-clinically infected cows (Streeter, 1995), which indicates that MAP is able to disseminate and invade the mammary epithelium.

Another concern is that yeast derivatives may influence epithelial cell viability. The cell viability of both MAC-T cells and BECs was therefore tested after a 6-hr exposure period with
the yeast derivatives. The results varied with the cell type and yeast derivative. MAC-T cell viability for example, appeared to be lower than that of BECs within the same treatment of CWCs from strain A at 16 mg/ml, and the autolyzed yeast from strain A at 16 mg/ml.

The decrease of cell viability might be related to changes in intracellular homeostasis and apoptosis-regulated gene expression. Higher concentrations of yeast derivatives for example, may induce cell apoptosis via altering intracellular calcium levels and the ratio of Bax and bcl-2 genes, as was demonstrated in human breast cancer cells exposed to heat-killed *S. cerevisiae* (Ghoneum et al., 2008). Bax and bcl-2 are two discrete members of a gene family involved in the regulation of cellular apoptosis; Bax protein is known as an apoptosis-promoting factor, whereas bcl-2 protein as an apoptosis-suppressing factor (Oltval et al., 1993; Hockenbery et al., 1990; Yang et al., 1996). Future studies will explore whether or not yeast derivatives from this study induced epithelial cell apoptosis.

It was interesting to note that not all of the yeast derivatives in this study reduced the cell viability. Inactive yeast for example, actually increased the viability of both MAC-T cells and BECs in a quadratic manner. It is possible that inactive yeast stimulated cell proliferation during the 6-hr exposure period. Increased cell proliferation was also observed with lymphocytes isolated from weaned pigs (Sonck et al., 2010); in this study, the cells were stimulated with different concentrations of β-glucans from a variety of sources, and the glucan from *S. cerevisiae* significantly induced the lymphocyte proliferation compared with the negative control.

Another concern about the following observations is that the reduction of MAP binding to epithelial cells was partially attributed to a decrease of epithelial cell viability. CWCs from strain B for instance, reduced MAC-T cell viability and MAP binding at the same concentrations (2 and 4 mg/ml); thus, the reduction of MAP binding could have in part been attributed to a
decrease in the number of viable epithelial cells. However, a reduction in MAP binding to epithelial cells was also observed at lower non-cytotoxic concentration of CWCs from strain B and with the two forms of dead yeast from strain A without a decrease of cell viability.

In addition to the observed decrease in MAP binding to epithelial cells in the presence of \textit{S. cerevisiae} derivatives, reduced MAP infection has also been demonstrated using mice fed the probiotic \textit{Lactobacillus animalis} (Karunasena et al., 2013). Both studies provided evidence that MAP infection may be reduced in ruminants by probiotic/prebiotic use. Similar observations have also been observed with other enteropathogenic bacteria such as \textit{E. coli}, \textit{Salmonella Typhimurium}, and \textit{Salmonella Typhi} when \textit{S. cerevisiae} was used as an anti-adhesive agent (Tiago et al., 2012; Becker & Galletti, 2008; Chaucheyras-Durand et al., 2012). A specific yeast-derived mannoprotein fraction has also shown to effectively block adherence of \textit{Campylobacter jejuni} to Caco-2 cells (Ganan et al., 2009).

The present \textit{in vitro} binding assay that involves mCherry-MAP and bovine epithelial cells offers a potentially high through-put platform for screening the anti-adhesive properties of probiotics and their bioactive derivatives that may be useful for controlling Johne’s disease. Another potential concern about this \textit{in vitro} study, however, is that the yeast derivatives may influence other mucosal cell types such as goblet cells and dendritic cells (DCs) that are not taken into consideration with this cell culture model. In previous \textit{in vivo} studies, dietary supplementation with mannan oligosaccharides (MOS) derived from \textit{S. cerevisiae} increased the number of cells secreting acid mucins in posterior gut of sea bass (Torrecillas et al., 2011). Increased mucus secretion induced by MOS may help prevent pathogenic bacteria adhesion via the anti-adhesive action of mucins (Carlstedt et al., 1997) as well as bulk physical properties of mucus that help to clear bacteria (Sandberg et al., 2000). However, a reduction in the number of
goblet cells that produce mucus has also been reported in the ileal sections of chicken pouls fed 0.02% *S. cerevisiae* var *boulardii* (Bradley et al., 1994). Yeast derivatives can also have interactions with DCs. Mannan, which is a fungal component derived from the *S. cerevisiae* or *Candida albicans* cell wall, has been reported to induce the maturation of mouse DCs in vivo (Sheng et al., 2006). The maturation of DCs is required for the priming of Th1 or Th2 cells, both of which are involved in the adaptive immune response. Sheng et al. (2006) also showed that two different types of mannan, oxidized mannan and reduced mannan, differentially stimulated Th1/Th2 cytokine production from murine bone-marrow-derived DCs. Th1 cytokines play a key role during the cell-mediated immune response, which is required to control MAP infection (Pieters, 2001); whereas, the stimulation of Th2 cytokines that support an antibody immune response is thought to be insufficient for controlling intracellular pathogens such as MAP (Alonso-Hearn et al., 2008). Given the potential limitation of the present binding assay using mCherry-MAP, in vivo studies also need to be conducted in order to validate the benefits of promising yeast derivatives that are identified through in vitro screening.

In addition to influencing host mucosal cells, yeast probiotics and their derivatives may also influence commensal bacteria and protozoa that exist within ruminant gastrointestinal tract. It was reported that MOS treatment for example, significantly increased the diversity and band number of bacteria in both ileal and colonic fermentum from piglets (Hang et al., 2012), which implies that MOS could stabilize the gastrointestinal tract microflora that may act as a protective barrier against MAP infection. Additionally, feeding yeast culture was previously reported to increase the number of protozoa in the rumen (Arakaki et al., 2000), since the yeast culture is used as a protein and energy source by protozoa (Dehority, 1986; Dehority & Orpin, 1997). Given that two commonly occurring environmental protozoa, *Acanthamoeba castellanii* and *A.
polyphaga, have been reported to be the vectors for MAP (Whan et al., 2000), it is still unclear if the oral administration of yeast derivatives would be beneficial to help reduce risk of MAP infection.

3.6 Summary

In conclusion, the results from the present study demonstrate that the adhesion of MAP to epithelial cells is significantly reduced by S. cerevisiae derivatives when MAP infection is carried out in vitro. Since MAP is an intracellular pathogen, this anti-adhesive action could potentially reduce uptake of MAP by epithelial cells. However, since the specific mechanism of reduced MAP adhesion remains to be elucidated, further characterization and investigation is warranted to determine mechanisms of action, the stability of the binding between yeast derivatives and MAP, and the efficacy of the tested yeast derivatives in vivo.
Figure 3.1: OD$_{600}$ versus fluorescent intensity of mCherry MAP Ge86 (Mead, 2013).
Figure 3.2: $OD_{600}$ versus colony-forming units (CFU) of mCherry MAP Gc86 (Mead, 2013).
Figure 3.3: Viability of MAC-T cells following 6-hr exposure to CWCs from yeast strains A (A) and B (B). Data are presented as least square mean +/- standard error.
Figure 3.4: Viability of MAC-T cells following 6-hr exposure to two forms of dead yeast from strain A (inactive yeast (A); autolyzed yeast (B)). Data are presented as least square mean +/- standard error.
Figure 3.5: Viability of BECs following 6-hr exposure to CWCs from yeast strains A (A) and B (B). Data are presented as least square mean +/- standard error.
Figure 3.6: Viability of BECs following 6-hr exposure to two forms of dead yeast from strain A (inactive yeast (A); autolyzed yeast (B)). Data are presented as least square mean +/- standard error.
Figure 3.7: Binding of mCherry-MAP to MAC-T cells in the presence of yeast CWCs from strains A (A) and B (B) after 6-hr exposure. Data are presented as least square mean +/- standard error. Significant differences relative to the control are indicated at $p<0.05$ (*), 0.01 (**), and 0.001 (***)
Figure 3.8: Binding of mCherry-MAP to MAC-T cells in the presence of two forms of dead yeast from strain A (inactive yeast (A); autolyzed yeast (B)) after 6-hr exposure. Data are presented as least square mean +/- standard error. Significant differences relative to the control are indicated at $p<0.05$ (*), 0.01 (**), and 0.001 (***).
Figure 3.9: Binding of mCherry-MAP to BECs in the presence of yeast CWCs from strains A (A) and B (B) after 6-hr exposure. Data are presented as least square mean +/- standard error. Significant differences relative to the control are indicated at $p<0.05$ (*), 0.01 (**), and 0.001 (***)
Figure 3.10: Binding of mCherry-MAP to BECs in the presence of two forms of dead yeast from strain A (inactive yeast (A); autolyzed yeast (B)) after 6-hr exposure. Data are presented as least square mean +/- standard error. Significant differences relative to the control are indicated at $p<0.05$ (*), 0.01 (**), and 0.001 (***).
Table 3.1: Concentrations of yeast derivatives used for binding assay

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Strain A CWCs</th>
<th>Strain B CWCs</th>
<th>Inactive yeast</th>
<th>Autolyzed yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAC-T cells</td>
<td>0.25, 0.5, 1, 2, 4, 8</td>
<td>0.25, 0.5, 1, 2, 4, 8</td>
<td>0.25, 0.5, 1, 2, 4, 8, 16</td>
<td>0.25, 0.5, 1, 2, 4, 8, 16</td>
</tr>
<tr>
<td>BECs</td>
<td>0.25, 0.5, 1, 2, 4, 8</td>
<td>0.25, 0.5, 1, 2, 4, 8</td>
<td>0.25, 0.5, 1, 2, 4, 8, 16</td>
<td>0.25, 0.5, 1, 2, 4, 8, 16</td>
</tr>
</tbody>
</table>
Chapter 4. *In vitro* bio-assessment of the immunomodulatory activity of *S. cerevisiae* derivatives using bovine macrophages and MAP

4.1 Abstract

Since yeast *S. cerevisiae* and its derivatives are being used for the prevention and treatment of enteric disease in different species, they may also be useful preventing Johne’s disease, a chronic inflammatory bowel disease of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The goal of this study was to identify potential immunomodulatory *S. cerevisiae* derivatives using a bovine macrophage cell line (BOMACs). BOMAC phagocytic activity, ROS production and immune-related gene (interleukin (IL)-6, IL-10, IL-12p40, IL-13, IL-23, and transforming growth factor β (TGF-β)) expression were investigated when BOMACs were co-cultured with CWCs from two different strains (A and B) and two forms of dead yeast from strain A. BOMAC phagocytosis of mCherry-MAP was concentration-dependently attenuated when BOMACs were co-cultured with yeast derivatives for 6 hr. Each yeast derivative also induced a concentration-dependent increase in BOMAC ROS production after 6-hr exposure. In addition, BOMAC mRNA expression of the immune-related genes was also altered after 6 and 24 hr of exposure to yeast derivatives. The CWCs from both strains and autolyzed yeast induced IL-6 expression after 6 hr. The CWCs from strain A reduced the expression of IL-13 and after 6 hr, whereas IL-13 was induced by the CWCs from strain B. The CWCs from strain A reduced the expression of IL-13, IL-23 and TGF-β at lower concentrations, but induced the expression of IL-13 and IL-23 at highest concentration after 24 hr. The CWCs from strain B increased the expression of IL-12p40, IL-13, IL-23 and TGF-β after 24-hr exposure. Lastly, inactive yeast increased the expression of IL-23 at 24 hr. Overall, all yeast derivatives were found to be immunomodulatory to BOMACs, however, the response varied among derivatives.
4.2 Introduction

Dairy producers have been using commercially available yeast probiotics and their derivatives as feed supplements for nearly two decades based on claims that these products will improve animal production, promote health, and reduce the need for antibiotic use. Studies demonstrate that supplementing the ruminant diet with specific strains of *S. cerevisiae* improves feed intake (Williams et al., 1991; Robinson & Garrett, 1999), weight gain (Salama et al., 2002), and fiber digestion (Wohlt et al., 1998; Kamel et al., 2004). It has also been reported that live yeast stabilize rumen pH (Mathieu et al., 1996; Doreau & Jouany, 1998; Jouany et al., 1998), and the number of anaerobic cellulolytic bacteria (Mosoni et al., 2007; Silberberg et al., 2013).

In addition to having nutritional value, there is evidence that *S. cerevisiae* and its derivatives (i.e. β-glucans) appear to have immunomodulatory properties that can affect both the innate and acquired immune system (Ganner & Schatzmayr, 2012). β-glucans, which are one the major derivatives from YCW, have been shown to stimulate immune responses inducing functional status of macrophages; up-regulating cytokine expression and increasing reactive oxygen species (ROS) production by macrophages, neutrophils, and dendritic cells; and promoting leukocyte activity including increasing host survival against pathogenic infections (Volman et al., 2008; Kogan & Kocher, 2007). Likewise, yeast mannan oligosaccharide derivatives are also able to modulate the immune system partly by acting as a non-pathogenic microbial antigen but also because it has adjuvant-like properties (Ferket et al., 2002). Given these properties of *S. cerevisiae* and its derivatives, we carried out a study to assess the bioactivity of CWCs from two strains of *S. cerevisiae* (A and B) and two different forms of dead strain A yeast (inactive and autolyzed yeast) using a bovine macrophage cell line (BOMACs). The aim of this study was to increase insight into the direct effects of yeast derivatives on bovine
macrophage functions by assessing phagocytic activity, ROS production and immune-related gene expression.

4.3 Materials and methods

4.3.1 Bacterial strain and culture conditions

The mCherry-labeled *Mycobacterium avium* spp. *paratuberculosis* (MAP) used in the present study was developed by Mead et al. (2014) using the clinical isolate Gc86 strain previously isolated in the laboratory of Dr. Lucy Mutharia by Melinda Raymond (Department of Molecular and Cellular Biology, University of Guelph).

4.3.2 Preparation of MAP infection stock

Liquid nitrogen frozen mCherry MAP Gc86 was thawed at 37 °C and was used to inoculate 5 ml of 7H9 broth (Difco laboratories, Franklin Lakes, NJ, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.25% v/v Tyloxopol (Sigma-Aldrich, St. Louis, MO, USA), 50 µg/ml kanamycin and 2 µg/ml of mycobactin J (Allied Laboratories, Wichita, KS, USA). The cultures were incubated at 37 °C, and once they reached a fluorescent intensity (FI) of 45000, equivalent to OD$_{600}$ = 0.8 (Mead, 2013), 5 ml aliquots were sub-cultured into 100 ml of media in a 250 ml sterile culture flask and incubated at 37 °C. When the cultures reached the logarithmic stage of growth (FI = 40000-50000 equivalent to OD$_{600}$ = 0.6-0.9), cells were centrifuged at 2000 × g for 30 min. The cells were resuspended to reach FI = 60000 equivalent to OD$_{600}$ = 1.0 using Fig. 3.1, then to establish Colony Forming Units (CFU/ml) using Fig. 3.2 (Mead, 2013). Quantification of fluorescence was based on the specific emission (587 nm) and excitation wavelengths (610 nm) for mCherry using the Wallac-1420 VICTOR3 Multilabel Counter (Perkin Elmer).
4.3.3 Bovine macrophage cell line (BOMACs) and culture conditions

The bovine macrophage cell line (BOMACs) was used in this study as an in vitro immunomodulation and infection model (Stabel & Stabel, 1995). BOMACs were cultured in T75 tissue culture flask (Corning, Tewksbury, MA, USA) at 37 °C with 5% CO₂, containing Roswell Park Memorial Institute-1640 medium (RPMI 1640; Invitrogen, Burlington, ON, Canada) supplemented with 2.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS; Invitrogen), 2.5 mM HEPES buffer (Invitrogen), 1 mM Sodium Pyruvate (Invitrogen) and 1% Antibiotic-Antimycotic (100 unit/ml of Penicillin, 100 µg/ml Streptomycin and 0.25 µg/ml Amphotericin B; Invitrogen).

4.3.4 Assessment of BOMAC viability

After reaching 80-100% confluence in flask, BOMACs were washed with warm phosphate buffered saline (PBS; Sigma-Aldrich), dislodged with TrypLE Express (Invitrogen) for 5 min, and counted with 0.4 % trypan blue (Invitrogen) using a hemocytometer chamber slide.

Cells were seeded into black 96-well flat bottom plates (50,000 cells per well), and incubated at 37 °C with 5% CO₂ overnight (17 hr). Cells were then exposed to a range of concentrations (0.25, 0.5, 1.2, 4, 6, 8 and/or 16 mg/ml) of yeast CWCs from strains A and B of S. cerevisiae and two forms of dead yeast from strain A provided by Lallemand Inc., Montreal, QC, Canada. The cell culture plate was centrifuged briefly at 200 × g for 3 min to ensure interaction between BOMACs and yeast derivatives before incubation at 37 °C with 5% CO₂. After a 6-hr period, BOMACs were washed with warm PBS, then incubated with calcein AM diluted in culture media (Invitrogen) at room temperature for 30 min to stain live cells. The number of live cells was estimated by measuring the fluorescence of calcein AM (excitation 494/ emission 517
nm) using a 1420 Victor2 Multilabel Counter (Beckman Coulter Inc.). The cell viability was calculated by using the formula below. Percent of cell viability = (X/Y) × 100, where X is the FI value in each well containing yeast derivative-treated cells and Y is the mean value of the FI of all control wells.

4.3.5 Assessment of BOMAC phagocytic activity

The BOMACs were seeded into black 96-well flat bottom plates (50,000 cells per well), and incubated for 17 hr at 37 °C with 5% CO2. The cells were then exposed to a range of concentrations of each yeast derivative (0.25, 0.5, 1, 2, and 4 mg/ml for CWCs, and 0.25, 0.5 and 1 mg/ml for the dead yeasts) from S. cerevisiae with MAP at a 10:1 (CFU: cell) multiplicity of infection. The cell culture plates were centrifuged at 200 × g for 3 min to ensure interactions between MAP and BOMACs before incubation at 37 °C with 5% CO2 for 6 hr. Following this, the plates were washed with warm PBS, and the uptake of MAP was estimated by measuring the fluorescent intensity (FI) of mCherry-MAP (excitation 587/ emission 610 nm) using a 1420 Victor2 Multilabel Counter. The amount of phagocytized of MAP was calculated for each yeast derivative using the formula, phagocytized MAP = A - B, where A is the FI value in each well of treatment groups with MAP infection, and B is the mean value of the FI of all wells of the corresponding control group with same concentration of yeast derivative without MAP.

4.3.6 Assessment of ROS production

The BOMACs were seeded into black 96-well flat bottom plates at 50,000 cells per well, incubated for 17 hr at 37 °C with 5% CO2, and then exposed to a range of concentrations of each S. cerevisiae yeast derivative (0.5, 1, 2, and 4 mg/ml for CWCs, and 0.25, 0.5 and 1 mg/ml for the dead yeasts). The cell culture plate was centrifuged at 200 × g for 3 min before incubation at 37 °C with 5% CO2. After a 6-hr period, the plates were washed with warm PBS, then incubated
with 100 µl/well CellROX® Green Reagent (Invitrogen), which is a fluorogenic probe for measuring oxidative stress in live cells, at 37\(^\circ\) C with 5% CO\(_2\) for 1 hr, and again washed with warm PBS. The production of ROS was estimated using a 1420 Victor2 Multilabel Counter by measuring the fluorescence of CellROX® Green Reagent (excitation 485/ emission 520 nm).

4.3.7 Real-time PCR quantification of BOMAC gene expression

4.3.7.1 Cell preparation and exposure to yeast derivatives

The BOMACs were seeded at 0.5 \(\times\) 10\(^6\) per well into 6-well flat-bottom plates (Corning Inc.) and incubated for 17 hr at 37 \(\circ\)C with 5% CO\(_2\). The cells then were exposed to either 10 µg/ml of Pam3CSK4 (Invitrogen) as a positive control, which is a TLR1/2 ligand synthetic triacylated lipoprotein, or a range of concentrations of yeast derivative (0.25, 0.5, 1, 2, and 4 mg/ml for CWCs; or 0.25, 0.5, and 1 mg/ml for the dead yeasts). A control group that did not contain either Pam3CSK4 or yeast derivatives was also included. For all treatments, the cells were incubated for 6 or 24 hr, and the cells were washed with PBS then lysed using 1 ml TRIzol per well. The cell lysates were aliquotted into 2.0 ml nuclease-free cryovials and stored at \(-80 \circ\)C for later RNA isolation.

4.3.7.2 Total RNA isolation and cDNA synthesis

Total RNA extraction and isolation from the BOMACs was carried out using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA concentration was measured using NanoDrop\textsuperscript{®} ND-8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and RNA integrity number (RIN) was assessed using an Agilent 2100a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA (5 µg) was reverse
transcribed to cDNA using oligo-dT primers and Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions.

4.3.7.3 Primer design and Real-time PCR quantification

Two candidate reference genes that included glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were evaluated in the present study. GAPDH was selected based on the gene expression stability appearing in each treatment, and it was used to normalize the target gene expression and to determine relative gene expression. The primers were designed based on the sequences of *Bos Taurus* extracted from GenBank using Primer3 software. All primer sequences and relevant information about the genes are presented in Table 4.1.

Target gene expression was evaluated by semi-quantitative real-time PCR (qPCR) using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Burlington, ON, Canada). The qPCR was performed in a 25-μl reaction containing 1 μl cDNA (15 ng/μl), 12.5 μl PerfeCTaTM SYBR® Green SupeMix, ROXTM (Quanta, Canada), and 400 nM of each primer. The qPCR running protocol was 50 °C for 3 min, 95°C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing temperatures for 30 s (Table 4.1), and extension at 72 °C for 30 s. A dissociation curve was performed following each qPCR to ensure the presence of a uniform and single PCR product. Each cDNA sample from each trial was carried out in quadruplicate for each gene. The cycle threshold (Ct) values for each sample were determined using the auto Ct function of the ABI Prism 7000 SDS Software (Applied Biosystems); these Ct values were used to calculate the quantity of PCR product using the standard curve analysis. Gene-specific standard curves were generated using pooled cDNA and run on each plate.
4.3.8 Statistical analysis

The BOMAC viability was analyzed as a randomized complete block design, in which the three independent trials represented the random blocks in each analysis. There were 7 or 8 treatments (one control and 6 and 7 different concentrations of CWCs and dead yeasts, respectively) and 6 replicates for each treatment within each block. The model included blocks as a random effect and yeast derivatives and concentration plus their interactions as fixed effects. All data were log-transformed prior to analysis in order to stabilize variances. Separate residual variances for each yeast derivative were incorporated in the model. Linear and quadratic orthogonal polynomial contrasts across concentration level were used to assess changes in viability over concentration using the mixed procedure SAS 9.4 (SAS Institute Inc., 2012).

The BOMAC phagocytic activity, ROS production, and gene expression data were analyzed separately as a randomized complete block design study with three independent trials representing each block. All data were log-transformed prior to analysis in order to stabilize variances. Concentration-dependent evaluations were compared between control and treatments by one-way ANOVA using Dunnett’s test for statistical significance with the mixed procedure from SAS 9.4 (SAS Institute Inc.).

Graphs were generated using the Graphpad Prism version 4.00 (GraphPad Software, 2003, San Diego California, USA), and all data were presented as least squares means of the log transformed data ± standard deviation. A $p$-value $\leq 0.05$ was considered statistically significant.
4.4 Results

4.4.1 Effect of yeast derivatives on BOMAC viability

BOMAC viability was significantly reduced by CWCs from both yeast strain A and B 
\(p<0.01\) as indicated by linear contrasts (Fig. 4.1). Both inactive and autolyzed yeast from strain 
A also affected BOMAC viability as indicated by quadratic contrasts \(p<0.01\) (Fig. 4.2).

4.4.2 Effect of yeast derivatives on BOMAC phagocytic activity

CWCs from both strains A and B reduced MAP uptake by BOMACs under co-cultured 
conditions for 6 hr. There was significant reduction in the number of MAP phagocytized by 
BOMACs after 6-hr exposure to CWCs from both strains at all tested concentrations (Fig. 4.3). 
Similarly, the number of MAP phagocytized by BOMACs appeared to decrease as the 
concentration of the both forms of dead yeast strain A increased; a significant reduction can be 
seen from 0.25 mg/ml and above for the inactive yeast (Fig. 4.4A), as well as 0.5 mg/ml and 
above for the autolyzed yeast (Fig. 4.4B).

4.4.3 Effect of yeast derivatives on ROS production in BOMACs

A significant increase in ROS production by BOMACs was observed at all tested 
concentrations of CWCs from strain A (Fig. 4.5A) during the 6-hr exposure period. Similarly, a 
significant concentration-dependent increase in ROS production was observed at 1 mg/ml and 
higher for CWCs from strain B (Fig. 4.5B). Additionally, the two forms of dead yeast also 
significantly and concentration-dependently induced ROS production by BOMACs during the 6-
hr exposure period at all tested concentrations (Fig. 4.6).
4.4.4 Effect of yeast derivatives on BOMAC cytokine gene expression

The effect of yeast derivatives on the relative expression of genes responsible for modulating immune function was evaluated with BOMACs using GAPDH as reference gene because the expression of GAPDH was not influenced by different treatment. There was a 22-fold increase in the expression of IL-6 after BOMACs were exposed to the Pam3CSK4 positive control at 6 hr, and the expression of IL-6 was also induced 14 fold by Pam3CSK4 at the 24-hr time point (Appendix 1).

At 6-hr, the expression of IL-6 was significantly induced by CWCs from both strain A at 2, 4 mg/ml and strain B at 1, 2, 4 mg/ml (Fig. 4.7A), as well as the autolyzed yeast at 0.5 mg/ml (Fig. 4.8A). The expression of IL-13 was significantly decreased by CWCs from strain A at 0.25 mg/ml, but increased by CWCs from strain B at 2 and 4 mg/ml (Fig. 4.7D). The expression of IL-10 appeared to be induced by CWCs from both yeast strains, but this did not show statistical significance due to extreme variation. The expression of IL-12p40, IL-23 and TGF-β was also not significantly affected by yeast CWCs from strains A and B, and the expression of IL-10, IL-12p40, IL-13, IL-23, and TGF-β was not significantly affected by both forms of dead yeast strain A.

At 24-hr, there was a significant increase in the expression of IL-12p40 following treatment with CWCs from strain B at 4 mg/ml (Fig. 4.9C). The expression IL-13 in contrast, was significantly reduced by CWCs from strain A at 0.25 mg/ml, but significantly increased at 4 mg/ml (Fig. 4.9D); increased IL-13 expression was also observed in response to CWCs from strain B at 0.5, 2, 4 mg/ml (Fig. 4.9D). A significant reduction in the expression of IL-23 occurred in response to CWCs from strain A at 0.5 mg/ml; however, the expression of IL-23 was significantly induced by CWCs from both strains at 4 mg/ml (Fig. 4.9E), as well as autolyzed
yeast at 0.25 mg/ml (Fig. 4.10E). A similar observation was also observed for TGF-β expression (Fig. 4.9F), with decreased expression occurring in response to CWCs from strain A at 0.5 mg/ml, and increased expression occurring in response to CWCs from strain B at 4 mg/ml. Significant changes in IL-6 and IL-10 were not observed in response to CWCs from either yeast strain; IL-6, IL-10, IL-12p40, IL-13, IL-23 and TGF-β appeared to be unaffected by exposure to both forms of dead yeast strain A.

4.5 Discussion

Here we report that bovine macrophage function which included BOMAC phagocytic activity, ROS production and immune-related gene expression was influenced by S. cerevisiae cell wall derivatives. A number of studies have demonstrated that yeast derivatives are potent immunostimulants in fish (Andrews et al., 2011; Kunttu et al., 2009; Jha et al., 2007). In addition, yeast derivatives have also been shown to affect the immune response in pigs by altering T cell priming and cytokine secretion (Zhou et al., 2013; Ganner et al., 2010a). However, the immunomodulating effect of yeast derivatives on ruminants has not been fully explored. The present study contributes to this research field by demonstrating that bovine macrophage function is also altered by S. cerevisiae derivatives.

A bovine macrophage cell line (BOMAC) was used in this study because of ease of culture and because it is challenging to obtain sufficient numbers of primary macrophages from peripheral blood mononuclear cells for high through-put in vitro screening of bioactive compounds. One concern with in vitro exposure was that yeast derivatives may alter macrophage viability, therefore, BOMAC viability was tested after a 6-hr exposure period, and the results varied with yeast derivative. Although significant changes in cell viability were detected, only CWCs from strain B reduced cell viability below 95%, and this was only at the highest
concentration. BOMAC viability was also tested in a preliminary study after 24-hr exposure with the same yeast derivatives, and similar results were observed (Appendix 2). The decrease in cell viability associated with CWCs from strain B might be related to changes in intracellular homeostasis and apoptosis-regulated gene expression. Higher concentrations of yeast derivatives may induce cell apoptosis via altering intracellular calcium levels and the ratio of bax and bcl-2 genes, as was demonstrated in human breast cancer cells exposed to heat-killed *S. cerevisiae* (Ghoneum et al., 2008). Bax and bcl-2 are two discrete members of a gene family involved in the regulation of cellular apoptosis; bax protein is known as an apoptosis-promoting factor, whereas bcl-2 protein is an apoptosis-suppressing factor (Oltval et al., 1993; Hockenbery et al., 1990; Yang et al., 1996). Future studies will explore whether or not CWCs from strain B induced macrophage apoptosis.

It was interesting to note that BOMAC viability was increased by both forms of dead yeast from strain A at lower concentration, but not by the CWCs. We also observed this response when two different types of epithelial cells were exposed to inactive yeast from chapter 3. Since the dead forms of yeast strain A are more crude derivatives than the CWCs, it is likely that these changes reflect increased cell proliferation were induced by yeast cellular components no longer present in the CWCs.

BOMAC phagocytic activity was also slightly but significantly inhibited by exposure to the yeast derivatives. Since we previously demonstrated that these same yeast derivatives reduced MAP binding to bovine epithelial cells from chapter 3, it is likely that similar interactions occurred with BOMACs, which subsequently reduced the number of MAP that could have been phagocytized by the BOMACs. Since the maximum reduction of MAP phagocytized by BOMACs ranged from 5-8 %, it is also possible that reduced cell viability may
have confounded results at higher concentrations, especially for CWCs from strain B at the highest concentration; however, we do not believe this to be the case, since phagocytosis was also reduced at lower concentrations where viability was unaffected. Since MAP has been reported to target, survive, and replicates within macrophages (Kuehnel et al., 2001; Pieters et al., 2001), the reduced number of MAP phagocytized by macrophage might be beneficial to reducing risk of MAP infection.

The present study also showed that *S. cerevisiae* derivatives induced ROS production by BOMACs. ROS are involved in phagosome acidification that helps kill intracellular mycobacteria (Ehrt et al., 2009), however, ROS can also induce cell apoptosis (Dumont et al., 1999), which implies that reduced cell viability at the high CWC concentration from strain B may also be due in part to the generation of ROS.

It has previously been demonstrated that yeast β-glucans interact with a lectin-like moiety on dectin-1 (Willment et al., 2001), which is a transmembrane pattern-recognition receptor on macrophages (Brown et al., 2002) that initiates macrophage phagocytosis and generation of inflammatory mediators, ROS for example, in association with TLR2 (Brown et al., 2003). The induction of ROS was previously observed with neutrophils and monocytes in response to stimulation with β-glucans (Rubin-Bejerano et al., 2007; Vetvicka et al., 1996), and in aquaculture research, dietary administration of β-glucans also induced Asian catfish (Kumari et al., 2006) and zebrafish (Rodríguez et al., 2009) macrophage ROS production. Increased ROS production was also detected when human whole blood was pre-incubated with poly-[1-6]-D-glucopyranosyl-[1-3]-D-glucopyranose-glucan derived from the cell wall of *S. cerevisiae* (Wakshull et al., 1999). In future studies, the dectin-1 receptor can be blocked with an antagonist
such as laminarin for example, to determine if ROS production by the tested yeast derivatives was mediated through dectin-1.

The expression of macrophage genes encoding immune-related cytokines was also altered in this study by various yeast derivatives. The expression of pro-inflammatory cytokine, IL-6 for example, was significantly induced by exposure to CWCs from both strains and autolyzed yeast. This is in agreement with a study by Volman et al. (2008), who showed that IL-6 expression was increased when human whole blood was incubated with β-glucans derived from S. cerevisiae. IL-6 has been found to be associated with inflammation, and one of the most common symptoms of inflammation is fever, which has been reported during bacterial infection due to the generation of IL-6 (Akira et al., 1990). In addition, IL-6 has also been shown to modulate T cell and B cell activation and proliferation; therefore, increased IL-6 production may augment cellular immune events of the adaptive immune system (Van Seventer et al., 1991).

The increased production of various cytokines such as anti-inflammatory IL-10 and pro-inflammatory IL-12p40 from macrophages has also been reported after the stimulation of β-glucans (Rogers, et al., 2005; Gantner et al., 2003). Although, changes in IL-10 expression were not observed in the present study, significant induction in the expression of IL-12p40 was detected by treatment with CWCs from strain B at the highest concentration at 24 hr. Increased IL-12p40 expression is consistent with another report, in which a human monocyte cell line was challenged by Agaricus blazei mushroom extracts with high content of β-glucans (Ellertsen et al., 2006). IL-12p40 has been shown to promote cell-mediated immunity against mycobacteria; for example, the IL-12p40 deficient mice were susceptible to chronic Mycobacterial infection and a higher rate of mortality was observed after pulmonary infection with M. tuberculosis (Hölscher et al., 2001).
In addition to inducing IL-6 and IL-12p40 production, dectin-1-mediated ligand binding has been suggested to preferentially induce IL-17 expressing T helper cells (Th17) in both humans and mice (Palm et al., 2007). Th17 cell differentiation depends on the presence of TGF-β, IL-6, and IL-23. Increased IL-23 secretion has been reported in DCs after stimulation with a fungal cell wall component (LeibundGut-Landmann et al., 2007). Consistent with this observation, significant induction of IL-23 expression was also observed in the present study. Additionally, expression of another Th17-related cytokine, TGF-β, was significantly induced in this study. These results are consistent with observations with yeast zymosan-treated macrophages and DCs in mice (Dillon et al., 2006). Increased production of IL-23 and TGF-β has been shown to be positively associated with the differentiation of Th17 cells, which are potent inflammatory cells (Dubin et al., 2008). Th17 cells have been shown to be associated with increased protection against Mycobacteria challenge in mice; where an increased Th17 cell population that was triggered after vaccination induced the production of chemokines, that led to the recruitment of effector T cells and the production of interferon-γ, subsequently restricting bacterial growth (Khader et al., 2007). The reduction of both cytokines, IL-23 and TGF-β was also observed due to the exposure of lower concentrations of yeast derivatives in the present study. We were unable to find any research to explain this observation, and further experiments need to be conducted including assessment of protein expression to explore this in further detail.

The expression of IL-13 was also significantly induced by CWCs from strain B at 6 and 24 hr. In contrast, a significant change in the expression of this Th2 cytokines could not be detected when human DCs were exposed to fungal zymosan (Dillon et al., 2006). IL-13 has been shown to regulate Th2 responses that promote immunoglobulin synthesis. Th2 responses are not effective for preventing MAP infection, since MAP is an intracellular pathogen. In addition, IL-
13 has also been found to associate with inducing development of alternatively activated macrophages (Gordon, 2003). The switch from classically activated macrophage to alternatively activated macrophages has been reported in a study using intracellular pathogen, *Leishmania major*; this switch could be associated with susceptibility to this pathogen (Hölscher et al., 2006; Kropf et al., 2005). However, a reduction of this cytokine was also observed in this study, and similar to the observation of reduced IL-23 and TGF-β expression, relevant studies supporting this are rare. Future experiments are also warranted to explore this in further detail.

### 4.6 Summary

In summary, our results demonstrated that the exposure of yeast derivatives affected macrophage function by (*i*) inhibiting the phagocytic activity, (*ii*) inducing the production of ROS and (*iii*) altering the immune-related gene expression. Given that yeast derivatives, particularly β-glucans are commonly used as immunostimulants, the tested yeast derivatives may contribute to inducing host immunity. Therefore, application of yeast derivatives could be a novel strategy for preventing some ruminant diseases, such as Johne’s disease. Indeed, to gain further insight into immune regulation by these yeast derivatives, future experiments combining cytokines assays and gene expression analysis with disease models would be useful.
Figure 4.1: Viability of BOMACs following 6-hr exposure to CWCs from yeast strain A (A) and B (B) Data are presented as least square mean +/- standard error.
Figure 4.2: Viability of BOMACs following 6-hr exposure to two forms of dead yeast from strain A (inactive yeast (A); autolyzed yeast (B)). Data are presented as least square mean +/- standard error.
Figure 4.3: BOMAC phagocytosis of MAP in the presence of yeast CWCs from strains A (A) and B (B) after 6-hr exposure. Data are presented as least square mean +/- standard error. Significant differences relative to the control are indicated at $p < 0.05$ (*), 0.01 (**), and 0.001(***).
Figure 4.4: BOMAC phagocytosis of MAP in the presence of two forms of dead yeast from strain A (inactive yeast (A); autolyzed yeast (B)) after 6-hr exposure. Data are presented as least square mean +/- standard error. Significant differences relative to the control are indicated at $p<0.05$ (*), 0.01 (**), and 0.001(***).
Figure 4.5: Reactive oxygen species (ROS) production in BOMACs in the presence of yeast CWCs from strain A (A) and B (B) after 6-hr exposure. Data are presented as least square mean +/- standard error. Significant differences relative to the control are indicated at $p<0.05$ (*), 0.01 (**), and 0.001(**).
Figure 4.6: Reactive oxygen species (ROS) production in BOMACs in the presence of two forms of dead yeas from strain A (inactive yeast (A); autolyzed yeast (B)) after 6-hr exposure. Data are presented as least square mean +/- standard error. Significant differences relative to the control are indicated at $p< 0.05$ (*), 0.01 (**), and 0.001(***).
Figure 4.7: The effect of 6-hr exposure to yeast CWCs from strains A and B on the expression of BOMAC immune-related genes. Data are presented as least square mean +/- standard error of the normalized values relative to the GAPDH reference gene. Significant differences relative to the control are indicated at p<0.05 (*) and 0.01(**).
Figure 4.8: The effect of 6-hr exposure to two forms of dead yeast from strain A (inactive and autolyzed yeast) on the expression of BOMAC immune-related genes. Data are presented as least square mean +/- standard error of the normalized values relative to the GAPDH reference gene. Significant differences relative to the control are indicated at $p<0.05$ (*) and 0.01(**).
Figure 4.9: The effect of 24-hr exposure to yeast CWCs from strains A and B on the expression of BOMAC immune-related genes. Data are presented as least square mean +/- standard error of the normalized values relative to the GAPDH reference gene. Significant differences relative to the control are indicated at p<0.05 (*) and 0.01(**).
Figure 4.10: The effect of 24-hr exposure to two forms of dead yeast from strain A (inactive and autolyzed yeast) on the expression of BOMAC immune-related genes. Data are presented as least square mean +/- standard error of the normalized values relative to the GAPDH reference gene. Significant differences relative to the control are indicated at $p<0.05$ (*) and 0.01(**).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene function</th>
<th>Primer sequences (forward above reverse)</th>
<th>PCR product size (bp)</th>
<th>Annealing Temperature (°C)</th>
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</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>House-keeping</td>
<td>5'-GTTCGACAGATAGCCGTAACTTCT-3' 5'-GACCATGTAAGTGAGGTCAATGAA-3'</td>
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<td>β-actin</td>
<td>House-keeping</td>
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<td>60</td>
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<td>IL-6</td>
<td>Pro-inflammatory</td>
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<td>60</td>
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<td>IL-10</td>
<td>Anti-inflammatory T regulatory</td>
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<td>141</td>
<td>64</td>
</tr>
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<td>IL-12p40</td>
<td>Pro-inflammatory Th1</td>
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<tr>
<td>IL-13</td>
<td>Th2</td>
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<td>62</td>
</tr>
<tr>
<td>IL-23</td>
<td>Th17</td>
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<tr>
<td>TGF-β</td>
<td>T regulatory</td>
<td>5'-AATTCAGGTCTGGGAATCAGCAG-3' 5'-GGGATGGTCTCTCTTCTTTCTTA-3'</td>
<td>173</td>
<td>60</td>
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</table>
Chapter 5. General Discussion and Conclusions

The beneficial effects of yeast derivatives on consumer well-being and health have been shown in a number of studies using different species including humans, livestock and and companion animals. Interest in this field has grown lately as the potential mechanisms of action of yeast derivatives, which involve anti-adhesive and immunomodulation, have become clearer. Both properties may help to promote livestock production and survival by reducing risk of infection and enhancing immunity. It has been reported for example, that yeast derivatives reduce \textit{E. coli} infection by blocking attachment of this bacteria to mucosal surfaces in piglets (Jensen et al., 2013). Similarly yeast derivative administration can also decrease \textit{E. coli} load in broilers after challenge (Baurhoo et al., 2007). In addition, administration of yeast derivatives, especially $\beta$-glucans, has been reported to enhance protection of shrimp against \textit{Vibrio vulnificus} infection (Soltanian et al., 2007); this increased resistance was attributed to stimulation of haemocyte phagocytic activity (Itami et al., 1994). In addition, injection of $\beta$-glucans has also been found to significantly increase zebrafish survival against \textit{Aeromonas hydrophila} infection due to the enhanced kidney cell effector killing function (Rodríguez et al., 2009).

In order to apply these beneficial properties of yeast derivatives further, we carried out a series of \textit{in vitro} studies to investigate the effects of \textit{S. cerevisiae} derivatives on adherence of MAP to bovine epithelial cells, and bovine macrophage (BOMAC) function including macrophage phagocytic activity, ROS production and cytokine secretion. These \textit{in vitro} assays may be used in future studies as a potential high throughput means of screening potential bioactive yeast derivatives, and for monitoring product quality.

Bovine mammary epithelial cells (MAC-T cells) were used in this study because of ease of culture and lack of a suitable bovine small intestinal epithelial cell line, and because it is
challenging to obtain sufficient numbers of primary intestinal epithelial cells for high through-
put *in vitro* screening of bioactive compounds. In addition, MAP has previously been detected
within the mammary gland of sub-clinically infected cows (Streeter et al., 1995), which indicates
that MAP is able to disseminate and invade the mammary epithelium. We also found that MAP
binding was largely similar between MAC-T cells and primary BECs, which supports their
continued use in future binding assays. Chapter 3 of this thesis demonstrated that *S. cerevisiae*
yeast derivatives significantly inhibited the binding of MAP to bovine epithelial cells. A similar
observation has been reported in the literature (Karunasena et al., 2013), where probiotic *L.
animalis* administration also reduced concentrations of MAP cells in both intestinal and liver
tissues in murine model infected with MAP; the maximum reduction was observed in female
mice group fed killed-probiotic with 50% of MAP cells compared to the control group treated
with MAP only. Although the reduction of MAP binding to epithelial cells in this study was
partially attributed to a decrease of epithelial cell viability, as both MAC-T cell and BEC
viability and MAP binding was reduced due to the exposure to the same concentrations of CWCs
from strain B; significant binding was also observed at yeast derivative concentrations where cell
viability was greater than 95%. Interestingly, the inactive yeast from strain A also inhibited MAP
binding to both MAC-T cells and BECs, while cell viability of both these cell types appeared to
increase at lower concentrations, but this likely reflected increased cell proliferation at these
concentrations. In future studies, it would be interesting to investigate the mechanisms of action
by which inactive yeast from strain A stimulated epithelial cell proliferation.

In addition to the inhibitory effects of yeast derivatives on MAP binding to epithelial
cells, BOMAC function also appeared to be altered by yeast derivatives. In the present study,
macrophage phagocytic activity, ROS production and immune-related gene expression was
assessed to demonstrate the effect of yeast derivatives on BOMAC function. It has been seen that when gilthead sea bream were fed β-glucan-supplemented diets, their head-kidney leucocyte phagocytic activity was increased (Guzmán-Villanueva et al., 2014). In contrast, results from Chapter 4 showed that the number of MAP phagocytized by BOMACs was decreased in the presence of yeast derivatives when compared to the control group. In the case of CWCs from strain B, this observation was partially attributed to reduced cell viability at the highest concentration. However, it is more likely that reduced MAP binding to BOMACs, as was the case with the epithelial cells, meant that a smaller number of MAP were phagocytized by BOMACs. Since MAP is an intracellular pathogen, this could potentially reduce uptake of MAP into their host macrophages and therefore protect the host against disease.

In chapter 4, our in vitro studies also revealed that ROS production was significantly induced by the tested yeast derivatives. Increased ROS production has been reported to be associated with phagosome acidification, which is required to kill intracellular mycobacteria (Ehrt et al., 2009). Future studies should consider whether or not this increased macrophage effector function affects the viability and proliferation of MAP within macrophages. However, since MAP is such a slow growing pathogen, this would require a much longer exposure period than was used in the present study.

Lastly, in chapter 4 we also reported that yeast derivatives altered BOMACs gene expression. These findings are important because they showed that yeast derivatives differentially affected BOMACs function. These findings need to be confirmed by experiments combining cytokine protein and gene expression, and the biological significance of these results should be explored under in vitro co-culture conditions and in vivo, since macrophages are
unlikely to come into direct contact with yeast derivatives unless there is damage to the epithelium.

Overall, this study provided an in vitro evaluation of the anti-adhesive and immunomodulatory activity of yeast derivatives. This study demonstrated that S. cerevisiae derivatives significantly reduced the adhesion of MAP to bovine epithelial cells when MAP infection is carried out in vitro. It also demonstrated that yeast derivatives reduced macrophage BOMAC phagocytic activity, induced ROS production and altered immune-related gene expression. There are number of studies that demonstrate that yeast derivatives influence gastrointestinal infections with E. coli and Salmonella species, where the pathogenic bacterial loads were reduced in presence of yeast derivatives, which subsequently leads to a decrease in the incidence of enteric disease. These studies help to support the further hypothesis that yeast derivatives may help reduce risk of MAP infection in vivo, but this still remains to be tested.
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A>G in the macrophage migration inhibitory factor (MIF) gene. *10th World Congress of Genetics Applied to Livestock Production, Vancouver, 541.*


Appendix 1: Effect of 6-hr and 24-hr exposure to Pam3CSK4 on the expression of IL-6. Data are presented as least square mean +/- standard error of the normalized values relative to the GAPDH reference gene. Significant differences relative to the control are indicated at $p<0.05$ (*) and 0.01(**).
Appendix 2: Viability of BOMACs following 24-hr exposure to CWCs from yeast strain A and B, inactive yeast (IY) and autolyzed yeast (AY). Data are presented as least square mean +/- standard error.